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ARTICLE

Identification and RP-HPLC-ESI-MS/MS Quantitation of Bitter-Tasting β-Acid Transformation Products in Beer

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Thermal treatment of the hop β-acid colupulone under wort boiling conditions, followed by LC-TOF-MS and 1D/2D NMR spectroscopy, revealed cohulupone, hulupinic acid, nortricyclocolupone, two tricyclocolupone epimers, two dehydrotricyclocolupone epimers, two hydroxytricyclocolupone epimers, and two hydroperoxytricyclocolupone epimers as the major bitter-tasting β-acid transformation products. Among these compounds, the chemical structures of the hydroxy- as well as the hydroperoxytricyclocolupone epimers have not previously been confirmed by 1D/2D NMR experiments. Depending on their chemical structure, these compounds showed rather low recognition thresholds ranging from 7.9 to 90.3 μmol/L. The lowest thresholds of 7.9 and 14.7 μmol/L were found for cohulupone, imparting a short-lasting, iso-α-acid-like bitter impression, and for hydroxytricyclocolupone, exhibiting a long-lasting, lingering, and harsh bitterness perceived on the posterior tongue and throat. Furthermore, HPLC-ESI-MS/MS analysis allowed for the first time a simultaneous detection and quantitation of these bitter-tasting β-acid transformation products in a range of commercial beer samples without any sample cleanup. Depending on the type of beer, these studies revealed remarkable differences in the concentrations of the individual β-acid transformation products.

KEYWORDS: Beer; hop; β-acids; bitter taste; ECHO technique; colupulone; cohulupone; hulupinic acid; tricyclocolupone; hydroxytricyclocolupone; hydroperoxytricyclocolupone

INTRODUCTION

For several centuries cones, pellets, or extracts of hop (*Humulus lupulus* L.) have been used as important ingredients in beer manufacturing to impart the typical bitter taste as well as the attractive aroma to the final beverage. On the basis of their chemical structures, the bitter compounds in hop are divided into two groups of prenylated polyketides, namely, α-acids and β-acids, as well as prenylated chalcones, such as xanthohumol (*1*, *2*). During the wort boiling process, xanthohumol and α-acids are well-known to be converted into isoxanthohumol and the corresponding *cis*/*trans*-iso-α-acids, respectively (*3*, *4*), and the latter have been identified as the main contributors to the bitter taste of beer (*5*, *6*). In comparison, information available on bitter compounds generated during wort boiling from the parent β-acids colupulone (**1a**), lupulone (**1b**), and adlupulone (**1c**) is rather scarce (**Figure 1**).

Previous studies have revealed that the so-called hulupones, which occur as the congeners cohulupone (**2a**), hulupone (**2b**), and adhulupone (**2c**), as well as hulupinic acid (**3**) are formed upon β-acid degradation (**Figure 1**). Among these compounds, the hulupones have been found in aged hops as well as in beer at levels of 2−10 ppm and are believed to contribute to the bitter taste of beer (*7*−*9*). Although hulupinic acid (**3**), proposed to be generated upon degradation of the hulupones, was identified in beer, this compound does not seem to exhibit any distinctive bitterness (*9*, *10*).

Previously, in order to screen for bitter compounds generated upon degradation of β-acids, purified colupulone (**1a**) was thermally treated under wort boiling conditions and then investigated for bitter-tasting degradation products by application of the taste dilution analysis (*11*). Besides cohulupone (**2a**), five previously not reported bitter-tasting colupulone degradation products were isolated and their structures determined as nortricyclocolupone (**4a**), two dehydrotricyclocolupone epimers, **5a** and **5′a**, and two tricyclocolupone epimers, **6a** and **6′a**, respectively, by means of LC-TOF-MS and 1D/2D NMR spectroscopy. Sensory studies revealed a lingering and harsh bitter taste of the compounds **4a**−**6a**/**6′a** with low recognition thresholds between 37.9 and 90.3 μmol/L, but the occurrence of these bitter compounds in beer was not yet confirmed. Furthermore, two additional degradation products were found to be formed upon cohulupone degradation, but their low yields as well as their instability did not allow the successful determination of their chemical structures (*11*).

The objectives of the present investigation were, therefore, to isolate these unknown colupulone degradation products in model wort boiling experiments in suitable amounts to enable their chemical structure determination and to quantify these molecules as well as the previously identified compounds **4a**−**6a**/**6′a** in authentic beer samples by means of HPLC-MS/MS using

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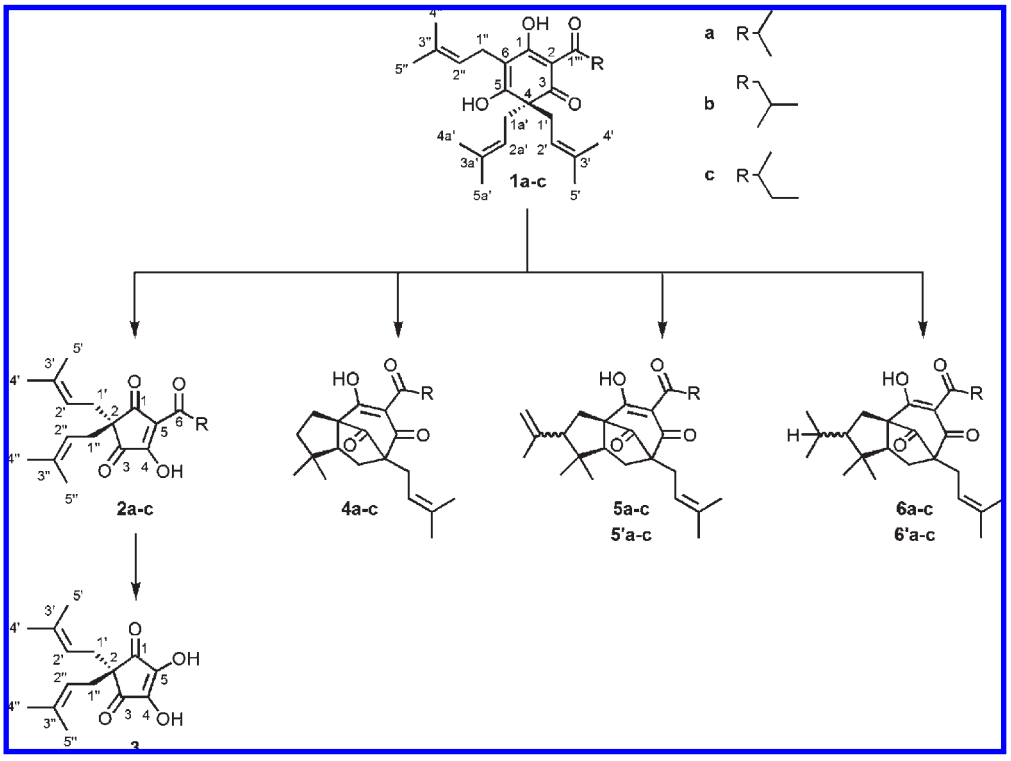

