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Oxidation of Isohumulones Induces the Formation of Carboxylic Acids by Hydrolytic Cleavage

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ABSTRACT: The degradation of isohumulones in mechanistic experiments was investigated. Incubation of *trans*-isohumulone in the presence of L-proline led to the formation of carboxylic acids and their corresponding proline amides. In the context of isohumulones unknown amides were verified first in model incubations and then in beer for the first time by comparison with authentic reference standards via LC-MS analyses. Carboxylic acids and amides were formed preferably under oxidative conditions and increasing pH. Stable isotope experiments excluded the incorporation of molecular oxygen into carboxylic acids, strongly indicating a hydrolytic mechanism via β-dicarbonyl cleavage. The proposed mechanism includes oxidation and thereby incorporation of molecular oxygen to the isohumulone ring structure followed by hydrolytic cleavage leading to acids and amides.

KEYWORDS: *hop bitter acids, isohumulones, carboxylic acids, carboxylic acid amides, β-dicarbonyl cleavage, amino acid modifications, beer*

INTRODUCTION

Beer is one of the most popular beverages worldwide due to its unique flavor. The particular flavor is the result of a tremendous number of volatile and nonvolatile substances. The nature and reactivity of these substances are important subjects to the scientific community. Both raw materials and chemical alterations are responsible for the final flavor composition. Although chemical reactions occurring in bottled beer generally are of no risk to human health, they are often related to the generation of negatively perceived flavors. Vanderhaegen et al. published a review on aging-related reactions in beer.[1] Especially hop-based flavor compounds, in particular bitter acids, are of great interest to researchers. During wort boiling the α-acids (humulones) are isomerized to iso-α-acids (isohumulones), the main bitter compounds in beer, resulting in a *trans*/*cis* mixture for each α-acid. The *trans*/*cis* ratio is typically around 0.4 due to the higher thermodynamic stability of the *cis* isomer and depends on various parameters, such as pH and temperature.[2] Upon beer aging the *trans* isomers are depleted significantly more quickly.[3,4] De Cooman et al. assumed a higher electron density leading to an increased susceptibility toward oxidative degradation.[3] In contrast, Intelmann et al. showed the formation of polycyclic compounds under nonoxidative conditions exclusively from *trans*-iso-α-acids and related them to a harsh, lingering bitter taste.[4,5] Furthermore, various oxidation products of the intact iso-α-acid structure were identified.[6,7] The formation of volatile, aroma active substances from iso-α-acids is of major concern. In the case of the so-called light-struck flavor, a photo-oxidative mechanism was presented.[8] Also, the hop-originated precursor 3-methyl-2-buten-1-ol is known to generate onion-like off-flavors.[9] Hashimoto and Eshima found evidence for the formation of staling aldehydes from the alkanoyl side chain by oxidative degradation of iso-α-acids.[10] However, the formation of these aldehydes by iso-α-acid degradation was not supported by mechanistic investigations and seems to play only a minor role in beer aging.[11]

The formation of carboxylic acids from iso-α-acids was reported under oxidative conditions, apparently also from the alkanoic and alkenoic side chain.[12−14] No mechanism for their formation is given in the literature. As a matter of fact, iso-α-acids can also be recognized as β-dicarbonyl structures and may show the same reactions as other β-dicarbonyls mentioned in the literature. In this context Davidek et al. showed that acetic acid is the main cleavage product from 1-deoxyglucosone formed by hydrolytic β-dicarbonyl cleavage in Maillard reaction systems.[15] Today, hydrolytic β-dicarbonyl cleavage is considered to be one of the major degradation pathways for carbohydrates.[16,17]

The scope of the present work was to elucidate the molecular mechanisms leading to flavor active carboxylic acids from degradation of iso-α-acids. The formation of these acids was studied under defined conditions. As a strong proof for hydrolytic β-dicarbonyl cleavage the corresponding carboxylic acid amides from the reaction with amines were found in such incubations.[18] This included the syntheses of authentic reference compounds to unambiguously verify these amides. Thus, the interaction of iso-α-acids and amines was established for the first time in a mechanistic experiment. In particular, *trans*-isohumulone, the most relevant *trans*-iso-α-acid in beer, was incubated in the presence of L-proline, which represents approximately 50% of the total free amino acids in beer.[19]

MATERIAL AND METHODS

Chemicals. Chemicals of the highest available grade were obtained from Sigma-Aldrich (Munich, Germany) unless otherwise indicated.

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Acetonitrile and methanol (HPLC gradient grade) were purchased from VWR (Darmstadt, Germany). Silica gel for column chromatography (0.06−0.2 mm) was obtained from Roth (Karlsruhe, Germany). Diethylenetriaminepentaacetic acid (DTPA) was obtained from Merck (Darmstadt, Germany). Methanol-d_4 (CD_3OD) and trichloromethane-d ($CDCl_3$) were purchased from Armar Chemicals (Döttingen, Switzerland). 4-Methyl-2-pentenoic acid and isovaleryl chloride were obtained from Alfa Aesar (Karlsruhe, Germany). The hop extract was kindly provided by the Hasseröder brewery (Wernigerode, Germany).

4-Methyl-3-pentenoic Acid. 4-Methyl-3-pentenoic acid was prepared according to the literature with some modifications.[20] One milliliter of 4-methyl-2-pentenoic acid (8.4 mmol) was dissolved in 20 mL of 6 M potassium hydroxide solution and heated to 105 °C for 48 h. After cooling to room temperature, the reaction mixture was acidified to pH 1 with 2 M hydrochloric acid and extracted three times with dichloromethane (30 mL). The combined organic layers were dried with sodium sulfate, and the solvent was removed under reduced pressure to afford a mixture of the target compound and the educt as a colorless liquid (90% 4-methyl-3-pentenoic acid, determined by gas chromatography as its trimethylsilyl derivative and NMR). ^{1}H NMR (400 MHz, $CDCl_3$), δ 1.65 (s, 3H), 1.79 (d, 3H, ^4J = 0.9 Hz), 3.08 (d, 2H, ^3J = 7.2 Hz), 5.30 (m, 1H); ^{13}C NMR (400 MHz, $CDCl_3$), δ 18.1, 25.8, 33.6, 115.2, 136.5, 178.3.

