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In-Depth Mechanistic Study on the Formation of Acrylamide and Other Vinylogous Compounds by the Maillard Reaction

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The formation of acrylamide was studied in low-moisture Maillard model systems (180 °C, 5 min) based on asparagine, reducing sugars, Maillard intermediates, and sugar degradation products. We show evidence that certain glycoconjugates play a major role in acrylamide formation. The *N*-glycosyl of asparagine generated about 2.4 mmol/mol acrylamide, compared to 0.1−0.2 mmol/mol obtained with α-dicarbonyls and the Amadori compound of asparagine. 3-Hydroxypropanamide, the Strecker alcohol of asparagine, generated only low amounts of acrylamide (∼0.23 mmol/mol), while hydroxyacetone increased the acrylamide yields to more than 4 mmol/mol, indicating that α-hydroxy carbonyls are much more efficient than α-dicarbonyls in converting asparagine into acrylamide. The experimental results are consistent with the reaction mechanism based on (i) a Strecker type degradation of the Schiff base leading to azomethine ylides, followed by (ii) a β-elimination reaction of the decarboxylated Amadori compound to afford acrylamide. The β-position on both sides of the nitrogen atom is crucial. Rearrangement of the azomethine ylide to the decarboxylated Amadori compound is the key step, which is favored if the carbonyl moiety contains a hydroxyl group in β-position to the nitrogen atom. The β-elimination step in the amino acid moiety was demonstrated by reacting under low moisture conditions decarboxylated model Amadori compounds obtained by synthesis. The corresponding vinylogous compounds were only generated if a β-proton was available, for example, styrene from the decarboxylated Amadori compound of phenylalanine. Therefore, it is suggested that this thermal pathway may be common to other amino acids, resulting under certain conditions in their respective vinylogous reaction products.

KEYWORDS: Acrylamide; asparagine; Maillard reaction; mechanisms; synthesis; Strecker degradation; β-elimination; decarboxylated Maillard intermediates; azomethine ylides; LC-MS/MS; GC-MS; NMR.

INTRODUCTION

The recent discovery of relatively high amounts of acrylamide in carbohydrate-rich foods obtained by thermal processing (review in ref *1*) has led to numerous studies to help understand how acrylamide is formed. Investigations published or submitted for publication in 2002 have revealed the Maillard reaction as one major reaction pathway, in particular in the presence of asparagine, which directly provides the backbone of the acrylamide molecule (*2*−*7*). However, other reaction pathways and precursors have also been suggested, such as acrolein released by oxidative lipid degradation leading to acrylic acid, which can react with ammonia to give acrylamide (*8*, *9*). Acrylic acid can also be generated from aspartic acid by the Maillard reaction, in analogy to the formation of acrylamide from asparagine (*10*).

There are basically two major hypotheses published so far pertaining to the formation of acrylamide from asparagine in foods by the Maillard reaction. Mottram and co-workers (*3*) have suggested that α-dicarbonyls are necessary coreactants in the Strecker degradation reaction affording the Strecker aldehyde as the precursor of acrylamide. We have proposed glycoconjugates, such as *N*-glycosides and related compounds formed in the early phase of the Maillard reaction, as key intermediates leading to acrylamide (*4*). This hypothesis is supported by the work recently published by Yaylayan et al. (*11*) and Zyzak and co-workers (*12*). Both groups have shown evidence for the importance of the Schiff base of asparagine, which corresponds to the dehydrated *N*-glucosyl compound. The key mechanistic step is decarboxylation of the Schiff base leading to Maillard intermediates that can directly release acrylamide. However, the key intermediates were not or only partially characterized, and therefore the chemical reactions leading to acrylamide remained largely hypothetical.

The objective of this study was to further clarify the mechanism of acrylamide formation in food by comparing the

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two major hypothetical pathways, that is, via (i) the Strecker aldehyde route and (ii) glycoconjugates of asparagine. Gaps in the currently proposed routes are evident, and consequently we have synthesized appropriate model intermediates and employed these in model systems to gain a deeper insight into the key precursors and the salient reaction steps. Finally, the validity of the reaction mechanism was evaluated to explain the formation of vinylogous compounds from amino acids in general.

EXPERIMENTAL PROCEDURES

Materials. L-Asparagine, potassium hydroxide, trifluoroacetic acid (TFA), D-fructose, D-glucose, benzylamine, 2-phenylethylamine, triflic anhydride, *tert*-butyl asparaginate, butane-2,3-dione, pentane-2,3-dione, pentane-2,4-dione, hexane-2,3-dione, hydroxyacetone (acetol), methylglyoxal, glyoxal, acrylamide, and α,*β*,*β*-$^2H_3$-styrene (isotopic purity, 98%) were from Fluka/Aldrich (Buchs, Switzerland). Methanol, formic acid, dichloromethane, water for HPLC, and sodium hydroxide were from Merck (Darmstadt, Germany). The NMR solvents deuterated water (^2H_2O) and chloroform (C^2HCl_3) were from Aldrich. 3-Hydroxypropanamide was custom synthesized by Toronto Research Chemicals (Toronto, Canada). 1,2,3-$^{13}C_3$-Acrylamide (isotopic purity, 99%) was purchased from Cambridge Isotope Laboratories (Andover, MA). Solid-phase cartridges (OASIS HLB 6 cm^3, 0.2 g) were from Waters (Rupperswil, Switzerland). The filter units Spartan 13/0.2 RC were purchased from Schleicher & Schuell (Dassel, Germany). All other reagents were of analytical grade and were used without further purification. *Caution*: *Acrylamide (CAS 79-06-1) is classified as toxic and may cause cancer. Wear suitable protective clothing, gloves, and eye/face protection when handling this chemical.*

Synthesis of Amadori Compounds. Maillard intermediates related to the synthesis of Amadori compounds were prepared following the general procedure described by Lopez and Gruenwedel (*13*).

*2,3:4,5-Di-O-isopropylidene-β-*D*-fructopyranose* ***2***. The synthesis has recently been reported including characterization by NMR (*14*).

*2,3:4,5-(Di-O-isopropylidene-1-O-trifluoromethanesulfonyl)-β-*D*-fructopyranose* ***3***. The synthesis has recently been reported including characterization by NMR (14).

*tert-Butyl N-(2,3:4,5-Di-O-isopropylidene-1-deoxy-*D*-fructos-1-yl)-*L*-asparaginate* ***4***. The synthesis procedure was performed as described in ref *14*. Purity: 95% by NMR. ^1H NMR (360 MHz, C^2HCl_3): δ 1.12, 1.16, 1.27, 1.30 (4 s, 3H each, Me_2C); 1.24 (s, 9H, 3 CH_3); 1.92−1.95 (s, 1H, NH); 2.18 (dd, 1H, CH_2, ^3J = 9.6 Hz, ^2J = 16.0 Hz); 2.34 (dd, 1H, ^3J = 3.3 Hz, ^2J = 16.0 Hz); 2.53 (d, 1H, NCH, ^2J = 12.1 Hz); 2.82 (d, 1H, NCH, ^2J = 12.1 Hz); 3.26 (dd, 1H, ^2J = 9.6 Hz, ^3J = 3.3 Hz); 3.56 (dd, 1H, H_6, ^2J = 13.1 Hz, ^3J = 0.9 Hz); 3.65 (dd, 1H, H_6, ^2J = 13.1 Hz, ^3J = 2.0 Hz); 3.98 (ddd, 1H, H_5, ^3J = 0.9 Hz, ^3J = 2.0 Hz, ^3J = 7.9 Hz); 4.05 (d, 1H, H_3, ^3J = 2.4 Hz); 4.34 (dd, 1H, H_4, ^3J = 7.9 Hz, ^3J = 2.4 Hz); 5.40 (sl, 1H, NH); 7.8 (sl, 1H, NH). ^{13}C NMR (90 MHz, C^2HCl_3): δ 24.3, 25.5, 26.2, 26.8 (4 CH_3); 28.3 (3 CH_3); 35.2 (CH_2); 53.3 (NCH_2); 59.5 (CH); 62.9 (1 CH_2, C_6); 70.7−72.9 (3 CH, C_3, C_4, C_5); 82.4 (Cq); 103.5 (1 Cq, C_2); 108.4, 109.2 (2 Cq); 172.5 (1 C═O); 173.7 (1 C═O).