Figure 1. Structures of the β-acids colupulone (**1a**), lupulone (**1b**), and adlupulone (**1c**) and their transformation products cohulupone (**2a**), hulupone (**2b**), adhulupone (**2c**), hulupinic acid (**3**), nortricyclocolupone (**4a**), nortricyclolupone (**4b**), nortricycloadlupone (**4c**), and the epimers of dehydrotricyclocolupone (**5a**, **5′a**), dehydrotricyclolupone (**5b**, **5′b**), dehydrotricycloadlupone (**5c**, **5′c**), tricyclocolupone (**6a**, **6′a**), tricyclolupone (**6b**, **6′b**), and tricycloadlupone (**6c**, **6′c**), respectively.

the so-called ECHO technique to compensate for the effect of coextracted matrix components during LC-MS/MS analysis (*12*−*15*).

MATERIALS AND METHODS

Chemicals and Materials. The following chemicals were obtained commercially: formic acid, magnesium chloride, hexane, platinum/charcoal (10% Pt) (Merck, Darmstadt, Germany); hydrochloric acid, sodium hydroxide (Riedel-de-Haen, Seelze, Germany); hulupinic acid (Phytolab, Vestenbergsgreuth, Germany). Solvents were of HPLC grade (Merck); deuterated solvents were from Euriso-top (Saarbrücken, Germany). Deionized water used for chromatography was purified by means of a Milli-Q Gradient A10 system (Millipore, Billerica, MA). An ethanolic hop extract (Hopsteiner ethanolic extract, Hallertauer Taurus) was obtained from the German hop industry. Twelve fresh commercial beer samples (<4 weeks after manufacturing date) were obtained from the German brewing industry, five samples of dark beer (I−V), a country lager (VI), two Pilsner-type beers (VII, VIII), two bitter beers (X, XI), and one wheat beer sample (XII). Beer samples X and XI exhibited 100 bitter units according to a method of the European Brewing Convention (*16*), whereas the bitter units of the other beers ranged from 22 to 34. A fresh sample of hop-free beer, coined "zero beer", was provided by the German brewing industry. For sensory analysis, bottled water (Evian, low mineralization = 405 mg/L) was adjusted to pH 4.4 with trace amounts of formic acid prior to use. Reference samples of nortricyclocolupone (**4a**) and the dehydrotricyclocolupone epimers **5a** and **5′a**, as well as the tricyclocolupone epimers **6a** and **6′a**, were prepared and purified closely following the protocol reported recently (*11*).

Isolation of Pure β-Acids (1a−1c). An aliquot (2.0 g) of the ethanolic hop extract was dissolved in methanol (5 mL) and, after filtration, was placed onto the top of a glass column filled with a slurry of LiChroprep, 25−40 μm, RP-18 material (Merck) in acetonitrile/water (20:80, v/v). Chromatography was performed by flushing the column with acetonitrile/water mixtures (150 mL each) to give fraction I (20:80, v/v; yield = 5.9%), fraction II (30:70, v/v; yield = 0.6%), fraction III (40:60, v/v; yield = 0.9%), fraction IV (50:50, v/v; yield = 0.9%), fraction V (60:40, v/v; yield = 9.6%), fraction VI (70:30, v/v; yield = 41.4%), fraction VII (80:20, v/v; yield = 16.1%), fraction VIII (90:10, v/v; yield = 11.0%), and fraction IX (100:0, v/v; yield = 11.5%), respectively. Fraction VIII containing the β-acids **1a**−**1c** was collected in ice-cooled brown glass vials and separated from solvent under vacuum, and the residue was suspended in water, freeze-dried twice, and kept at −18 °C until use. An aliquot (0.5 g) of the lyophilized fraction VIII was dissolved in methanol (5 mL) and, after filtration, separated by semipreparative RP-HPLC using a 250 × 10 mm i.d., 5 μm, ODS-Hypersil C18 column (ThermoHypersil, Kleinostheim, Germany). Monitoring the effluent flow at 272 nm, chromatography was performed starting with a mixture (40:60, v/v) of aqueous formic acid (0.1% in water; solvent A) and acetonitrile (solvent B), thereafter increasing solvent B to 85% within 5 min, holding for 10 min, then increasing solvent B within 5 min to 100%, and, finally, keeping solvent B at 100% for an additional 2 min. The effluents of the three major peaks were collected individually, separated from solvent under vacuum, and freeze-dried twice. By means of UV−vis, LC-MS/MS, and 1D/2D NMR experiments, colupulone (**1a**) was unequivocally confirmed in the first eluting fraction, followed by lupulone (**1b**) in the second eluting fraction and adlupulone (**1c**) in the last fraction.

Colupulone (**1a**, **Figure 1**): UV−vis (1% formic acid in water/acetonitrile; 20:80, v/v) λ_{max} = 275 and 332 nm; LC-TOF-MS, *m*/*z* 399.2547 ([M − H]$^-$, measured), *m*/*z* 399.2547 (([M − H]$^-$, calculated for [$C_{25}H_{36}O_4$−H]$^-$); LC-MS (ESI$^-$), *m*/*z* (%) 399 (100) [M − H]$^-$; MS/MS (−30 V), *m*/*z* (%) 287 (100), 399 (75), 330 (30); ^{1}H NMR (400 MHz CD_3OD, COSY) δ 1.09 [d, 6H, *J* = 7.3 Hz, H−C(3‴,4‴)], 1.55 [s, 6H, H−C(5′,5a′)], 1.57 [s, 6H, H−C(4′,4a′)], 1.67 [s, 3H, H−C(4″)], 1.73 [s, 3H, H−C(5″)], 2.59 [m, 4H, H−C(1′,1a′)], 3.10 [d, 2H, *J* = 6.8 Hz, H−C(1″)], 3.98 [m, 1H, *J* = 7.3 Hz, H−C(2‴)], 4.77 [dd, 2H, *J* = 7.6, 7.6 Hz, H−C(2′,2a′)], 5.02 [dd, 1H, *J* = 6.7; 6.7 Hz, H−C(2″)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) δ 16.5 [C(4′,4a′,5″)], 17.8 [C(3‴,4‴)], 20.2 [C(1″)], 24.2 [C(4″,5′,5a′)], 36.0 [C(2‴)], 37.3 [C(1′,1a′)], 57.6 [C(6)], 110.9 [C(2)], 117.7 [C(2′,2a′)], 121.5 [C(2″)], 131.0 [C(3″)], 134.3 [C(3′,3a′)], 172.8 [C(1)], 189.3 [C(3)], 196.8 [C(5)], 207.0 [C(1‴)].