General Method for the Preparation of *N*-Acyl-L-proline Derivatives. The preparation was based on a method described in the literature with some modifications.[21] One equivalent of L-proline was suspended with 1.1 equiv of triethylamine in 10 mL of dry dichloromethane. The mixture was flushed with argon and cooled to 0 °C. Then 1 equiv of carboxylic acid chloride dissolved in 5 mL of dry dichloromethane was added dropwise. The reaction mixture was then stirred at room temperature for 15 h. After completion, the reaction mixture was washed two times with 15 mL of 1 M hydrochloric acid. The aqueous phase was re-extracted with 25 mL of dichloromethane. The combined organic layers were dried with sodium sulfate, and the solvent was removed under reduced pressure. The crude product was purified using flash chromatography (silica gel 60, *n*-hexane/acetone/trifluoroacetic acid 66:33:1). Fractions containing the desired product (TLC (silica gel 60, *n*-hexane/acetone/trifluoroacetic acid 66:33:1); detection, mixture of ninhydrine and *p*-toluenesulfonic acid (1%, 3%) in isopropanol) were collected and concentrated in vacuo.

***N*-Isovaleryl-L-proline (3-Methylbutyric Acid Proline Amide).** Six hundred and thirty-three milligrams of L-proline (5.5 mmol), 837 μL of triethylamine (6 mmol), and 671 μL of isobutyryl chloride (5.5 mmol) were used according to the general procedure. The product (TLC, R_f 0.31) was yielded as a yellowish oil (98%) from which the *trans* derivative was precipitated as colorless crystals at room temperature. ^{1}H NMR (500 MHz, $CDCl_3$, *trans* derivative), δ 0.97−0.99 (2d, 6H, ^3J = 6.4 Hz), 1.85−2.35 (m, 7H), 3.45−3.68 (m, 2H), 4.54−4.59 (m, 1H), 11.00 (s br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, *trans* derivative), δ 22.6. 22.7, 24.8, 25.7, 43.2, 48.0, 59.6, 173.9, 174.4; HR-MS, m/z 198.1135 (found), m/z 198.1135 (calcd for $C_{10}H_{17}NO_3$ $[M - H]^-$).

***N*-4-Methyl-3-pentenoyl-L-proline (4-Methyl-3-pentenoic Acid Proline Amide).** One milliliter of 4-methyl-3-pentenoic acid (7.6 mmol) was dissolved in 1 mL of thionyl chloride (13.8 mmol) and refluxed for 2 h. Excess thionyl chloride was removed under reduced pressure. The product, 967 mg of L-proline (8.4 mmol), and 1.25 mL of triethylamine (9 mmol) were used according to the general procedure. The product (TLC, R_f 0.31) was yielded as a brown oil (74%). ^{1}H NMR (500 MHz, $CDCl_3$, *trans* derivative), δ 1.63 (s, 3H), 1.73 (s, 3H), 1.88−2.17 (m, 2H), 2.18−2.35 (m, 2H), 3.10 (d, 2H, ^3J = 6.5 Hz), 3.38−3.68 (m, 2H), 4.48−4.57 (m, 1H), 5.23 (t, 1H, ^3J = 6.3 Hz), 10.30 (s br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, *trans* derivative), δ 18.2, 24.8, 25.7, 28.4, 34.5, 47.8, 59.7, 115.2, 136.3, 173.7, 174.2; HR-MS, m/z 210.1135 (found), m/z 210.1136 (calcd for $C_{11}H_{17}NO_3$ $[M - H]^-$).

Preparation of *n*-Humulone and *trans*-Isohumulone. A mixture of α-acids was obtained by multilayer coil countercurrent chromatography (MLCCC) using *n*-hexane, methanol, and water as tertiary system (10:8.2, v/v/v). The crude MLCCC product was further purified by preparative HPLC as described below (water/methanol (25:75, v/v)) to give pure humulone. To obtain *trans*-isohumulone, humulone was isomerized by using a modified literature protocol.[22] One gram of pure humulone was dissolved in 250 mL of methanol and flushed with argon before the isomerization was begun. The solution was radiated with a mercury-vapor lamp until the educt was completely vanished (monitored by HPLC). After removal of the solvent under reduced pressure, the crude product was purified by preparative HPLC as described below (water/methanol (50:50, v/v)) to yield *trans*-isohumulone with a purity of 98% (HPLC-DAD). Structure was confirmed by NMR experiments (^{1}H, ^{13}C; CD_3OD) in accordance with literature data.[23]

Preparation of *trans*-Humulinic Acid. Pure humulone was converted into *trans*- and *cis*-humulinic acid by using a modified literature protocol.[24] Humulone was stirred in 2 M aqueous sodium hydroxide at 95 °C for 2 h to give a mixture of *trans*- and *cis*-humulinic acid (89:11). After acidification with hydrochloric acid, the solution was extracted with diethyl ether and concentrated under reduced pressure. The individual isomers were isolated by preparative HPLC as described below (water/methanol (65:35, v/v)) to yield pure *trans*-humulinic acid. NMR spectra (^{1}H, ^{13}C; CD_3OD) were in line with the literature.[24,25]

Preparation of Hydroxy-*trans*-alloisohumulone. Pure *trans*-isohumulone was incubated in 0.1 M phosphate buffer at pH 7 after the addition of 10% vol ethanol for 1 week at 50 °C. After acidification with hydrochloric acid, the incubation was extracted with ethyl acetate, dried over sodium sulfate, and concentrated in vacuo. The crude extract was further processed by preparative HPLC (water/methanol (80:20, v/v)) to give hydroxy-*trans*-alloisohumulone with a purity of 95% (HPLC-DAD). NMR spectra (^{1}H, ^{13}C; CD_3OD) were in line with the literature.[6]

MLCCC. The MLCCC system (Ito, multilayer separator-extractor model, P. C. Inc., Potomac, MD, USA) was equipped with a constant-flow pump (Waters 510, Waters Corp., Milford, MA, USA) and a Jasco UV-2075 detector (Jasco, Gross-Umstadt, Germany) operating at 250 nm. Eluted liquids were collected in fractions of 16 mL with a fraction collector (LKB Ultrorac 7000). Chromatograms were recorded on a plotter (Servogor 200). The multilayer coil was prepared from a 1.6 mm inner diameter polytetrafluoroethylene (PTFE) tubing. The total capacity was 270 mL. The MLCCC was run at a revolution speed of 790 rpm and a flow rate of the mobile phase of 2 mL/min in head-to-tail modus. Separation was performed by using binary solvent systems (upper phase as stationary phase and lower phase as mobile phase). The sample was dissolved in a 1:1 mixture of upper and lower phase (10 mL) and injected into the system. The collected fractions were dried for further processing.