*Sodium N-(2,3:4,5-Di-O-isopropylidene-1-deoxy-*D*-fructos-1-yl)-*L*-asparaginate* ***5***. Purity: 90% by NMR. ^{13}C NMR (90 MHz, ^2H_2O): δ 23.4, 24.3, 25.3, 25.4 (4 CH_3); 32.3 (CH_2); 53.5 (NCH_2); 59.5 (CH); 61.5 (1 CH_2, C_6); 69.8−72.2 (3 CH, C_3, C_4, C_5); 100.3 (1 Cq, C_2); 110.3, 111.1 (2 Cq); 172.1 (1 C═O); 174.9 (1 C═O).

*N-(1-Deoxy-*D*-fructos-1-yl)-*L*-asparagine* ***6***. Yield: 80%. Purity: 90% by NMR. ^{13}C NMR (90 MHz, ^2H_2O): δ 33.5 (CH_2); 53.5 (NCH_2); 59.5 (CH); 64.4 (1 CH_2, C_6); 69.3−70.7 (3 CH, C_3, C_4, C_5); 95.6 (1 Cq, C_2); 170.6 (1 C═O); 173.7 (1 C═O). HRMS (composition $C_{10}H_{18}N_2O_8Na$): calculated mass molecular ion 317.0961, measured 317.0960.

*N-(2,3:4,5-Di-O-isopropylidene-1-deoxy-*D*-fructos-1-yl)-benzylamine* ***7***. Yield: 67%. Purity: 95% by NMR. ^1H NMR (360 MHz, C^2HCl_3): δ 1.15, 1.20, 1.22, 1.35 (4 s, 3H each, Me_2C); 1.50 (sl, 1H, NH); 2.63 (d, 1H, CH_2, ^2J = 12.5 Hz); 2.81 (d, 1H, ^2J = 12.5 Hz); 3.58 (d, 1H, H_6, ^2J = 12.9 Hz); 3.68 (s, 2H, CH_2N); 3.71 (dd, 1H, H_6, ^2J = 12.9 Hz, ^3J = 2.0 Hz); 4.03 (dd, 1H, H_5, ^3J = 2.0 Hz, ^3J = 7.9 Hz); 4.19 (d, 1H, H_3, ^3J = 2.6 Hz); 4.40 (dd, 1H, H_4, ^3J = 7.9 Hz, ^3J = 2.6 Hz); 7.00−7.20 (m, 5H, aromatic). ^{13}C NMR (90 MHz, C^2HCl_3): δ 24.4, 25.9, 26.2, 26.9 (4 CH_3); 54.3 (NCH_2); 55.3 (NCH_2, C_1); 61.5 (1 CH_2, C_6); 70.7−72.0 (3 CH, C_3, C_4, C_5); 103.9 (1 Cq, C_2); 108.5, 109.3 (2 Cq); 127.2 (CH, aromatic); 128.4−128.7 (2 CH, aromatic); 140.7 (1 Cq, aromatic).

*N-(2,3:4,5-Di-O-isopropylidene-1-deoxy-*D*-fructos-1-yl)-2-phenylethylamine* ***8***. Yield: 70%. Purity: 95% by NMR. ^1H NMR (360 MHz, C^2HCl_3): δ 1.37, 1.39, 1.50, 1.52 (4 s, 3H each, Me_2C); 1.50 (sl, 1H, NH); 2.75−3.04 (m, 6H, 3 CH_2); 3.78 (d, 1H, H_6, ^2J = 13.1 Hz); 3.90 (dd, 1H, H_6, ^2J = 13.1 Hz, ^3J = 2.0 Hz); 4.25 (dd, 1H, H_5, ^3J = 2.0 Hz, ^3J = 7.9 Hz); 4.38 (d, 1H, H_3, ^3J = 2.5 Hz); 4.62 (dd, 1H, H_4, ^3J = 7.9 Hz, ^3J = 2.5 Hz); 7.20−7.33 (m, 5H, aromatic). ^{13}C NMR (90 MHz, C^2HCl_3): δ 24.4, 25.8, 26.3, 26.9 (4 CH_3); 36.8 (CH_2); 52.1 (NCH_2); 55.9 (NCH_2, C_1); 61.5 (1 CH_2, C_6); 70.8−71.8 (3 CH, C_3, C_4, C_5); 103.9 (1 Cq, C_2); 108.5, 109.3 (2 Cq); 126.5 (CH, aromatic); 128.8−129.2 (2 CH, aromatic); 140.6 (1 Cq, aromatic).

*N-(1-Deoxy-*D*-fructos-1-yl)-benzylamine* ***9***. Yield: 80%. Purity: 95% by NMR. ^{13}C NMR (90 MHz, ^2H_2O): δ 49.9 (NCH_2); 52.1 (NCH_2, C_1); 64.3 (1 CH_2, C_6); 69.3−70.1 (3 CH, C_3, C_4, C_5); 95.8 (1 Cq, C_2); 127.9 (CH, aromatic); 128.4−128.7 (2 CH, aromatic); 136.7 (1 Cq, aromatic).

*N-(1-Deoxy-*D*-fructos-1-yl)-2-phenylethylamine* ***10***. Yield: 80%. Purity: 95% by NMR. ^{13}C NMR (90 MHz, ^2H_2O): δ 31.8 (CH_2); 49.9 (NCH_2); 53.4 (NCH_2, C_1); 64.4 (1 CH_2, C_6); 69.3−70.2 (3 CH, C_3, C_4, C_5); 95.8 (1 Cq, C_2); 127.9 (CH, aromatic); 129.3−129.6 (2 CH, aromatic); 136.8 (1 Cq, aromatic).

Synthesis of *N*-Glycosides of Amino Acids. These compounds were prepared by adapting the general procedure for obtaining *N*-glycosides by condensation of reducing sugars and amino acids in anhydrous methanol under basic pH conditions, first described by Weitzel et al. (*15*).

*Potassium N-(*D*-Glucos-1-yl)-*L*-asparaginate* ***11***. A solution of potassium hydroxide (8.34 mmol) in absolute methanol (5 mL) was added to L-asparagine (8.34 mmol). The mixture was stirred for 15 min at room temperature. Then methanol (10 mL) and anhydrous D-glucose (5 mmol) were added, and the suspension was stirred and heated under reflux for 1 h at 75 °C until the solution turned yellowish. The reaction mixture was cooled to ambient temperature and filtered. The solid was washed with methanol and dried under a vacuum, affording 1.1 g of a yellow solid (64% yield). The purity was >80% by NMR, containing a residual amount of the amino acid mainly. ^{13}C NMR (90 MHz, ^2H_2O): δ 40.0 (1 CH_2, $C_{3'}$); 57.9 (1 CH, $C_{2'}$); 61.4 (1 CH_2, C_6); 70.3, 73.3, 77.1, 77.2 (4 CH, C_2, C_3, C_4, C_5); 89.4 (C_1); 177.0 (1 Cq, $C_{4'}$); 181.0 (1 Cq, $C_{1'}$).