Lupulone (**1b**, **Figure 1**): UV−vis (1% formic acid in water/acetonitrile; 20:80, v/v) λ_{max} = 274 and 331 nm; LC-TOF-MS, *m*/*z* 413.2707 ([M − H]$^-$, measured), *m*/*z* 413.2697 ([M − H]$^-$, calculated for [$C_{26}H_{38}O_4$−H]$^-$); LC-MS

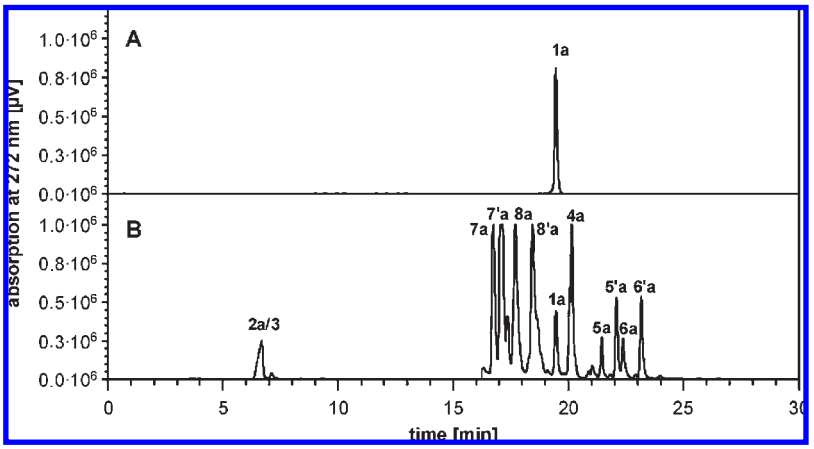

Figure 2. RP-HPLC chromatogram (λ = 272 nm) of purified colupulone (**1a**) before (**A**) and after wort boiling (**B**).

(ESI$^-$), *m/z* (%) 413 (100) [M − H]$^-$; MS/MS (−30 V), *m/z* (%) 413 (100), 301 (44), 233 (11); ^{1}H NMR (400 MHz CD_3OD, COSY) δ 0.93 [d, 6H, J = 6.7 Hz, H−C(4‴,5‴)], 1.52 [s, 6H, H−C(5′,5a′)], 1.55 [s, 6H, H−C(4′,4a′)], 1.65 [s, 3H, H−C(4″)], 1.71 [s, 3H, H−C(5″)], 2.07 [m, 1H, H−C(3‴)], 2.34 [m, 4H, H−C(1′,1a′)], 2.82 [m, 2H, H−C(2‴)], 3.07 [d, 2H, J = 6.8, H−C(1‴)], 4.74 [dd, 2H, J = 7.6; 7.6 Hz, H−C(2′,2a′)], 5.00 [dd, 1H, J = 6.8, 6.8 Hz, H−C(2″)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) δ 16.5 [C(4′,4a′,5″)], 20.2 [C(1″)], 21.6 [C(4‴,5‴)], 24.5 [C(4″,5′,5a′)], 32.6 [C(3‴)], 37.2 [C(1′,1a′)], 48.6 [C(2‴)], 57.2 [C(6)], 111.0 [C(2)], 117.5 [C(2′,2a′)], 121.4 [C(2″)], 131.3 [C(3″)], 134.4 [C(3′,3a′)], 172.8 [C(1)], 189.4 [C(3)], 197.6 [C(5)], 208.9 [C(1‴)].

Adlupulone (**1c**, **Figure 1**): UV−vis (1% formic acid in water/acetonitrile; 20:80, v/v) λ_{max} = 280 and 332 nm; LC-TOF-MS, *m/z* 413.2715 ([M − H]$^-$, measured), *m/z* 413.2697 ([M − H]$^-$, calculated for $[C_{26}H_{38}O_4-H]^-$); LC-MS (ESI$^-$), *m/z* (%) 413 (100) [M − H]$^-$; MS/MS (−30 V), *m/z* (%) 413 (100), 301 (44), 233 (11); ^{1}H NMR (400 MHz CD_3OD, COSY) δ 0.89 [dd, 3H, J = 7.4, 14.8 Hz, H−C(5‴)], 1.07 [d, 3H, J = 6.8 Hz H−C(3‴)], 1.29 [m, 2H, H−C(4‴)], 1.54 [s, 6H, H−C(4′,4α′)], 1.57 [s, 6H, H−C(5′,5α′)], 1.67 [s, 3H, H−C(5″)], 1.73 [s, 3H, H−C(4″)], 2.59 [d, 4H, J = 7.9 Hz, H−C(1′,1α′)], 3.09 [m, 2H, H−C(1″)], 3.89 [m, 1H, H−C(2‴)], 4.76 [m, 2H, H−C(2′,2α′)], 5.02 [m, 1H, H−C(2″)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) δ 12.4 [C(5‴)], 16.9 [C(3‴)], 18.0 [C(4″,4′,4α′), 21.7 [C(1″)], 26.1 [C(5′,5α′,5″)], 30.8 [C(4‴)], 38.9 [C(1′,1α′)], 43.9 [C(2‴)], 81.7 [C(6)], 112.0 [C(2)], 119.4 [C(2′,2α′)], 123.3 [C(2″)], 132.6 [C(3″)], 136.0 [C(3′,3α′)], 174.8 [C(1)], 191.5 [C(3)], 198.4 [C(5)], 208.6 [C(1‴)].