Preparative HPLC-UV. A Besta HD 2-200 pump (Wilhelmsfeld, Germany) was used at a flow rate of 20 mL/min. Elution of material was monitored by a UV detector (Jasco UV-2075). Detection wavelength for α-acids, iso-α-acids, and *trans*-humulinic acid was set at 270 and 250 nm for hydroxy-*trans*-alloisohumulone. Chromatographic separations were performed on a stainless steel column (KNAUER, Berlin, Germany; Eurospher-100-10 C18, 250 × 20 mm, 10 μm). All target substances were separated isocratically with mixtures of water and methanol containing 0.2% formic acid. The fractions containing target material were combined and dried under reduced pressure.

***trans*-Isohumulone Incubation Setup.** Individual iso-α-acids were incubated in 0.1 M ethanolic phosphate buffer (10% vol. ethanol) at two different pH values (5 and 7) with 25 mM L-proline for up to 21 days at 50 °C. The final iso-α-acids concentration was 5 mM. The incubations were performed under aerated and deaerated conditions. Deaerated conditions were achieved by adding diethylenetriaminepentaacetic acid (1 mM), degassing the buffer with helium, and flushing the residual headspace with argon. All samples were prepared in triplicate.

Incubation of *trans*-Isohumulone with $H_2{}^{18}O$. The buffer (300 μL) described above (aerated, pH 7) was lyophilized and redissolved with 270 μL of $H_2{}^{18}O$ (97 atom % ^{18}O). Thirty microliters of an ethanolic *trans*-isohumulone solution (50 mM) was added to meet the

conditions described above. Samples were incubated for 21 days at 50 °C.

Analytical HPLC-UV for Determination of Residual Iso-α-acid Levels. Analyses were performed on a Jasco HPLC system (PU-2089plus, AS-2055plus, UV-2075plus; Gross-Umstadt, Germany). Chromatographic separations were performed on a stainless steel column (KNAUER; Eurospher 100-5 C18, 250 × 4.0 mm, 5 μm) using a flow rate of 1 mL/min at 20 °C. The mobile phase used consisted of eluent A (water, 20 mg/L DTPA, 0.2% formic acid) and eluent B (acetonitrile/water (90:10, v/v), 0.2% formic acid). The incubation solutions were diluted with methanol and injected at 50% B. After 20 min, B was raised to 100% within 5 min (held for 5 min) and returned to starting conditions within 2 min (held for 8 min). The detection wavelength was set at 270 nm.

Extraction and Derivatization of Carboxylic Acids from Iso-α-acid Incubations. First, all of the samples (200 μL) were spiked with 100 μL of aqueous butyric acid solution (0.1 mM) as internal standard. After acidification with concentrated hydrochloric acid (25 μL), the precipitate was removed by centrifugation. An aliquot of the supernatant (200 μL) was saturated with sodium chloride and extracted with 300 μL of toluene. Two hundred microliters of the organic layer was dried with sodium sulfate. An aliquot was then derivatized with an equal volume of hexamethyldisilazane at 50 °C for 3 h prior to GC-MS analysis. External calibration samples were prepared in the same manner.

GC-LCI-MS Determination of Carboxylic Acid Trimethylsilyl Derivatives. One microliter of the samples described above was injected to a Thermo Finnigan Trace GC Ultra coupled to a Thermo Trace DSQ (Thermo Fisher Scientific, Bremen, Germany). Separation was carried out on a DB-5MS GC column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies, Palo Alto, CA, USA). Helium 5.0 was used as carrier gas in constant flow mode (linear velocity = 28 cm/s, flow = 1 mL/min). The split flow was set at 20 mL/min. Injector and transfer lines were heated to 220 and 250 °C, respectively. The oven program was as follows: 40 °C (1 min), 4 °C/min to 100 °C (0 min), 20 °C/min to 270 °C (10 min). LCI mass spectra were obtained at 70 eV (source, 190 °C; emission current, 50 μA) in SIM mode using methanol as reagent gas. The retention times and SIM parameters for the silylated acid derivatives were as follows: butyric acid at 7.7 min (m/z 161), isovaleric acid at 9.2 min (m/z 175), and 4-methyl-3-pentenoic acid at 14.3 min (m/z 187).

LC-MS/MS Analyses of Humulinic Acid and Hydroxy-*trans*-alloisohumulone. Analyses were performed on a Jasco HPLC system (AS2057plus and PU-2080plus). Chromatographic separations were performed on a stainless steel column (KNAUER; Eurospher 100-5 C18, 250 × 4.6 mm) using a flow rate of 1 mL/min at 25 °C. The mobile phase used consisted of eluent A (30 mM ammonium formate buffer, pH 5.5) and eluent B (methanol). For analyses of incubations, samples were diluted with methanol (1:1). The respective samples (20 μL) were injected to the LC system at 25% B. After 10 min, B was raised to 30% within 12 min and then to 70% within 3 min (held for 5 min) and returned to starting conditions within 1 min (held 5 min). The LC system was connected to the probe of the mass spectrometer. The mass analyses were performed using an Applied Biosystems API 4000 quadrupole instrument (Applied Biosystems, Foster City, CA, USA) equipped with an API source using an electrospray ionization (ESI) interface. Nitrogen was used as sheath and auxiliary gas. For electrospray ionization in negative mode the following specifications were used: sprayer capillary voltage, −4.5 kV; nebulizing gas flow, 50 mL/min; heating gas, 60 mL/min at 500 °C; and curtain gas, 40 mL/min. For multiple reaction monitoring (MRM), declustering potential (DP), collision energy (CE), and cell exit potential (CXP) were optimized by syringe injection of reference compounds. Quantitation was based on matrix-assisted calibration. The LC-MS/MS parameters are given in Table 1A.