*Potassium N-(*D*-Fructos-2-yl)-*L*-asparaginate* ***12***. This was prepared using the procedure and conditions described for **11** by replacing D-glucose by D-fructose. A yellow solid was obtained (874 mg, 52% yield). The purity was >80% by NMR, containing a residual amount of the amino acid mainly. ^{13}C NMR (90 MHz, ^2H_2O): δ 37.8 (1 CH_2, $C_{3'}$); 52.5 (1 CH, $C_{2'}$); 63.9, 64.3 (2 CH_2, C_6, C_1); 68.0, 69.6, 70.1 (3 CH, C_3, C_4, C_5); 98.5 (1 C_q, C_2); 176.0 ($C_{4'}$); 177.7 ($C_{1'}$).

Nuclear Magnetic Resonance (NMR) Spectroscopy. The samples for NMR spectroscopy were prepared in WILMAD 528-PP 5 mm Pyrex NMR tubes, using deuterated water or deuterated chloroform as the solvent (0.7 mL). The NMR spectra were acquired on a Bruker AM-360 spectrometer, equipped with a quadrinuclear 5 mm probe head, at 360.13 MHz (^1H) and 90.03 MHz (^{13}C) spectra. All chemical shifts (δ) are cited in ppm measured relative to the solvent signal. ^1H NMR, ^{13}C NMR, and distortionless enhancement by polarization transfer (DEPT 135) were acquired as described earlier using standard conditions (*16*). The spectra were interpreted using the MestRe-C 2.3 software.

High-Resolution Mass Spectrometry (HRMS). High-resolution mass spectra were acquired on a Micromass QToF-2 instrument (Micromass, Manchester, U.K.). Data acquisition and data evaluation were performed using the Micromass MassLynx 3.5 software, SP367. Survey mass spectra were acquired by directly infusing sample extracts in methanol into the electrospray ion source of the instrument at a flow rate of 5 μL/min. The electrospray (needle) voltage was set at 3 kV, and the ion source block temperature at 120 °C. The cone gas (nitrogen)

was set to a flow rate of 50 L/min and the desolvation gas to 300 L/min with a temperature of 140 °C. Accurate mass measurements were realized by mixing the respective extracts obtained with a calibrant solution (1 mg/mL NaI, 0.05 mg/mL CsI in water/2-propanol, 60/40, v/v) and infusing as described above. About 20 scans were averaged at different cone voltages (1 min total acquisition time at ca. 1 scan/s), and accurate mass data were obtained using a suitable lockmass from the calibrant. The elemental composition of the resulting values was calculated using a program as described previously (*17*).

Gas Chromatography−Mass Spectrometry (GC−MS). Mass spectra of volatile compounds were acquired using a gas chromatograph GC 5890 (Agilent, Palo Alto, CA) equipped with two splitless injectors heated at 260 °C and coupled with a quadrupolar mass spectrometer MS 5970 (Agilent) operating in the electron impact (EI) ionization mode at 70 eV. Acquisitions were carried out over a mass range of 10−350 Da. Separations were performed on a poly(ethylene glycol) polar stationary phase (DB-WAX, 60 m × 0.25 mm i.d., 0.5 μm film thickness; J&W, Folsom, CA). Helium was used as the carrier gas with a constant flow rate of 0.6 and 1 mL/min, respectively. The oven was programmed as follows: 20 °C (0.5 min), 70 °C/min to 60 °C, and 4 °C/min to 240 °C. The temperature of the transfer line was held at 280 °C during the chromatographic run.

The analyses of styrene were performed using a GC 6890A coupled to an MSD 5973N (both Agilent) equipped with a DB-Wax capillary column (J&W Scientific): 60 m × 0.25 mm; film thickness, 0.25 μm. Helium was used as a carrier gas (2.4 mL/min constant flow). Samples were introduced via splitless injection at 250°C (1 μL). The oven temperature program was as follows: 35 °C (2 min) and 6 °C/min to 240 °C (10 min). The EI-MS spectra were generated at 70 eV. The temperature of the ion source was 280 °C. Quantification of styrene by isotope dilution assay (IDA) was performed in the EI-MS mode by measuring the molecular ions of the analyte and labeled internal standard at *m*/*z* 104 and 107, respectively.

Liquid Chromatography Tandem Mass Spectrometry (LC−MS/MS). Mass spectrometry measurements were performed using a Waters separation module Alliance 2690 (Rupperswil, Switzerland) coupled with a Quattro LC mass spectrometer (Micromass, Manchester, U.K.). Analytical separation was achieved by using a Shodex RSpak DE-613 HPLC column (poly(methacrylate) gel, 150 × 6 mm i.d.; Showa Denko K.K., Japan). The elution mode was isocratic, using a mixture of methanol and water (60:40, v/v) containing 0.06% (v/v) of concentrated formic acid as the LC eluent. The initial flow rate was set at 0.75 mL/min and reduced by postcolumn splitting after the LC column to 0.25 mL/min. The column temperature was maintained at 40 °C with a column heater. The injection volume was 50 μL.

The analytes were detected by multireaction monitoring (MRM) in positive electrospray ionization mode (*18*). Three different fragment ion transitions were monitored for both acrylamide (*m*/*z* 72 → 55, *m*/*z* 72 → 54, and *m*/*z* 72 → 27) and the internal standard (*m*/*z* 75 → 58, *m*/*z* 75 → 57, and *m*/*z* 75 → 29), with t_R = 5.2 min. 3-Hydroxypropanamide (t_R = 4.2 min) was monitored using transitions *m*/*z* 90 → 72 and *m*/*z* 90 → 44; the collision energy was set to −14 eV, and the cone voltage to 26 V.

For all analytes, the dwell time was set to 0.2 s. The MS settings were 3.2 kV needle voltage and for acrylamide 26 V for the cone voltage. The ion energy was set to 0.9 and 1.0 V for the first and second quadrupole, respectively. The desolvation and ion block temperatures were set at 350 and 100 °C, respectively. Nitrogen was used as nebulizer (100 L/h) and desolvation gas (600 L/h). The collision energy was set at −19 eV for all acrylamide transition reactions, except the fragmentation transitions *m*/*z* 72 → 55 and *m*/*z* 75 → 58 that were set at −11 eV. Argon was used as the collision gas at a pressure of 1.7 mTorr (2.3 mbar).

Quantification of Acrylamide. Acrylamide standards were prepared in Millipore-grade water from a stock solution (0.01 mg/mL) and stored in the refrigerator for 1 week. External calibration curves (6 point) were established in the concentration range from 5 to 1000 pg/μL acrylamide, containing a fixed amount of internal standard (20 pg/μL). All data evaluation (using the MassLynx software) was normalized to the area response analyte to internal standard. For all standard curves, good linearity was obtained (r^2 > 0.998).