Synthesis of Cohulupone (2a). A portion (1.0 g) of colupulone (**1a**) was dissolved in hexane (40 mL), extracted twice with aqueous NaOH (0.5 mol/L; 20 mL), and, after the addition of platinum/charcoal (10% Pt; 0.15 g) to the combined aqueous layer, the reaction mixture was heated for 1 h at 80 °C while oxygen was bubbled through the solution. After cooling to room temperature, the catalyst was removed by filtration and, after acidification with aqueous formic acid (0.1 mol/L) to pH 3.0, the solution was extracted with hexane (3 × 30 mL). The combined organic layer was freed from solvent, the residue was dissolved in methanol, and cohulupone (**2a**; yield = 30%) was isolated by means of semipreparative RP-HPLC in a purity of > 98% .

Cohulupone (**2a**, **Figure 1**): UV−vis (1% formic acid in water/acetonitrile; 20:80, v/v) λ_{max} = 255 and 327 nm; LC-TOF-MS, *m/z* 317.1761 ([M − H]$^-$, measured); *m/z* 317.1758 ([M − H]$^-$, calculated for $[C_{19}H_{26}O_4-H]^-$; LC-MS (ESI$^-$), *m/z* (%) 317 (100) [M − H]$^-$; MS/MS (−54 V), *m/z* (%) 248 (100), 180 (75), 317 (49); ^{1}H NMR (400 MHz CD_3OD, COSY) δ 1.09 [d, 6H, J = 6.9 Hz, H−C(8,9)], 1.56 [s, 6H, H−C(5′,5″)], 1.57 [s, 6H, H C(4′,4″)], 2.40 [d, 4H, J = 7.8 Hz, H−C(1′,1″)], 3.48 [m, H, H−C(7)], 4.84 [m, 2H, H−C(2′,2″)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) δ 16.1 [C(5′,5″)], 16.5 [C(8,9)], 24.7 [C(4′,4″)], 32.5 [C(1′,1″)], 38.9 [C(7)], 55.4 [C(2)], 117.2 [C(2′,2″)], 135.6 [C(3′,3″)], 200.1 [C(3)], 202.9 [C(6)], 205.1 [C(1)].

Preparative Model Wort Boiling of Colupulone (1a) and Identification of Reaction Products. A portion (720 mg) of pure colupulone (**1a**) was dissolved in methanol (65 mL), mixed with an aqueous solution of $MgCl_2$ (1.55 mol $MgCl_2$/L; 65 mL), and, after the pH value had been adjusted to 5.8 with aqueous NaOH solution (0.1 mol/L in water), thermally treated in a closed flask (250 mL) in a laboratory oven for 1 h at 110 °C. After cooling to room temperature, the solvent was removed under vacuum, and the residue was taken up in methanol (5 mL) and then separated by preparative HPLC using a 250 × 10 mm i.d., 5 μm, ODS-Hypersil C18 column (ThermoHypersil, Kleinostheim, Germany). With the effluent monitored at 272 nm, chromatography was performed by starting with a mixture (50:50, v/v) of aqueous formic acid (1% in water, solvent A) and acetonitrile (solvent B), then increasing the content of solvent B to 100% within 25 min, and, finally, maintaining solvent B at 100% for additional 5 min. The effluents of the individual peaks detected in the chromatogram (**Figure 2**) were collected, separated from solvent under vacuum, and then freeze-dried twice. Comparison of the spectroscopic data (UV−vis, LC-MS/MS, 1D/2D NMR) with those obtained for reference compounds led to the identification of cohulupone (**2a**) and hulupinic acid (**3**) in the HPLC fraction eluting between 6 and 7 min. In addition, the five major peaks eluting between 20 and 24 min were unequivocally identified as the previously identified nortricyclocolupone (**4a**) and the dehydrotricyclocolupone epimers **5a** and **5′a**, as well as the tricyclocolupone epimers **6a** and **6′a** (*11*). The four compounds eluting between 16 and 19 min were isolated in a purity of > 98%, and their structures were determined as the hydroxytricyclocolupone epimers **7a** and **7′a** as well as the hydroperoxytricyclocolupone epimers **8a** and **8′a** by means of UV−vis, LC-MS/MS, LC-TOF-MS, and 1D/2D NMR experiments, respectively.

Hydroxytricyclocolupone epimer A (**7a**, **Figure 3**): UV−vis (0.1% aqueous formic acid/acetonitrile; 20:80, v/v) λ_{max} = 283 nm; LC-TOF-MS, *m/z* 415.2480 ([M − H]$^-$, measured), *m/z* 415.2489 ([M − H]$^-$, calculated for $[C_{25}H_{36}O_5-H]^-$); LC-MS (ESI$^-$), *m/z* (%) 415 (100) [M − H]$^-$; MS/MS (−50 V), *m/z* (%) 277 (100), 111 (80), 287 (40), 415 (35); ^{1}H NMR (500 MHz, CD_3OD, COSY) δ 0.76 [s, 3H, H−C(15)], 1.06 [s, 3H, H−C(16)], 1.15 [d, 3H, J = 6.8 Hz, H−C(19)], 1.15 [d, 3H, J = 6.8 Hz, H−C(20)], 1.29 [s, 3H, H−C(1)], 1.32 [s, 3H, H−C(2)], 1.69 [s, 3H, H−C(15′)], 1.71 [s, 3H, H−C(16′)], 1.85 [m, 1H, H−C(13)], 2.09 [m, 1H, H−C(5α)], 2.20 [m, 1H, H−C(4)], 2.28 [m, 1H, H−C(12α)], 2.43 [m, 1H, H−C(12′α)], 2.51 [m, 1H, H−C(12β)], 2.57 [dd, 1H, J = 7.5, 13.5 Hz H−C(5β)], 2.68 [dd,1H, J = 9.0; 14.3 Hz H−C(12′β)], 4.04 [m, 1H, J = 6.8 Hz, H−C(18)], 5.21 [m, 1H, H−C(13′)]; ^{13}C NMR (125 MHz, CD_3OD, HMQC, HMBC) δ 17.6 [C(15)], 18.0 [C(15′)], 19.2 [C(19)], 19.2 [C(20)], 23.7 [C(5)], 26.1 [C(16′)], 26.2 [C12′]), 29.4 [C(16)], 30.2 [C(2)], 31.0 [C(1)], 35.0 [C(12)], 35.9 [C(18)], 46.4 [C(14)], 57.6 [C(4)], 62.5 [C(13)], 71.0 [C(10)], 71.5 [C(3)], 73.3 [C(6)], 109.1 [C(8)], 120.4 [C(13′)], 136.2 [C(14′)], 208.4 [C(11)], 209.8 [C(17)].