LC-MS/MS Analyses of Amides. Analyses were performed on a Jasco HPLC system (AS2057plus and PU-2080plus). Chromatographic separations were performed on a stainless steel column (Vydac 218TP54, RP18, 5 μm 250 × 4.6 mm; Hesperia, CA, USA) using a flow rate of 1 mL/min at 25 °C. The mobile phase used consisted of eluent A (water, 1.2 mL/L heptafluorobutyric acid) and eluent B (methanol/water (70:30, v/v), 1.2 mL/L heptafluorobutyric acid). For analyses the samples were diluted with methanol (1:1) and injected to the LC system at 20% B. After 10 min, B was raised to 70% within 20 min and then to 100% within 2 min (held for 13 min) and returned to starting conditions within 5 min for equilibration (10 min). The LC system was connected to the probe of the mass spectrometer. The mass analyses were performed using an Applied Biosystems API 4000 quadrupole instrument equipped with an API source using an electrospray ionization (ESI) interface. Nitrogen was used as sheath and auxiliary gas. For electrospray ionization in positive mode the following specifications were used: sprayer capillary voltage, 4 kV; nebulizing gas flow, 70 mL/min; heating gas, 70 mL/min at 500 °C; and curtain gas, 50 mL/min. For MRM, declustering potential (DP), collision energy (CE), and cell exit potential (CXP) were optimized by syringe injection of reference compounds. Quantitation was based on matrix-assisted calibration. The LC-MS/MS parameters are given in Table 1B. Proline amides were extracted from Pilsener type beer after acidification to pH 2 with hydrochloric acid by means of solid phase extraction (Bakerbond C_{18} Polar Plus; Avantor, Deventer, The Netherlands). After elution with methanol, the extract was concentrated and redissolved in methanol/water (7:3, v/v). The crude extract was filtered and fractionated by analytical HPLC runs with the setup described above to isolate 3-methylbutyric acid proline amide. Fractions were collected by an automated fraction collector (1 min/fraction; CHF122SB, Advantec, Japan). Fractions containing the desired amide were dried and redissolved in a mixture of methanol and water (1:1) for LC-MS analysis. MS^2 spectra were generated by using the parameters described above (collision energy = 19 eV). The same experiment was performed with the authentic reference compound.

Table 1. Mass Spectrometer Parameters for Quantitation (MRM Mode)

	t_R (min)	mass (amu) Q1	mass (amu) Q3	DP (V)	CE (eV)	CXP (V)
		A				
trans-humulinic acid	21.2	265.1	154.9	−75	−30	−24
hydroxy-*trans*-alloisohumulone	23.5	377.1	195.2	−90	−41	−16
		B				
3-methylbutyric acid proline amide	22.0	200.2	116.1	50	20	6
4-methyl-3-pentenoic acid proline amide	24.7	212.1	116.2	56	25	10

Nuclear Magnetic Resonance Spectroscopy (NMR). NMR spectra were recorded on a Varian VXR 400 spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C or on a Varian Unity Inova 500 instrument operating at 500 MHz for ^1H and 125 MHz for ^{13}C, respectively. Chemical shifts are given relative to external $SiMe_4$.

Accurate Mass Determination (HR-MS). The high-resolution positive ion ESI mass spectra (HR-MS) were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with an Infinity cell, a 7.0 T superconducting magnet (Bruker, Karlsruhe, Germany), a radio frequency (RF)-only hexapole ion guide, and an external electrospray ion source (Apollo; Agilent, off-axis spray). Nitrogen was used as drying gas at 150 °C. The samples were dissolved in methanol, and the solutions were introduced continuously via a syringe pump at a flow rate of 120 μL/h. The data were acquired with 256K data points and zero filled to 1024K by averaging 32 scans.

RESULTS

Preparation of *N*-Acyl-L-proline Derivatives. For the analyses of the amino acid amides authentic reference material was necessary. In the case of proline the synthesis led to *cis*- and *trans*-configured amides due to the partial double character of

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the amide moiety. In this context the *trans* isomer is considered to be the thermodynamically more stable species, which was confirmed by the performed syntheses.[26,27] The proline-originated α-methine proton was used as a probe for the *cis*/*trans* ratio (1:5.7−6.5, Figure 1). The crystals precipitated from

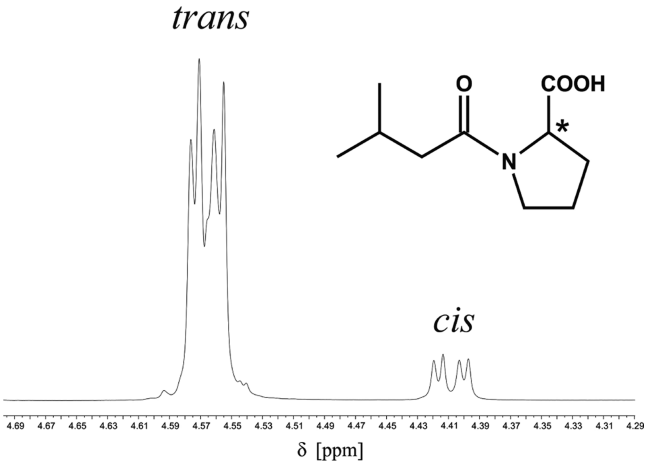

Figure 1. 3-Methylbutyric acid proline amide ^{1}H NMR spectra (excerpt) showing the α-methine proton of the *trans* (4.57 ppm) and *cis* (4.41 ppm) isomer.

3-methylbutyric acid proline amide were identified as the *trans* derivative by crystal structure analysis (data not shown). Interestingly, redissolution of *trans* crystals in deuterated chloroform, followed by NMR analysis, resulted in the reconstitution of the isomeric ratio stated above. Furthermore, the isomers could not be distinguished by LC-MS analyses.

Determination of Carboxylic Acids in Incubations. For quantitation the carboxylic acids had to be separated from the matrix. Residual *trans*-isohumulone led to artifacts during derivatization with hexamethyldisilazane and thus had to be removed by precipitation through acidification with hydrochloric acid. The carboxylic acids were extracted from the isohumulone-free supernatant (verified by HPLC-UV) with toluene. Butyric acid was used as internal standard because it cannot be formed from *trans*-isohumulone. The optimized workup provided a minimization of matrix effects and led to reproducible results.

Formation of *trans*-Isohumulone Degradation Products. *trans*-Isohumulone was incubated from 3 to 21 days at two different pH values under aerated and deaerated conditions. An overview of the substances analyzed in incubations is given in Figure 2.