Pyrolysis Procedures. The chemicals of interest were heated in a temperature-controlled heating module (Brouwer) at 180 °C in tightly closed 6 mL Pyrex vacuum hydrolysis tubes (16 cm × 0.9 mm) that were immersed in silicone oil. After a defined heating period (e.g., 5 min), the tubes were cooled on ice. For quantification of acrylamide, the pyrolysates were spiked with $^{13}C_3$-acrylamide (50 ng) and suspended in hot water (>90 °C, 2.5 mL), vortexed (1 min), and sonicated in an ultrasonic water bath (5 min). After suspension, the extracts were filtered through Schleicher & Schüll, 597½ folded filters. A portion of the clear extract (1 mL) was loaded onto an SPE cartridge, preconditioned with each two-bead volumes of first methanol and then water. After gravity-induced penetration, the column was rinsed with 0.5 mL of water (short vacuum-induced suction to remove residual water from the columns) and then acrylamide eluted (gravity-induced) with 0.5 mL of 20% methanol in water (v/v). The eluate was filtered (0.2 μm syringe filters) prior to analysis of an aliquot (0.05 mL) by LC−MS/MS as described above.

For ^{13}C NMR experiments, $^{13}C_3$-labeled acrylamide (20 μg) was added to the samples prior to pyrolysis.

The model Maillard intermediates **9** and **10** were heated at 180 °C for 15 min. The reaction sample was dissolved in water and extracted with dichloromethane. The organic phase was dried over sodium sulfate and analyzed by GC−MS.

Fructose (0.2 mmol) and phenylalanine (0.2 mmol) were placed in a 20 mL crimp cap vial (Chromacol) and were heated in a silicone bath at 180 °C for 15 min. After cooling, the reaction sample was dissolved in water (2 mL), spiked with α,β,β-$^2H_3$-styrene (5.12 μg in MeOH), and extracted with diethyl ether (2 mL). The organic phase was dried over sodium sulfate and analyzed by GC−MS. The experiments were performed in duplicate.

RESULTS

Synthesis of Early Maillard Intermediates. The Amadori compound *N*-(1-deoxy-D-fructos-1-yl)-L-asparagine **6** was prepared starting with β-D-fructose **1** (**Figure 1**), which was protected in the positions C-2/3 and C-4/5 with acetone (*13*). The intermediate 2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranose **2** was then activated at the C-1 position by a triflate group to give the intermediate 2,3:4,5-di-*O*-isopropylidene-1-*O*-(trifluoromethansulfonyl)-β-D-fructopyranose **3** in 80% yield. The leaving group was substituted by the *tert*-butylester of asparagine in 57% yield. The substitution reaction resulted in *tert*-butyl *N*-(2,3:4,5-di-*O*-isopropylidene-1-deoxy-D-fructos-1-yl)-L-asparaginate **4**. Saponification of ester **4** afforded the acetal-protected asparagine derivative sodium *N*-(2,3:4,5-di-*O*-isopropylidene-1-deoxy-D-fructos-1-yl)-L-asparaginate **5**. Deprotection of the acetal groups in **5** under mild acidic conditions yielded the target product **6** in 80% yield.

In analogy, triflate **3** and benzylamine or 2-phenylethylamine resulted in the intermediates *N*-(2,3:4,5-di-*O*-isopropylidene-1-deoxy-D-fructos-1-yl)-benzylamine **7** and *N*-(2,3:4,5-di-*O*-isopropylidene-1-deoxy-D-fructos-1-yl)-2-phenylethylamine **8** in 67 and 70% yield, respectively. Their deprotection by trifluoroacetic acid led to the model Amadori compounds **9** and **10** in about 80% yield. The compounds were characterized by NMR and used as such for pyrolysis experiments.

Finally, two *N*-glycosides of L-asparagine were prepared, namely, the potassium salts of *N*-(D-glucos-1-yl)-L-asparagine **11** and *N*-(D-fructos-2-yl)-L-asparagine **12**. The Maillard intermediates **4**−**12** are suitable compounds to study the reaction mechanisms leading to acrylamide under food processing conditions.

Pyrolysis of Asparagine-Based Maillard Intermediates. As shown in **Table 1**, the early Maillard intermediate *N*-(D-glucos-1-yl)-L-asparagine **11** released significant levels of acrylamide upon heating at 180 °C for 5 min (sample A). Prolonged heating for 20 min led to reduced amounts of acrylamide and may be

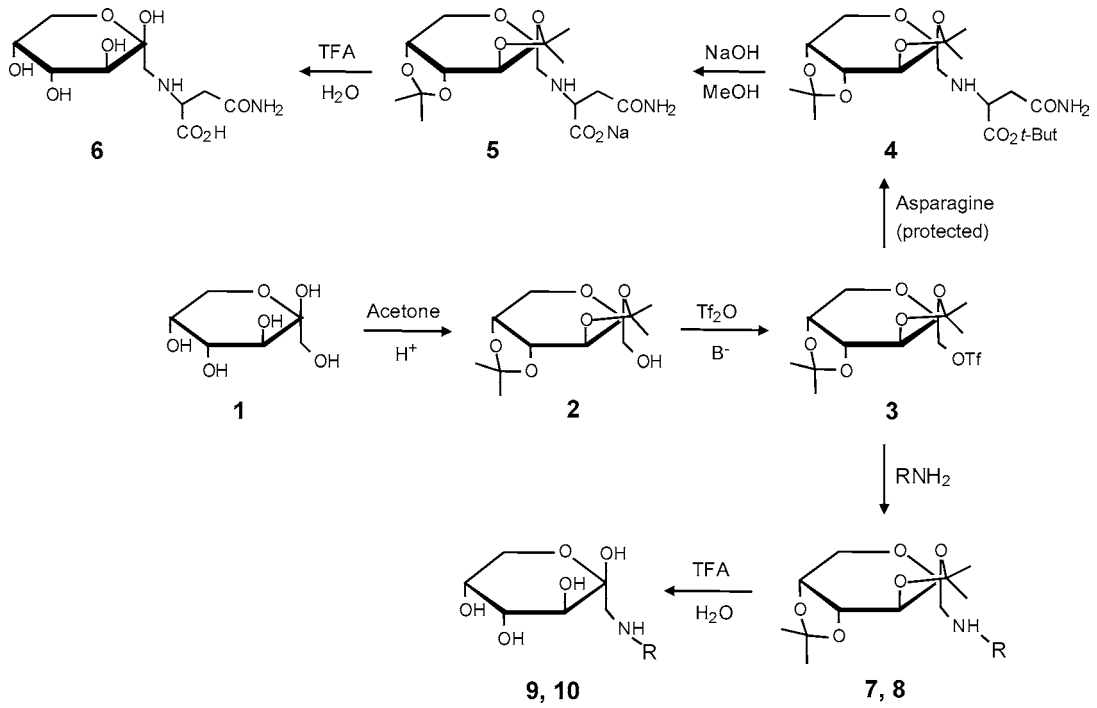

Figure 1. Synthetic pathway to the Amadori compounds of asparagine (**6**) and protected derivatives thereof (**4**, **5**) as well as of benzylamine (**9**) and 2-phenylethylamine (**10**). **7** and **9**: R = CH_2Ph. **8** and **10**: R = CH_2CH_2Ph. For an explanation, see the text.