Hydroxytricyclocolupone epimer B (**7′a**, **Figure 3**): UV−vis (0.1% aqueous formic acid/acetonitrile; 20:80, v/v) λ_{max} = 283 nm; LC-TOF-MS, *m/z* 415.2474 ([M − H]$^-$, measured), *m/z* 415.2489 ([M − H]$^-$, calculated for $[C_{25}H_{36}O_5-H]^-$); LC-MS (ESI$^-$), *m/z* (%) 415 (100) [M − H]$^-$; MS/MS (−30 V), *m/z* (%) 111 (100), 277 (40), 287 (16), 415 (10); ^{1}H NMR (500 MHz, CD_3OD, COSY) δ 1.06 [s, 3H, H−C(16)], 1.07 [s, 3H, H−C(15)], 1.14 [m, 3H, H−C(19)], 1.16 [m, 3H, H−C(20)], 1.28 [s, 3H,

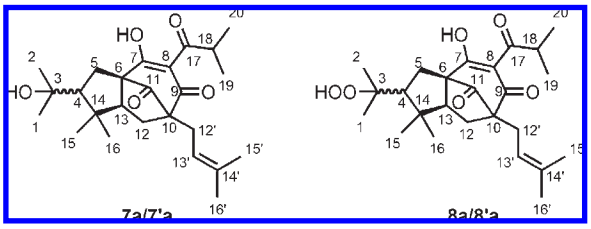

Figure 3. Chemical structures of hydroxytricyclocolupone epimers (**7a**, **7′a**) and hydroperoxytricyclocolupone epimers (**8a**, **8′a**) isolated from a colupulone (**1a**) solution treated under wort boiling conditions.

H−C(1)], 1.30 [s, 3H, H−C(2)], 1.70 [s, 3H, H−C(15′)], 1.70 [s, 3H, H−C(16′)], 1.79 [m, 1H, H−C(4)], 1.91 [m, 1H, H−C(13)], 2.08 [m, 1H, H−C(5α)], 2.20 [m, 1H, H−C(12α)], 2.32 [m, 1H, H−C(12*β*)], 2.42 [m, 1H, H−C(12′α)], 2.56 [m, 1H, H−C(5*β*)], 2.67 [m, 1H, H−C(12′*β*)], 3.99 [m, 1H, J = 6.8 Hz, H−C(18)], 5.21 [t, 1H, J = 7.7 Hz, H−C(13′)]; ^{13}C NMR (125 MHz, CD_3OD, HMQC, HMBC) δ 17.7 [C(15′)], 18.9 [C(19)], 18.9 [C(20)], 23.3 [C(5)], 24.6 [C(12)], 25.7 [C(16′)], 26.2 [C12′]), 26.6 [C(15)], 29.0 [C(16)], 29.9 [C(2)], 29.9 [C(1)], 35.6 [C(18)], 46.7 [C(14)], 56.8 [C(4)], 60.5 [C(13)], 71.4 [C(6)], 71.4 [C(10)], 73.2 [C(3)], 120.2 [C(13′)], 135.6 [C(14′)], 207.9 [C(11)], 209.6 [C(17)].

Hydroperoxytricyclocolupone epimer A (**8a**, **Figure 3**): UV−vis (0.1% aqueous formic acid/acetonitrile; 20:80, v/v) λ_{max} = 283 nm; LC-TOF-MS, m/z 431.2426 ([M − H]$^-$, measured), m/z 431.2439 ([M − H]$^-$, calculated for [$C_{25}H_{36}O_6$−H]$^-$); LC-MS (ESI$^-$), m/z (%) 431 (100) [M − H]$^-$; MS/MS (−10 V), m/z (%) 341 (100), 287 (43), 356 (27), 431 (14); ^{1}H NMR (500 MHz, CD_3OD, COSY) δ 1.04 [s, 3H, H−C(15)], 1.04 [s, 3H, H−C(16)], 1.11 [d, 3H, J = 6.8 Hz, H−C(19)], 1.14 [d, 3H, J = 6.8 Hz, H−C(20)], 1.30 [s, 3H, H−C(1)], 1.31 [s, 3H, H−C(2)], 1.68 [s, 3H, H−C(15′)], 1.68 [s, 3H, H−C(16′)], 1.77 [dd, 1H, J = 6.4, 11.5 Hz, H−C(4)], 1.89 [dd, 1H, J = 8.6, 13.1 Hz, H−C(12α)], 1.99 [t, 1H, J = 11.4 Hz, H−C(12*β*)], 2.18 [dd, 1H, J = 6.5, 13.4 Hz, H−C(5α)], 2.33 [t, 1H, J = 11.5, 13.4 Hz, H−C(13)], 2.40 [dd, 1H, J = 11.6, 13.4 Hz, H−C(5*β*)], 2.56 [m, 2H, H−C(12′α;*β*)], 3.93 [m, 1H, H−C(18)], 5.21 [m, 1H, H−C(13′)]; ^{13}C NMR (125 MHz, CD_3OD, HMQC, HMBC) δ 18.0 [C(15′)], 19.2 [C(19)], 19.3 [C(20)], 23.6 [C(5)], 24.8 [C(1,2)], 25.5 [C16′]), 26.1 [C(15)], 26.9 [C(12′)], 27.0 [C(16)], 31.7 [C(12)], 36.0 [C(18)], 44.3 [C(14)], 56.1 [C(4)], 59.1 [C(13)], 67.1 [C(10)], 73.4 [C(6)], 85.0 [C(3)], 109.1 [C(8)], 119.4 [C(13′)], 135.9 [C(14′)], 208.5 [C(17)], 208.9 [C(11)].