Figure 3 (**1**) shows the degradation of *trans*-isohumulone. At acidic pH values it was almost completely vanished after 3 weeks (up to 97% degradation), although slightly more slowly at deaerated conditions (95%). On the contrary, relatively high amounts of unaltered *trans*-isohumulone were remained at pH 7, at which 72% was degraded in deaerated samples and 85% under oxidative conditions.

Along with the degradation of *trans*-isohumulone, the levels of 3-methylbutyric acid (MBA) increased over the incubation time in all incubation setups, preferably at neutral and aerated conditions with concentrations up to 86 mmol/mol *trans*-isohumulone (Figure 3, **2a**). The concentrations found in deaerated samples (35 mmol/mol) were approximately 3 times lower. At pH 5 the highest concentrations were also observed under oxidative conditions (34 mmol/mol), that is, about one-

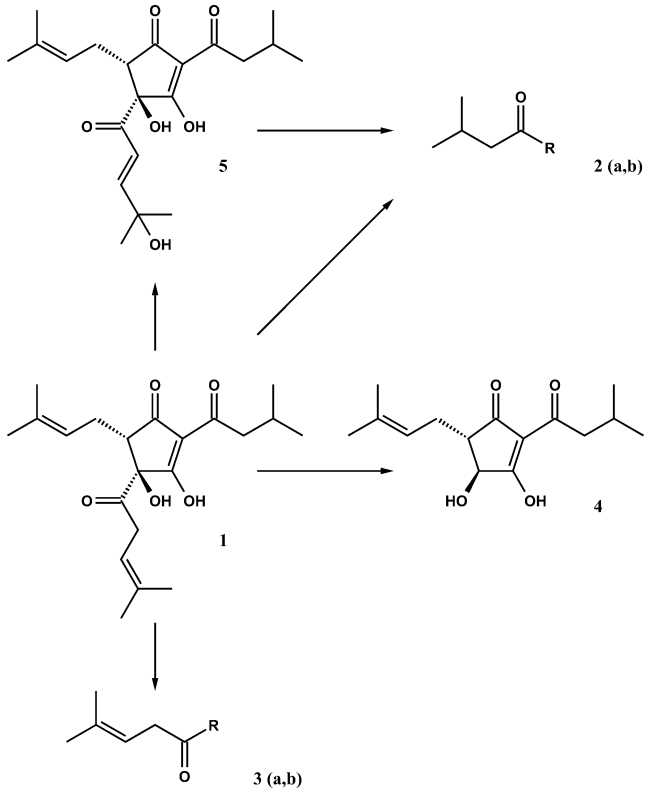

Figure 2. Overview of the structures analyzed in *trans*-isohumulone (**1**) incubations: 3-methylbutyric acid (**2a**, R = OH), 3-methylbutyric acid proline amide (**2b**, R = L-proline), 4-methyl-3-pentenoic acid (**3a**, R = OH), 4-methyl-3-pentenoic acid proline amide (**3b**, R = L-proline), *trans*-humulinic acid (**4**), hydroxy-*trans*-alloisohumulone (**5**).

third of the level determined at pH 7. A pH of 5 and deaeration led to concentrations of 24 mmol/mol after 3 weeks. The results for the formation of 4-methyl-3-pentenoic acid (MPA) (Figure 3, **3a**) revealed an almost identical pattern as seen for MBA. However, MPA levels were about 4−6 times lower compared to MBA concentrations. Again, the maximum was found at pH 7 and oxidative conditions (20 mmol/mol), whereas under deaeration half as much MPA was formed (10 mmol/mol). At pH 5 the levels were about 3 times lower after 3 weeks (4.1 mmol/mol for deaerated vs 6.6 mmol/mol for aerated samples). The carboxylic acids in the stable isotope experiment with $H_2{}^{18}O$ were analyzed analogously to the regular incubations. The incorporation of ^{18}O was monitored by analyzing additionally the [M + 2] molecular ion. For pH 7 and oxidative conditions about 90% of both carboxylic acids incorporated ^{18}O. As an example, the SIM chromatograms for the unlabeled (m/z 175) and labeled (m/z 177) MBA are given in Figure 4. Additional incubations were also performed without proline. However, there were no significant differences in the levels of carboxylic acids (data not shown).

On the basis of isolated authentic reference compounds, *trans*-humulinic acid and hydroxy-*trans*-alloisohumulone were also quantitated in the incubations. *trans*-Humulinic acid was preferentially formed at a pH of 7 under deaerated conditions (Figure 3, **4**; 11.6 mmol/mol after 3 weeks). Oxidative conditions led to levels up to 9.4 mmol/mol at the same pH. In contrast, in acidic incubations *trans*-humulinic levels were significantly higher at oxidative conditions (4.9 vs 3.0 mmol/mol in deaerated incubations). Hydroxy-*trans*-alloisohumulone had its maximum for pH 5 and oxidative conditions after 3 days