Table 1. Acrylamide Generated from Maillard Precursors upon Pyrolysis

sample[a]	heating time (min)	precursors	acrylamide[b] (mmol/mol)
A	5	potassium *N*-(D-glucos-1-yl)-L-asparaginate **11**	2.38
A	20	potassium *N*-(D-glucos-1-yl)-L-asparaginate **11**	1.31[c]
B	20	potassium *N*-(D-fructos-2-yl)-L-asparaginate **12**	1.42[c]
C	5	*N*-(1-deoxy-D-fructos-1-yl)-L-asparagine **6**	0.10
D	5	sodium *N*-(2,3:4,5-di-*O*-isopropylidene-1-deoxy-D-fructos-1-yl)-L-asparaginate **5**	0.17
E	5	*tert*-butyl *N*-(2,3:4,5-di-*O*-isopropylidene-1-deoxy-D-fructos-1-yl)-L-asparaginate **4**	0.06
F	30	D-fructose + L-asparagine (1:1)	1.13[d]
G	30	D-glucose + L-asparagine (1:1)	0.37[c,d]
H	5	butane-2,3-dione + L-asparagine (1:1)	0.12
I	5	pentane-2,3-dione + L-asparagine (1:1)	0.20
J	5	hexane-2,3-dione + L-asparagine (1:1)	0.19
K	5	pentane-2,4-dione + L-asparagine (1:1)	0.02
L	5	glyoxal + L-asparagine (1:1)	0.11
M	5	methylglyoxal + L-asparagine (1:1)	0.14
N	5	acetol + L-asparagine (1:1)	4.31
O	5	3-hydroxypropanamide	0.24

[a] Samples (each 0.2 mmol reactant, 0.05 mL water in the hydrolysis tubes unless otherwise stated) were heated at 180 °C for 5–30 min. [b] Acrylamide concentration expressed in mmol/mol precursor (asparagine or its derivative). Entries are averages of $n = 2$–6 independent determinations. [c] Values taken from ref *4*. Quantification was performed using $^{13}C_3$-acrylamide as the internal standard (transition *m/z* 72 → 27; see Experimental Procedures). The coefficients of variation were <20% within an experimental series. [d] Mixtures heated in a dry state.

attributed to the accelerated decomposition of acrylamide at high temperatures. The fructose-derived *N*-glycosyl of asparagine **12** was as efficient as **11** in generating acrylamide (sample B).

The formation of acrylamide by thermal decomposition of the *N*-glycoside **11** is depicted in **Figure 2**. The formation of acrylamide was evident already at a relatively low temperature of 100 °C (60 min heating period), increasing near linearly up to 180 °C (**Figure 2A**). The reaction proceeds at a very fast rate even at 100 °C and reaches a maximum after 20 min (**Figure 2B**), declining again over the following 30 min and reaching a more stable level up to a total incubation period of 180 min (0.199 ± 0.02 mmol/mol *N*-glycoside **11**, $n = 3$).

As shown in **Table 1**, thermolysis of the Amadori compound **6** (sample C) and its protected derivatives **5** and **4** (samples D and E) led to the formation of acrylamide, albeit in moderate amounts (0.06−0.17 mmol/mol) compared to sample A (5 min). These results and the fact that heating reducing sugars and asparagine tends to furnish higher amounts of acrylamide (samples F and G) rule out the Amadori compound of asparagine as a major precursor of acrylamide. The lower amounts of acrylamide recorded in glucose mixtures versus fructose might be due to the lower melting point of fructose and consequently faster interaction of the precursors to furnish the early Maillard intermediates. A more detailed discussion of this phenomenon will be reported elsewhere.

Decomposition of Acrylamide. As indicated by NMR experiments, acrylamide was easily polymerized. The stability of acrylamide at 190 °C was evaluated by pyrolysis of $^{13}C_3$-labeled acrylamide in a glucose/asparagine model reaction. The ^{13}C NMR spectra showed three signals at 173.7, 51.7, and 34.9

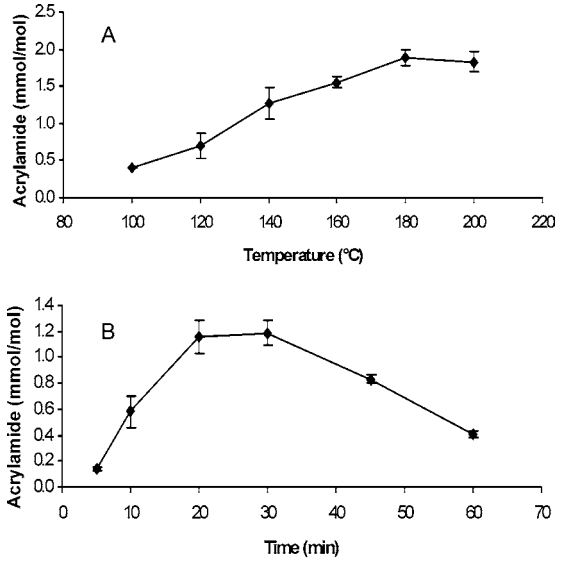

Figure 2. Formation of acrylamide from potassium *N*-(D-glucos-1-yl)-L-asparaginate **11**; 0.05 mmol incubated: (**A**) impact of the temperature after an incubation period of 1 h; (**B**) formation of acrylamide over time at 100 °C. Each data point is the average of three independent measurements.

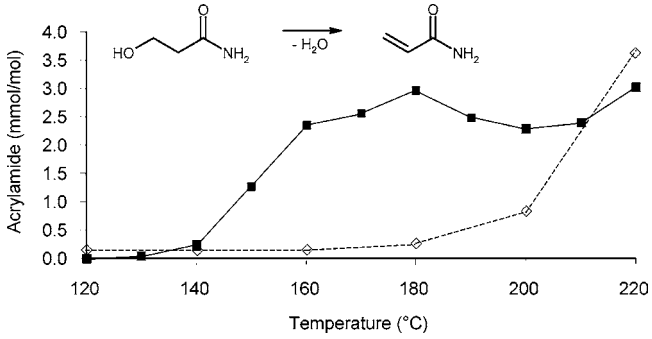

Figure 3. Formation of acrylamide from equimolar fructose/asparagine mixtures (■) and 3-hydroxypropanamide (◇) upon pyrolysis for 5 min. Each data point is the average of two independent measurements.

ppm representing a $C{=}ONH_2$, an aliphatic CH, and an aliphatic CH_2 group, respectively. These data suggest that residual acrylamide in the matrix at this temperature mainly occurs in the polymeric form with ($CH_2CHCONH_2$) as the monomeric unit. However, acrylamide may also react with soft nucleophiles according to the hard and soft acid base (HSAB) theory and can, therefore, be consumed in Michael type addition reactions.

Formation of Acrylamide by α-Dicarbonyl-Assisted Strecker Degradation. To further investigate the mechanisms of acrylamide formation, the role of specific Maillard degradation products was studied. Co-pyrolysis of asparagine with various dicarbonyls, such as for example α-diketones (**Table 1**, samples H−J), α-keto aldehydes (sample M), and glyoxal (sample L), gave rise to relatively lower acrylamide concentrations (0.1−0.2 mmol/mol) versus the sugar/asparagine binary mixtures (samples F and G). Interestingly, no pronounced difference was found between α-diketones and aldehydes bearing a carbonyl function in the α-position. However, pentane-2,4-dione (sample K) incubated with equimolar amounts of asparagine afforded only spurious amounts of acrylamide under the same temperature/time conditions. Surprisingly, acetol generated about 4.3 mmol/mol acrylamide (sample N), which represents the highest amount found so far in binary mixtures of asparagine.