Hydroperoxytricyclocolupone epimer B (**8′a**, **Figure 3**): UV−vis (0.1% aqueous formic acid/acetonitrile; 20:80, v/v) λ_{max} = 283 nm; LC-TOF-MS, m/z 431.2434 ([M − H]$^-$, measured), m/z 431.2439 ([M − H]$^-$, calculated for [$C_{25}H_{36}O_6$−H]$^-$); LC-MS (ESI$^-$), m/z (%) 431 (100) [M − H]$^-$; MS/MS (−30 V), m/z (%) 341 (100), 356 (42), 287 (30), 399 (15), 431 (14); ^{1}H NMR (500 MHz, CD_3OD, COSY) δ 0.73 [s, 3H, H−C(15)], 1.06 [s, 3H, H−C(16)], 1.15 [d, 6H, J = 6.8 Hz, H−C(19,20)], 1.33 [s, 3H, H−C(1)], 1.34 [s, 3H, H−C(2)], 1.69 [s, 3H, H−C(15′)], 1.71 [s, 3H, H−C(16′)], 1.91 [m, 1H, H−C(12α)], 2.07 [dd, 1H, J = 1.5, 7.5 Hz, H−C(12*β*)], 2.07 [m, 1H, H−C(5α)], 2.07 [m, 1H, H−C(13)], 2.28 [m, 1H, H−C(4)], 2.42 [dd, 1H, J = 5.3, 14.7 Hz, H−C(12′α)], 2.57 [m, 1H, H−C(5*β*)], 2.68 [dd, 1H, J = 9.0, 14.8 Hz, H−C(12′*β*)], 4.01 [m,1H, H−C(18)], 5.21 [m, 1H, H−C(13′)]; ^{13}C NMR (125 MHz, CD_3OD, HMQC, HMBC) δ 18.0 [C(15′)], 18.2 [C(15)], 19.2 [C(19,20)], 23.6 [C(5)], 24.8 [C(1,2)], 25.5 [C16′]), 26.1 [C(12′)], 26.4 [C(12)], 29.2 [C(16)], 36.1 [C(18)], 46.2 [C(14)], 58.6 [C(4)], 60.0 [C(13)], 70.6 [C(10)], 73.4 [C(6)], 85.1 [C(3)], 109.3 [C(8)], 120.8 [C(13′)], 135.9 [C(14′)], 208.4 [C(11)], 209.9 [C(17)].

Analytical Wort Boiling of Colupulone (1a) and Lupulone (2a). Samples (20.0 mg) of colupulone (**1a**) and lupulone (**1b**), respectively, were individually dissolved in methanol (10 mL), mixed with an aqueous solution (10 mL) of $MgCl_2$ (1.55 mol/L), and, after the pH value had been adjusted to 5.8 with an aqueous solution of NaOH (0.1 mol/L), the solutions were thermally treated in closed vials (50 mL) for 1 h at 110 °C. After cooling, the reaction mixtures were 1:5 diluted with water and, then, analyzed by means of LC-MS/MS.

High-Performance Liquid Chromatography (HPLC). The HPLC system consisted of two PU-2087 type pumps (Jasco, Gross-Umstadt, Germany), an Rh 7725i type Rheodyne injection valve (Bensheim, Germany), a DG-2080-53 type solvent degasser (Uniflows Co., Tokyo, Japan), a gradient mixer (Knauer ASI, Berlin, Germany), and a UV detector (Jasco UV PN-2075) monitoring the effluent flow at 272 nm. Data acquisition was performed by means of ChromPass V.1.8.6.1 software. Chromatography was performed at a flow rate of 4.5 mL/min; sample injection was carried out using a 0.5 mL sample loop and a 250 × 10.0 mm i.d., 5 μm RP-18, ODS-Hypersil column (ThermoHypersil, Kleinostheim, Germany) equipped with a guard column of the same type.

High-Performance Liquid Chromatography−Mass Spectrometry (HPLC-MS/MS). The Agilent 1100 series HPLC system, consisting of a pump, a degasser, and an autosampler (Agilent, Waldbronn, Germany), was connected to an API 4000 Q-TRAP mass spectrometer (AB Sciex Instruments, Darmstadt, Germany) equipped with the electrospray ionization (ESI) source running in the negative ion mode. The ion spray voltage was set to −4500 V, and the declustering potential as well as the MS/MS parameters were optimized for each substance to induce fragmentation of the pseudomolecular ion [M − H]$^-$ to the corresponding target product ions after collision-induced dissociation. The dwell time for each mass transition was 44 ms. Nitrogen was used as the collision gas (4 × 10^{-5} torr). Quantitative analysis was performed by means of the multiple reaction monitoring (MRM) mode with the fragmentation parameters optimized prior to analysis. Data processing and integration were performed by using Analyst software version 1.4.2 (AB Sciex Instruments, Darmstadt, Germany). Chromatography was performed using a 150 × 2.0 mm, 5 μm, Synergi 4u Hydro-RP column (Phenomenex, Aschaffenburg, Germany), acetonitrile containing 0.1% formic acid as solvent A, and aqueous formic acid (0.1% in water) as solvent B. Using a flow rate of 250 μL/min, chromatography was performed by increasing solvent A from 20 to 60% within 20 min, then to 70% within 15 min, to 92% within 28 min, and, finally, to 100% within 2 min.

Quantitative Analysis by HPLC-MS/MS Using the ECHO Technique. Prior to HPLC-MS/MS analysis, the beer samples were degassed by ultrasonification for 2 min in a glass beaker. For quantitative analysis of the target compounds **1a/b**−**8a/b** using the ECHO technique, the experiment was started by the injection of an aliquot (5 μL) of the beer sample, followed by a second injection of the ECHO standard solution (5 μL) containing purified colupulone (1 μmol/L; **e1a**) and nortricyclocolupone (1 μmol/L; **e4a**) after 50 min. External linear calibration curves were determined under the same conditions showing correlation coefficients of > 0.99 for all of the compounds. Colupulone (**e1a**) was used as ECHO standard for cohulupone (**2a**), hulupinic acid (**3**), and colupulone (**1a**); all tricyclic degradation products (**4a**−**8′a**) were quantified using the tricyclic nortricyclocolupone (**e4a**) as internal standard.

Investigation of Matrix Effects during HPLC-MS/MS. For the investigation of matrix effects caused by coeluting matrix compounds, the same HPLC-MS/MS parameters were used as given above, but, in addition, a constant flow of 10 μL/min of solutions (1 μmol/L) of colupulone (**1a**) and nortricyclocolupone (**4a**) was introduced by means of a PHD 4400 Hpsi type syringe pump (Harvard Apparatus, Cambridge, MA) connected to the solvent flow via a three-way valve. For LC-MS/MS analysis, an aliquot (5 μL) of hop-free "zero-beer" was injected.