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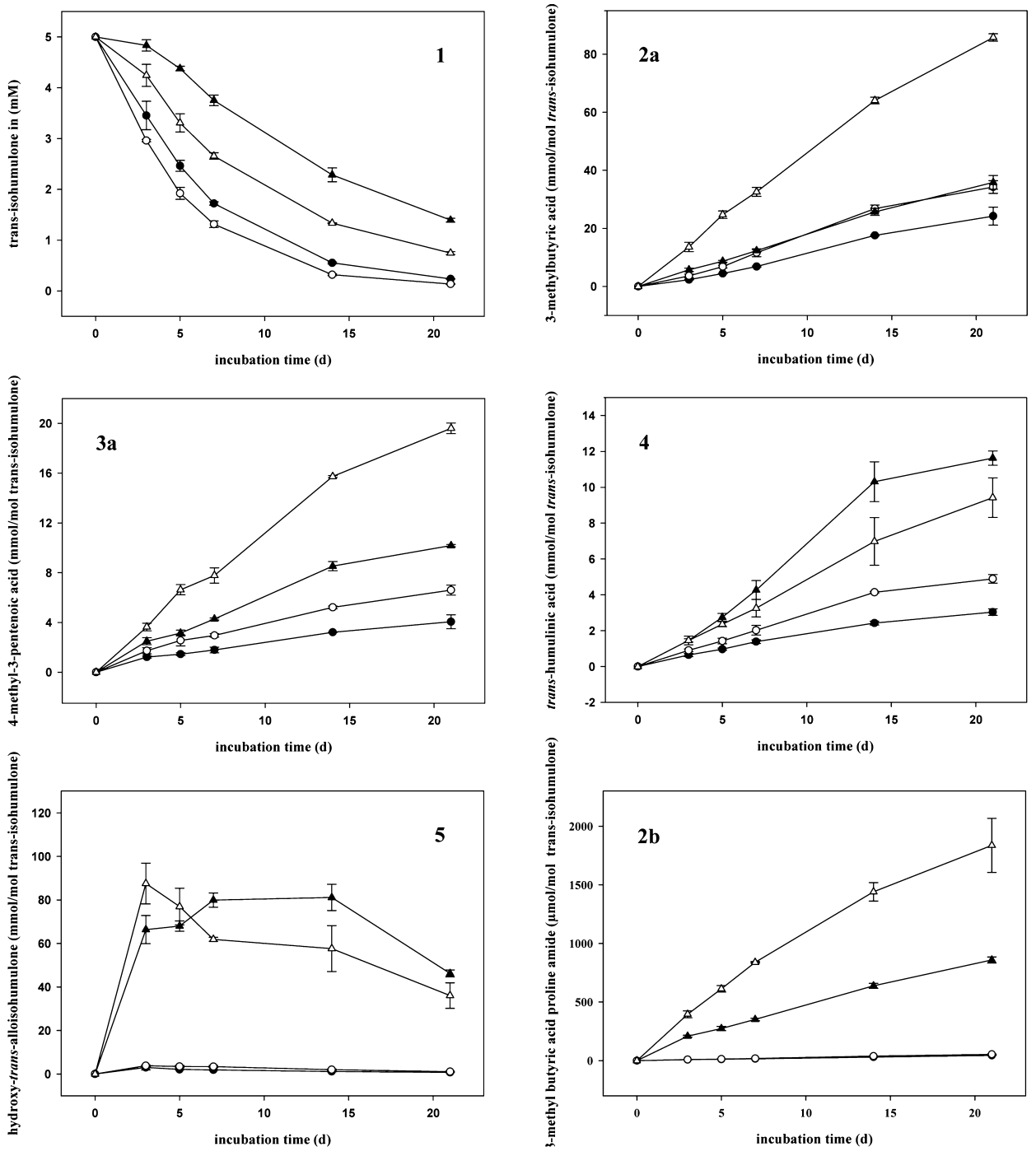

Figure 3. Degradation of *trans*-isohumulone (**1**) and formation of 3-methylbutyric acid (**2a**), 4-methyl-3-pentenoic acid (**3a**), *trans*-humulinic acid (**4**), hydroxy-*trans*-alloisohumulone (**5**), and 3-methylbutyric acid proline amide (**2b**) at pH 7 (aerated, △; deaerated, ▲) and pH 5 (aerated, ○; deaerated, ●).

(3.9 mmol/mol) and then started slowly to decrease to a final level of 1.1 mmol/mol (Figure 3, **5**). The curve for deaerated incubation was similar, although concentrations were slightly lower (maximum, 3.0 mmol/mol; 0.7 mmol/mol after 3 weeks). At pH 7 levels of hydroxy-*trans*-alloisohumulone were considerably higher. The maximum at aerated conditions was reached after 3 days at 87 mmol/mol. Thereafter, the level decreased to 36 mmol/mol within 3 weeks. At nonoxidative conditions the maximum was reached after 2 weeks at 80 mmol/mol and decreased to 46 mmol/mol afterward.

In parallel to the carboxylic acids, the analogous L-proline amides were found in the incubations. The results for 3-methylbutyric acid amide (MBA amide) are given in Figure 3 (**2b**). Whereas for pH 7 relatively high concentrations of MBA amide were found (up to 1800 µmol/mol), about 50 times lower compared to MBA itself, only minor amounts (up to 54 µmol/mol) were found at pH 5. The ratio of the MBA amide levels between aeration and deaeration at pH 7 were almost identical to the ratio found for the analogous acid (2.4 to 2.1). In contrast, 4-methyl-3-pentenoic acid proline amide (MPA amide) reached a plateau after 3 days regardless of the presence

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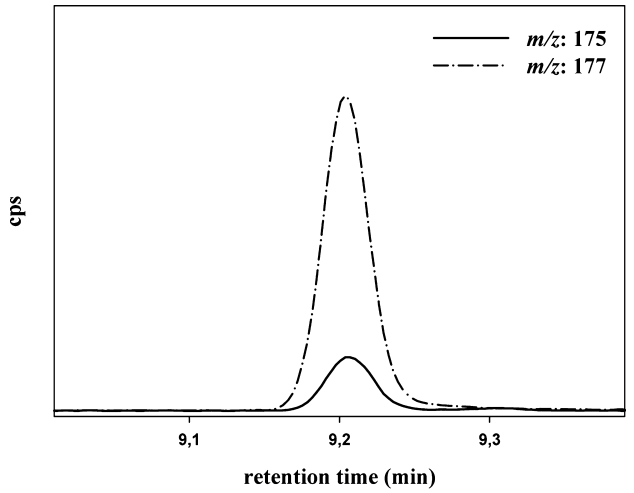

Figure 4. GC-LCI-MS chromatogram (SIM) of unlabeled (m/z 175; $[M + H]^+$) and labeled (m/z 177; $[M + H]^+$) 3-methylbutyric acid from *trans*-isohumulone incubation with $H_2{}^{18}O$. The ratio of 1:9 (unlabeled vs labeled) was calculated on the basis of the peak areas.

of oxygen and remained constant at levels around 170 μmol/mol at pH 7. An additional experiment showed that MPA amide was stable at these conditions. Analogous to MBA amide, MPA amide levels at pH 5 were extremely low (1.8 μmol/mol). MBA amide was also verified in beer by LC-MS/MS experiments (Figure 5). Although the spectrum of the isolate