The Strecker alcohol of asparagine, that is, 3-hydroxypropanamide, which may release acrylamide by a one-step dehydration reaction, was found to also generate acrylamide, albeit at levels not exceeding those recorded for sugar/asparagine mixtures under similar reaction conditions (**Table 1**, sample O). It must, however, be kept in mind that 3-hydroxypropanamide represents a direct precursor to acrylamide, whereas binary mixes of primary reactants must pass through several steps en route to acrylamide. As shown in **Figure 3**, dehydration of 3-hydroxypropanamide to release acrylamide follows different temperature kinetics as compared to the fructose/asparagine copyrolysates. The acrylamide concentration increased with temperature by favoring the thermally induced dehydration reaction. Concomitantly to monitoring the formation of acrylamide in the pyrolysates, the amount of unreacted 3-hydroxypropanamide was also determined and found to be in excess of >50% over all temperature ranges studied. Furthermore, monitoring of the characteristic trace *m*/*z* 90 → 72 for 3-hydroxypropanamide in the fructose/asparagine mixtures failed to identify the presence of the Strecker alcohol. These data suggest that the intermediacy of the Strecker aldehyde of asparagine via the alcohol is most likely of limited importance in the formation of acrylamide, which is well in line with the results obtained with the α-dicarbonyls (see **Table 1**).

Pyrolysis of Model Amadori Compounds. Pyrolysis experiments with the model Amadori compounds **9** and **10** were performed under the same pyrolysis conditions to study the formation mechanism. Qualitative GC−MS analysis indicated that **9** and **10** mainly furnished benzaldehyde and styrene as volatile reaction products, respectively (**Figure 4**). In addition, traces of benzyl alcohol were detected in the sample containing **9**. On the contrary, phenylacetaldehyde and 2-phenylethanol could not be detected in the sample based on compound **10**.

The formation of styrene from **10** proceeds via a Hofmann type elimination reaction due to the presence of a β-proton. In

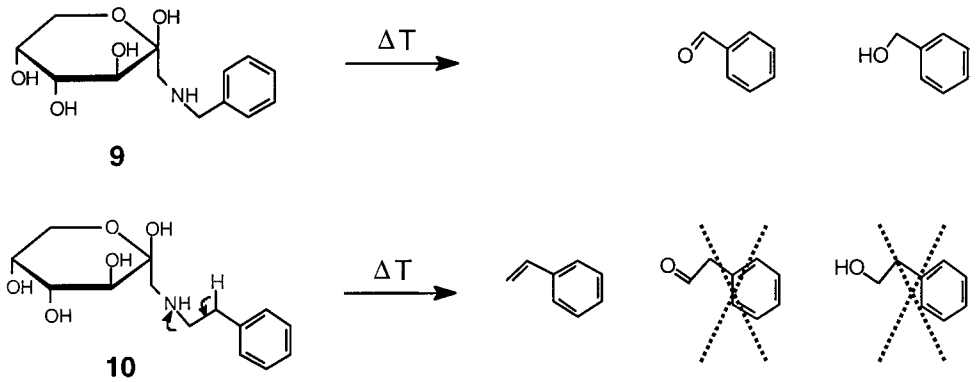

Figure 4. Formation of styrene and benzaldehyde through β-elimination of **10** and α-dicarbonyl-assisted Strecker type degradation of **9**, respectively.

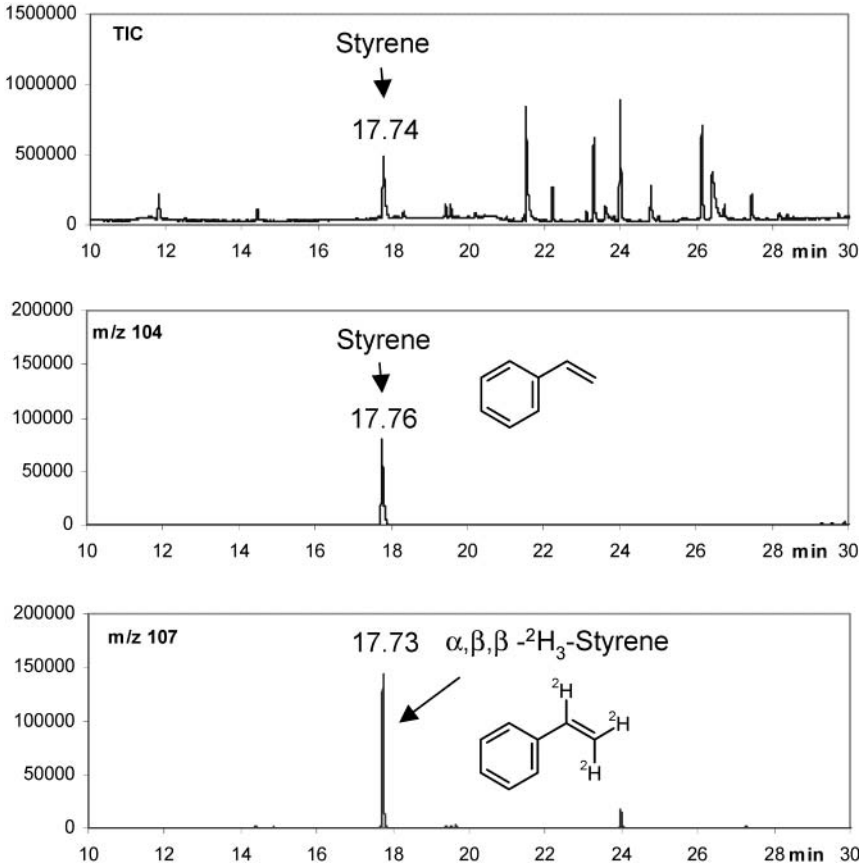

Figure 5. Formation of styrene by heating an equimolar mixture of fructose and phenylalanine at 180 °C for 15 min. The graph shows the total ion current (TIC) and the traces at *m/z* 104 and *m/z* 107 of the analyte and the internal standard (d_3-styrene), respectively.

contrast, compound **9** is not amenable to β-elimination but may undergo Strecker type reactions in the presence of α-dicarbonyl compounds. As compound **10** represents the decarboxylated Amadori compound of phenylalanine, acrylamide may, in analogy, be formed from the decarboxylated Amadori compound of asparagine. Therefore, these results support the formation mechanism suggested by Yaylayan et al. (*11*).

In fact, relatively high amounts of styrene were procured in dry-heated fructose/phenylalanine model systems (180 °C, 15 min). Quantification was performed by GC−MS using α,β,β-$^2H_3$-styrene as the internal standard, resulting in ∼0.2 mmol styrene per mol phenylalanine. The total ion chromatogram (TIC) and the ion chromatograms showing the traces of *m/z* 104 and 107 that correspond to unlabeled and labeled styrene, respectively, are shown in **Figure 5**. More details on the formation of styrene and other vinylogous compounds will be reported elsewhere.

DISCUSSION

Our preliminary model studies have shown that asparagine is the major amino acid source of acrylamide, the generation of which is drastically increased by copyrolysis with reducing sugars (*4*). We suggested sugar−asparagine conjugates, such as *N*-glycosylasparagines or related compounds, as direct precursors of acrylamide, as these early Maillard intermediates yield more acrylamide under milder reaction conditions compared to binary mixtures of the precursors. Similarly, the decarboxylated Schiff base and decarboxylated Amadori compound of asparagine have been proposed as direct Maillard precursors of acrylamide (*11*, *12*). At the same time, the Strecker aldehyde of asparagine has also been suggested as a possible direct intermediate of acrylamide (*3*).

According to our experimental results, the pathway of acrylamide formation in a glucose/asparagine system seems to occur prior to the Amadori rearrangement. Indeed, the amount of acrylamide released from *N*-glycosyl asparagine **11** is severalfold higher (20×) than from the Amadori compound **6** (**Table 1**, samples A and C). Furthermore, the similar results obtained for samples C, D, and E indicate that protection of the hydroxy groups in the sugar ring and the carboxyl group does not markedly affect acrylamide formation from Amadori type compounds of asparagine. It seems that the Amadori product does not easily decarboxylate.