Exact Mass Measurements. High-resolution mass spectra of the purified compounds were measured on a Bruker Micro-TOF (Bruker Daltronics, Bremen, Germany) mass spectrometer using sodium formate as the reference for calibration. The deviation of the measured from the calculated molecular mass was < 5 *m*amu.

Nuclear Magnetic Resonance Spectroscopy (NMR). The ^{1}H, ^{13}C, and 2D NMR experiments were performed using a Bruker 400 MHz DRX and a 500 MHz Avance III spectrometer (Bruker, Rheinstetten, Germany). Samples were dissolved in CD_3OD and placed into NMR tubes (Schott Professional 178 × 5 mm) prior to measurement. Chemical shifts were referenced to the solvent signal. Data processing was performed using XWin-NMR software (version 3.5, Bruker) as well as MestReNova V. 5.1.0−2940 (Mestrelab Research, La Coruña, Spain), respectively.

Analytical Sensory Experiments. *Panel Training*. To familiarize the subjects with the taste language used by the sensory group and to get them trained in recognizing and distinguishing different qualities of oral sensations, 12 assessors with no history of known taste disorders (5 women and 7 men, aged 25−40 years) participated in weekly training sessions using the reference materials reported recently (*11*).

Precautions Taken for Sensory Analysis of Isolated Substances. Prior to sensory analysis, the fractions isolated were suspended in water and, after removal of the volatiles under high vacuum (< 5 mPa), freeze-dried twice. GC-MS and ion chromatographic analysis revealed that the fractions treated by that procedure are essentially free of the solvents and buffer compounds used. Formic acid, which is considered to be "Generally Recognized as Safe" (GRAS) as a flavoring agent for food and feed applications, was used to adjust the pH value of solutions that should be sensorially analyzed, because trace amounts of this acid do not influence the sensory profile of the test solution.

Taste Recognition Threshold Concentrations. Twelve panelists determined the threshold concentrations of the purified bitter compounds in bottled water adjusted to pH 4.4 with trace amounts of aqueous formic acid (1% in water), using a triangle test with ascending concentrations of the stimulus as reported in detail in previous papers (*11*, *17*−*20*). The threshold value of the sensory group was approximated by averaging the threshold values of the individuals in three independent sessions. Values between individuals and separate sessions differed by not more than plus or minus one dilution step; that is, a threshold value of 0.5 mmol/L for the reference compound caffeine represents a range of 0.25−1.0 mmol/L.

RESULTS AND DISCUSSION

To identify previously unknown bitter compounds generated upon degradation of β-acids, colupulone (**1a**) and lupulone (**2a**) were isolated from an ethanolic hop extract, purified by means of semipreparative RP-HPLC as shown exemplarily for **1a** in **Figure 2A**, and then thermally treated by means of a laboratory wort boiling process at pH 5.8 to mimick the pH conditions during wort boiling. RP-HPLC analysis of the processed colupulone solution indicated the generation of multiple reaction products (**Figure 2B**). Comparison of the spectroscopic data (UV−vis, LC-MS/MS, 1D/2D NMR) with those obtained for the reference compounds led to the identification of cohulupone (**2a**) and hulupinic acid (**3**) in the HPLC fraction eluting between 6 and 7 min. In addition, the five major peaks eluting between 20 and 24 min were unequivocally identified as nortricyclocolupone (**4a**) and the dehydrotricyclocolupone epimers **5a** and **5′a**, as well as the tricyclocolupone epimers **6a** and **6′a** (**Figure 1**), thus confirming data reported previously (*11*).

In addition, the four compounds eluting between 16 and 19 min were isolated in a purity of > 98% and then analyzed by means of UV−vis, LC-MS/MS, LC-TOF-MS, and 1D/2D NMR experiments, respectively. Although all four compounds showed an UV absorption maximum at 283 nm, LC-MS/MS (ESI^-) analysis revealed a pseudomolecular ion ($[M - H]^-$) with m/z 415 for the first eluting compounds **7a** and **7′a**, but m/z 431 for the later eluting compounds **8a** and **8′a**. High-resolution mass spectrometry of the purified compounds revealed an empirical formula of $C_{25}H_{36}O_5$ for **7a** and **7′a** and an elemental composition of $C_{25}H_{36}O_6$ for **8a** and **8′a**, respectively. These data indicate that **7a**/**7′a** or **8a**/**8′a** differ from the starting material **1a** by an additional molecule of water or one molecule of water plus one oxygen atom as found for a hydroperoxide, respectively. The shorter retention times (RP18) of such compounds are well in agreement with their increased polarity when compared to the parent molecule **1a** (**B**, **Figure 2**).

Comparison of 1D/2D NMR spectroscopic data of **7a**/**7′a** or **8a**/**8′a** with those obtained for the parent colupulone (**1a**) as well as the recently identified degradation products showed large similarities of the unknown target compounds to the tricyclic carbon skeleton of **4**−**6a**/**6′a** (**Figure 1**). The ^1H and ^{13}C NMR data of the first eluting compound **7a** showed a total number of 18 resonance signals integrating for 34 protons and a total of 23 carbon atoms. The complete assignment of all proton and carbon atoms was successfully achieved by means of homo- and heteronuclear coupling experiments (COSY, HMQC, HMBC). For example, the HMBC experiment demonstrated the attachment of the isoprenyl carbon skeleton C(12′−16′) at the quaternary bridge atom C(10) and the presence of novel carbon−carbon bonds between C(6) and C(13) as well as between C(4) and C(14). Although these findings are well in line with the data found for the tricyclocolupone epimers (**6a**/**6′a**), an additional hydroxyl function was identified at position C(3), thus corroborating the chemical shift of 71.5 ppm found for C(3). On the basis of the careful consideration of all the 1D/2D NMR as well as LC-MS/MS data, the peak eluting at 16.5 min (**Figure 2**) could be unequivocally identified as the structure **7a**, coined hydroxytricyclocolupone (**Figure 3**).