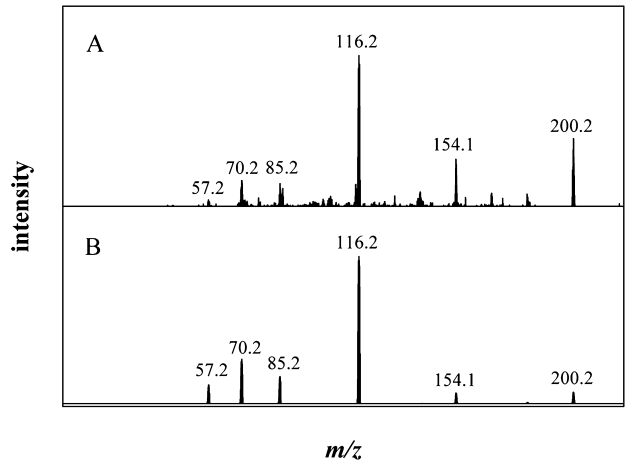

Figure 5. MS^2 of 3-methylbutyric acid proline amide (**2b**) isolated from beer (A) and synthesized reference standard (B) (product ion scan of m/z 200.2, collision energy of 19 eV).

(A) was much less intense, the fragmentation pattern was virtually identical to that of the authentic standard (B). For MPA amide, only traces were found in beer.

DISCUSSION

For the first time the degradation of *trans*-isohumulone in the presence of L-proline was thoroughly investigated in a model experiment. The clear impact of pH and oxidative conditions on the formation of carboxylic acids provided insights into the molecular mechanisms. In general, oxidative and hydrolytic reactions increased with higher pH. The question was whether the formation of carboxylic acids is based on hydrolytic or oxidative mechanisms or even a combination of both. Strong evidence for a hydrolytic cleavage was the formation of carboxylic acid amides. The nucleophilic L-proline nitrogen can react analogously to water or hydroxide, respectively. Obviously because of its weaker nucleophilicity compared to water, the amounts of the amides remain low. However, from a mechanistic point of view their formation is very important. As MBA amide was preferentially formed at oxidative conditions and the ratio between aerated and deaerated conditions was almost equal to that of the free acids, an identical mechanism must be assumed. To strengthen the postulated hydrolytic mechanism a stable isotope experiment with $H_2{}^{18}O$ was performed, revealing the incorporation of approximately 90% ^{18}O into the carboxylic acids, which supported the proposed hydrolytic cleavage. Incorporation of ^{18}O into free carboxylic acids was excluded by incubation of MBA and MPA with $H_2{}^{18}O$, resulting in no enrichment of the heavy isotope into the acids at these conditions. The remaining 10% must be explained by incorporation of molecular oxygen by yet unknown pathways, presumably including reactive oxygen species as intermediates. Thus, the difference in the formation of MBA under aeration versus deaeration is mainly not caused by the incorporation of molecular oxygen to the acid. Obviously there is an upstream oxidation step promoting the hydrolytic cleavage of *trans*-isohumulone.

trans-Isohumulone is a β-dicarbonyl structure and also a carboxylic acid with a pK_a of 3.23 and is almost completely deprotonated at pH 5 and entirely at pH 7.[28] Because of the β,β′-tricarbonyl system a negative charge after deprotonation is distributed over seven atoms. Thus, the anticipated carbonyl activity at the alkanoic side chain is limited in deprotonated isohumulones and does not support the preferred generation of carboxylic acid by hydrolytic β-dicarbonyl cleavage at neutral pH. Considering the given hydrolytic cleavage at oxidative conditions, we therefore postulate the mechanism shown in Figure 6.

After deprotonation, the isohumulate ion is oxidized via a one-electron oxidation, according to Huavere et al., giving a carbon-centered *trans*-isohumulone radical.[29] Reaction with molecular oxygen leads to the formation of the corresponding hydroperoxy radical. After abstraction of hydrogen, the hydroperoxide undergoes a Fenton-like redox reaction to form the alkoxy radical and subsequently the alcohol after a second abstraction of hydrogen. The now oxidized isohumulone has lost its ability to enolize, which comes along with a significantly increased carbonyl activity. Nucleophilic attack of water or L-proline at the carbonyl function at the alkanoic or alkenoic side chain then gives the free carboxylic acid or the carboxylic acid amide based on β-dicarbonyl cleavage, respectively. The difference between the concentrations of MBA and MPA is likely caused by lower reactivity of the alkenoic side chain compared to the β,β′-tricarbonyl system, a potential steric hindrance due to the prenyl side chain at the C5 of the isohumulone and the coinciding oxidation to hydroxy-*trans*-alloisohumulone. Hydrolytic cleavage of the latter would give 4-hydroxy-4-methyl-2-pentenoic acid.

The identification of the remaining cleavage products with intact ring structure would have been unambiguous proof for the present mechanism. Unfortunately, it was not possible to isolate these compounds. In contrast to the carboxylic acids and their amides, which represent stable reaction endproducts, the remaining isohumulone structures must be evaluated as highly reactive and thus can be further degraded. For instance, the cleavage of MBA gives a reductone structure, which is highly