It is generally accepted that Amadori compounds are the first stable intermediates generated in aqueous systems as a result of the early Maillard reaction cascade leading to 1- and 3-deoxyosones, which further decompose to generate color and flavor (*19*). However, in low-moisture systems limiting the reversibility of the initial step, the first stable intermediates are the *N*-glycosyl compounds **11**, which mainly rearrange via the corresponding Schiff base **13** to the Amadori compound **6**, the 1-deoxyfructosyl derivative of the amino acid (**Figure 6**, pathway I). As previously discussed, the latter, however, is not a favored Maillard intermediate to generate acrylamide (**Table 1**, sample C).

Alternatively, the Schiff base **13** may decarboxylate to the intermediary azomethine ylide **14** (*12*), which after tautomerization leads to the decarboxylated Amadori compound **15** (*11*) as shown in **Figure 6** (pathway II). The vinylogous compounds are then released, along with the corresponding aminoketone, by a β-elimination reaction and cleavage of the carbon−nitrogen covalent bond (*11*, *12*). This mechanistic pathway is supported by the fact that copyrolysis of a reducing sugar with asparagine,

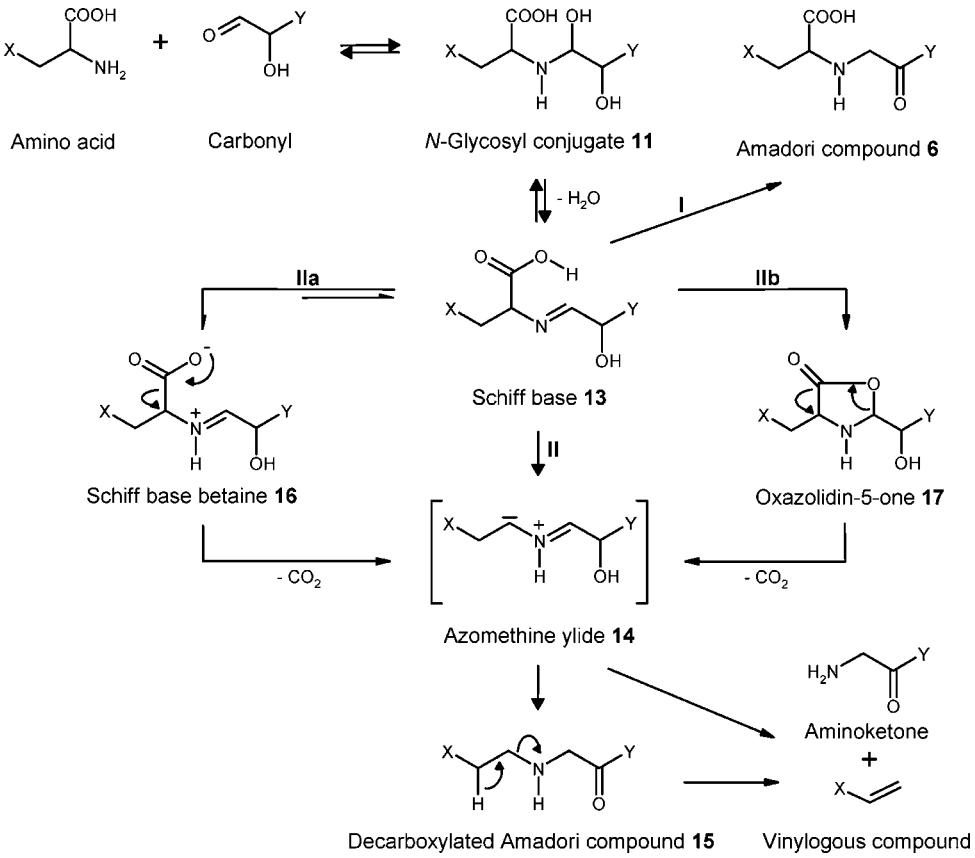

Figure 6. Formation of acrylamide from asparagine through the early Maillard reaction. Y = $(CHOH)_3$–CH_2OH for aldohexose sugars; X = $CONH_2$ for asparagine, X = Ph for phenylalanine, X = COOH for aspartic acid, and X = CH_2CONH_2 for glutamine, resulting in the vinylogous compounds acrylamide, styrene, acrylic acid, and 3-butenamide, respectively.

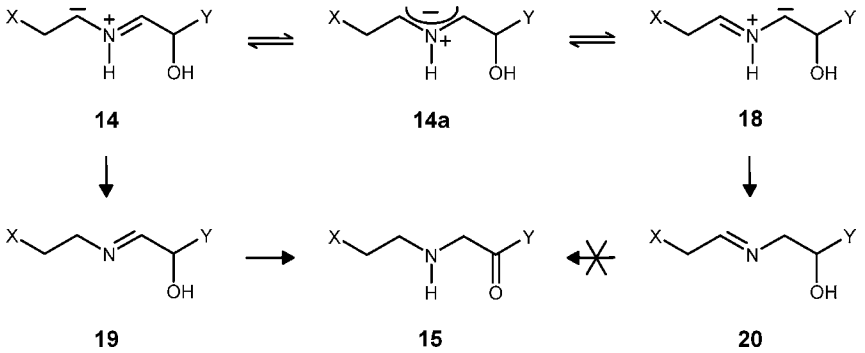

Figure 7. Isomerization of the decarboxylated Schiff base to the azomethine ylides **14** and **18** via the 1,3-dipole **14a** by 1,2-prototropy, leading to the neutral imines **19** and **20**, respectively.

aspartic acid, glutamine, and phenylalanine leads to acrylamide, acrylic acid (*9*), 3-butenamide (*9*), and styrene (*20*), respectively. Similarly, styrene can also be formed in aqueous glucose/phenylalanine solutions under boiling conditions (*21*).

Decarboxylation of the Schiff base **13** to **14** may proceed via the zwitterionic form **16** (**Figure 6**, pathway IIa), which was claimed by Grigg and co-workers (*22*, *23*) as more probable compared to the classical Strecker degradation mechanism (*24*). According to these authors, the decarboxylative transamination reaction goes through a 1,3-dipole intermediate **14a**, which may lead to the azomethine ylides **14** and **18** (**Figure 7**). The final location of the proton in the neutral imines (**19** and **20**) depends on the kinetically controlled proton transfer via 1,2-prototropy to the site of the dipole with the greatest electron density (*23*). A similar carbonyl-assisted decarboxylation of the *N*-alkyl α-amino acid sarcosine via a resonance-stabilized azomethine ylide has first been reported by Rizzi (*25*).

Zyzak and co-workers (*12*) have reported some evidence for the decarboxylated Schiff base of asparagine in model systems containing an excess of reducing sugar by MS(ESI+) measurement of *m*/*z* 251, which however may also represent the decarboxylated Amadori compound **15**. They suggested acrylamide to be formed directly from the Maillard intermediate **14** (**Figure 6**) and claimed the decarboxylation of the Schiff base to be the limiting step. Similarly, Yaylayan et al. (*11*) suggested a decarboxylated Maillard intermediate as a direct precursor of acrylamide (**Figure 6**, pathway IIb). The proposed pathway to the decarboxylated Amadori compound **15** is based on intramolecular cyclization of the Schiff base **13** to the oxazolidine-5-one derivative **17**. Such oxazolidine-5-ones have been reported to easily decarboxylate (*26*), thus giving rise to stable azomethine ylides (**14**), which after tautomerization lead to intermediate **15**. In this pathway, not the decarboxylation step has been claimed as the limiting step, but the cleavage of the strong

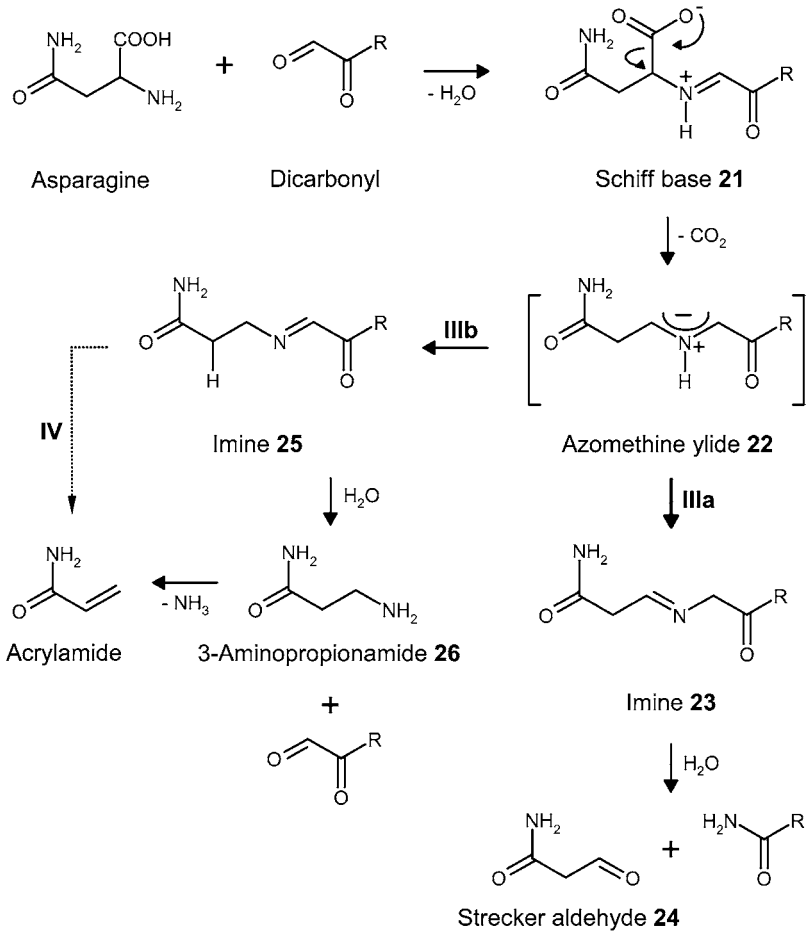

Figure 8. Formation of acrylamide, 3-aminopropanamide **26**, and the Strecker aldehyde **24** from asparagine in the presence of α-dicarbonyls. No experimental evidence has been found so far validating pathway IV.

carbon−nitrogen covalent bond in the β-elimination reaction (*11*). The authors failed to provide evidence for the decarboxylated Amadori compound. However, the Maillard intermediate with *m*/*z* 251 reported by Zyzak et al. (*12*) may, in fact, refer to compound **15**.

Although no direct evidence has been reported so far for the existence of the decarboxylated Amadori compound, we provide for the first time evidence for the validity of the β-elimination reaction of decarboxylated model Amadori compounds. Indeed, pyrolysis of compound **10** generated styrene by β-elimination without formation of phenylethanal, whereas **9** mainly gave rise to benzaldehyde via hydrolysis of the imine intermediate (**Figure 4**). These results confirm that β-elimination is favored compared to aldehyde formation, provided a β-proton is available for the Hofmann type elimination reaction.

In agreement with these findings, copyrolysis of binary mixtures of fructose and phenylalanine (180 °C, 15 min) furnished about 0.2 mmol styrene per mol phenylalanine. Similarly, acrylic acid and 3-butenamide have recently been detected in dry-heated sugar/aspartic acid and sugar/glutamine samples, respectively (*10*). Therefore, the reaction pathway via the decarboxylated Amadori compound might be extended to other amino acids having a β-proton. The concentration of the vinylogous compound released, however, depends on its reactivity under pyrolytic conditions. For example, 3-butenamide has been reported to further react to 2-pyrrolidinone by intramolecular cyclization (*10*).

As the "Strecker alcohol" of asparagine did not generate higher amounts of acrylamide (**Table 1**, sample O) than sugar/asparagine mixtures, the formation mechanism via the classical α-dicarbonyl-assisted Strecker reaction (*3*) can be considered as a marginal pathway. Concluding from the mechanistic results obtained so far, acrylamide formation as suggested by Zyzak et al. (*12*) and Yaylayan et al. (*11*) supports our hypothesis on the intermediacy of glycoconjugates as early Maillard intermediates as the critical step in acrylamide formation. The carbonyl compound may be a reducing sugar (*2*−*7*) or any other carbonyl compound, such as alkanals, glyoxal, and 2-deoxyglucose (*7*, *12*).

However, the type of carbonyl can significantly affect the yields of acrylamide, in particular the functional group in β-position to the nitrogen atom. The presence of a hydroxyl group favors the rearrangement **14** → **15** to the decarboxylated Amadori product to afford the vinylogous compound (**Figure 6**). On the basis of this assumption, hydroxyacetone (acetol) as a α-hydroxycarbonyl should generate higher amounts of acrylamide from asparagine than methylglyoxal. Indeed, the binary mixture of acetol and asparagine resulted in more than 4 mmol/mol acrylamide, compared to <0.2 mmol/mol acrylamide obtained with methylglyoxal (**Table 1**, samples N and M). These results show the crucial role of the β-position on both sides of the nitrogen atom in carbonyl−asparagine conjugates.

Overall, results obtained with binary mixtures composed of α-dicarbonyls and asparagine (**Table 1**, samples H−J, L, and M) clearly indicate that the pathway via the Strecker aldehyde is not decisive in acrylamide formation. In agreement with that, Becalski et al. (*7*) also found low acrylamide levels in dry systems containing diacetyl (0.2 mmol/mol). Mottram et al. (*3*) reported 0.5 mmol/mol; however, this concentration was almost 10 times lower compared to that of the glucose system.

According to **Figure 8**, the Schiff base **21** leads after decarboxylation to the azomethine ylide **22**. Assuming the higher tendency of the carbonyl group in β-position to the nitrogen atom to delocalize the negative charge, the intermediate **22** may preferably react to imine **23** (pathway IIIa), which upon hydrolysis furnishes the Strecker aldehyde **24**. Alternatively, intermediate **22** may react to imine **25** (pathway IIIb), which preferably hydrolyzes to the decarboxylated amino acid, that is, 3-aminopropionamide **26**. This compound has been reported to release acrylamide (*12*). A possible side reaction of imine **25**, however, may be the β-elimination reaction, also leading to acrylamide (pathway IV). Thus, the relatively low amounts of acrylamide reported in **Table 1** (samples H−M) might be explained by the preferred formation of imine **23** compared to imine **25**, due to the β-carbonyl group in the azomethine ylide **22**.

Several other minor pathways leading to the formation of acrylamide in foods have been proposed. For example, aspartic acid could afford acrylic acid by an analogous route, the latter reacting further by amino dehydroxylation in the presence of ammonium to generate acrylamide (*10*). Alternatively, the formation of acrylic acid from sugar degradation products has also been suggested (*27*). But the route via asparagine in the presence of reducing sugars or a suitable carbonyl source is most probably the major pathway in terms of yield of the corresponding vinylogous compound under low-moisture conditions and elevated temperatures. However, the acrylamide yields from asparagine will hardly exceed 1 mol %, even under favorable conditions.

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