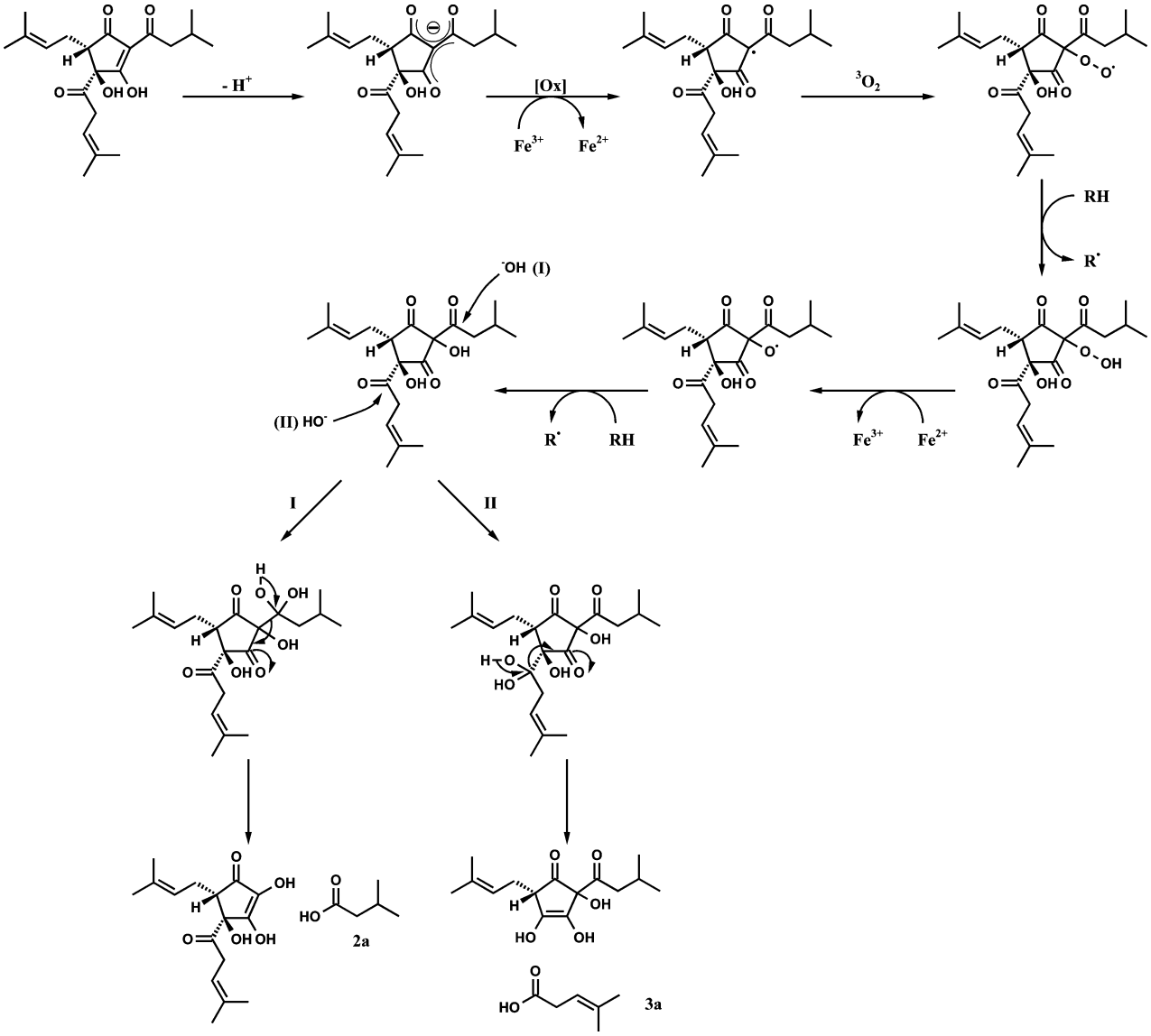

Figure 6. Proposed mechanism leading to the formation of 3-methylbutyric acid (**2a**) and 4-methyl-3-pentenoic acid (**3a**) from *trans*-isohumulone (**1**) by hydrolytic β-dicarbonyl cleavage after oxidation of the isohumulone ring structure.

susceptible to oxidation. This would also entail a loss or change of chromophoric properties to withdraw the products from the given detection systems. Therefore, in the present work, only *trans*-humulinic acid and hydroxy-*trans*-alloisohumulone were monitored as degradation products of *trans*-isohumulone.

Humulinic acids are known to form under harsh alkaline treatment. Here, we were able to detect *trans*-humulinic acid under mild conditions, preferably at pH 7. This per se nonoxidized structure must be formed by direct hydrolysis of the alkenoic side chain, which is not part of the tricarbonyl system and maintains its carbonyl activity. *trans*-Humulinic acid appeared to be stable under oxidative conditions and was not degraded significantly in an independent incubation (data not shown). Interestingly, higher amounts of *trans*-humulinic acid were generated at pH 7 under deaeration than in aerated incubations. This is reasonable considering the oxidation of *trans*-isohumulone according to above proposed mechanism leading to MBA and MPA. However, at pH 5 the picture is reversed and may be the result of a suppression of the initial oxidative step. Nevertheless, from our experiments the hydrolytic cleavage is evident at least for the alkenoic chain without previous oxidation. This is further supported by the fact that the levels of *trans*-humulinic acid and MPA were almost equal at pH 5, whereas MPA levels were significantly higher than *trans*-humulinic concentrations at pH 7 on the contrary. Obviously the oxidation of *trans*-isohumulone decreases the yield of *trans*-humulinic acid.

As a major degradation product under oxidative conditions at pH 7, 4-hydroxy-*trans*-alloisohumulone was isolated and analyzed in incubations. Together with its precursor, 4-hydroperoxy-*trans*-alloisohumulone, it has already been described by Intelmann et al.[6] The structure was relatively unstable and degraded after reaching a maximum more quickly under aerated conditions. In support, independent incubations of hydroxy-*trans*-alloisohumulone indeed revealed the formation of significant amounts of MBA, most likely according to our proposed mechanism (data not shown). We therefore must assume that MBA is not generated by only one precursor structure. As an alternative mechanism for the degradation of

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hydroxy-*trans*-alloisohumulone, Intelmann et al. assumed the formation of polycyclic compounds.[4]

In conclusion, we present a novel degradation mechanism that comprehensively explains the so far unknown formation of carboxylic acids from isohumulones. Stable isotope experiments with $H_2{}^{18}O$ as well as the generation of carboxylic acid amides unambiguously revealed a dominant hydrolytic mechanism. We showed that this reaction is relevant to brewing by the verification of MBA amide in beer. The lacking fragmentation counterparts and other reactive intermediates are subjects of current research. The actual influence of carboxylic acids derived from hops on the staling flavor of beer still has to be clarified. Nevertheless, our results are in good agreement with Williams and Wagner, who found increased levels of fatty acids during beer aging caused by the addition of hops.[14] Alternatively, MBA can also be formed by Strecker degradation of leucine, and molecular mechanisms may not be distinguishable in the aging of beer. It is very likely that the hydrolytic cleavage of isohumulones is also occurring during wort boiling after the addition of hops. High temperatures and exposure to oxygen would support the hydrolytic cleavage of isohumulones based on the proposed mechanism. Consequently, the formation of carboxylic acids from isohumulones at this stage would also promote the so-called staling esters during fermentation or beer storage. As we proved for L-proline, other nucleophiles besides water are able to react with isohumulones. Thus, also sulfur-containing substances may lead to potent odor-active compounds, such as *S*-methylthioisovalerate.[30]

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Notes

The authors declare no competing financial interest.

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