The spectroscopic data observed for compound **7′a** were rather similar to those found for **7a**, thus indicating the presence of a hydroxytricyclocolupone epimer. Interestingly, the methine carbon C(4) was found to be upfield shifted by 0.8 ppm from 57.6 (**7a**) to 56.8 ppm (**7′a**) and the methyl carbon C(15) strongly downfield shifted by 9 ppm from 17.6 (**7a**) to 26.6 ppm (**7′a**), whereas the resonances of the other carbon atoms in **7a** and **7′a** differed just to a minor extent. On the basis of these considerations, the structure of compound **7′a**, eluting after 17 min (**Figure 2**), was proposed as a hydroxytricyclocolupone epimer (**Figure 3**) differing from **7a** by the chirality at carbon C(4).

The presence of a hydroperoxy function in **8a** and **8′a** as indicated by means of high-resolution mass spectrometry was further strengthened by means of homo- and heteronuclear correlation spectroscopy. When compared to **7a**/**7′a**, carbon C(3) was strongly downfield shifted by approximately 13 ppm from 71.5/73.2 ppm (**7a**/**7′a**) to 85.0/85.1 ppm (**8a**/**8′a**), whereas the resonances of the other carbon atoms in **8a** and **8′a** were rather identical to those found in **7a**/**7′a**. On the basis of careful consideration of all 1D/2D NMR as well as LC-MS/MS data, the peaks eluting at 17.7 and 18.5 min (**Figure 2**) could be unequivocally identified as the epimeric structures **8a** and **8′a**, coined hydroperoxytricyclocolupones (**Figure 3**). Although these structures were already hypothesized by De Potter (*21*), who isolated such compounds from a colupulone sample thermally treated in the presence of oxygen, the NMR signal assignment of these compounds was not previously reported. Interestingly, incubation of the purified hydroperoxytricyclocolupone epimers **8a**/**8′a** in aqueous solutions at room temperature for 2 days revealed the formation of the corresponding alcohols **7a**/**7′a** (data not shown), thus confirming the instability of the parent hydroperoxide.

Sensory Activity of Colupulone and Its Transformation Products 2a−8a/8′a. To evaluate the sensory activity of colupulone (**1a**) as well as its wort boiling products **2a**−**8a**/**8′a**, the purity of the isolated substances was checked by LC-MS as well as ^1H NMR spectroscopy prior to sensory analysis. The human bitter taste threshold concentration of each individual compound was determined in 5% aqueous ethanol (pH 4.4) by means of a triangle test to overcome the limited water solubility of the bitter compounds and to evaluate their bitter thresholds in a beer-like medium.

Depending on their chemical structure, sensory analysis revealed a clear bitter taste of these compounds with rather low recognition thresholds between 7.9 and 90.3 μmol/L (**Table 1**). Whereas cohulupone induced a short-lasting, iso-α-acid-like bitter impression, all of the tricyclic compounds **4a**−**8a**/**8′a** were observed to exhibit a long-lasting, lingering, and harsh bitterness perceived on the posterior tongue and throat. The lowest threshold concentration of 7.9 μmol/L was found for the five-membered ring compound cohulupone (**2a**), which is somewhat lower when compared to the threshold concentration of 19.0 μmol/L found for the iso-α-acid *trans*-isocohumulone (*22*). In contrast, the

structurally related hulupinic acid (**3**) exhibited a comparatively high threshold value of 68.5 μmol/L (**Table 1**). Among the group of the tricyclic compounds **4**−**8a**/**8′a**, the lowest bitter threshold concentrations of 14.7−20.9 μmol/L were found for the more polar hydroxy- and hydroperoxytricyclocolupones **7a**/**7′a** and **8a**/**8′a**, whereas the more hydrophobic compounds **4a**−**6a**/**6′a** showed threshold levels above 37.9 μmol/L.

Identification of Colupulone Transformation Products (2a−8a/8′a) in Beer. To investigate the occurrence of the compounds **2a**−**8a**/**8′a** in beer by means of HPLC-MS/MS operating in the multiple reaction monitoring (MRM) mode, characteristic mass transitions were selected and instrument settings such as declustering potential, collision energy, and cell exit potential were optimized for each individual compound in tuning experiments. A degassed dark beer sample was then injected into the LC-MS/MS system, and the retention times as well as the characteristic mass transition of each individual degradation product detected in beer were compared to those obtained for a solution of the corresponding reference compound. As displayed in **Figure 4**, cohulupone (**2a**) and hulupinic acid (**3**), as well as the seven tricyclic compounds **4a**−**7a**/**7′a** were unequivocally identified in the beer sample. In contrast, the hydroperoxytricyclocolupone epimers **8a** and **8′a** were not detectable in beer, being well in line with the observed instability of these hydroperoxides and their ability to be further converted into the corresponding alcohols **7a** and **7′a**. To the best of our knowledge, the occurrence of the tricyclic colupulone transformation products **4a**−**7a**/**7′a** in beer has not been previously reported in the literature.

Table 1. Bitter Recognition Threshold Concentrations of Colupulone (**1a**) and Its Transformation Products **2a**−**8a**/**8′a**

compound (no.)[a]	threshold concentration[b] (μmol/L)
colupulone (**1a**)	39.3
cohulupone (**2a**)	7.9
hulupinic acid (**3**)	68.5
nortricylocolupone (**4**)	90.3
dehydrotricyclocolupone epimer A (**5a**)	40.5
dehydrotricyclocolupone epimer B (**5′a**)	40.8
tricyclocolupone epimer A (**6a**)	54.4
tricyclocolupone epimer B (**6′a**)	37.9
hydroxytricyclocolupone epimer A (**7a**)	17.1
hydroxytricyclocolupone epimer B (**7′a**)	14.7
hydroperoxytricyclocolupone epimer A (**8a**)	20.5
hydroperoxytricyclocolupone epimer B (**8′a**)	20.9

[a] Structure and numbering of the compounds refer to **Figures 1** and **3**; [b] Taste threshold concentrations were determined in 5.0% aqueous ethanol (pH 4.4) by means of a triangle test.

Quantitation of 1a−8a/8′a in Beer by Means of the ECHO Technique. As the synthesis of stable isotope-labeled internal standards of **1a**−**8a**/**8′a** is extremely challenging, the so-called ECHO technique should be applied as an alternative quantification strategy. To perform this technique and to compensate for the effect of coextracted matrix components in LC-MS/MS analysis, the isolated nonlabeled reference compounds of the analytes are used as quasi-internal standards and are injected into

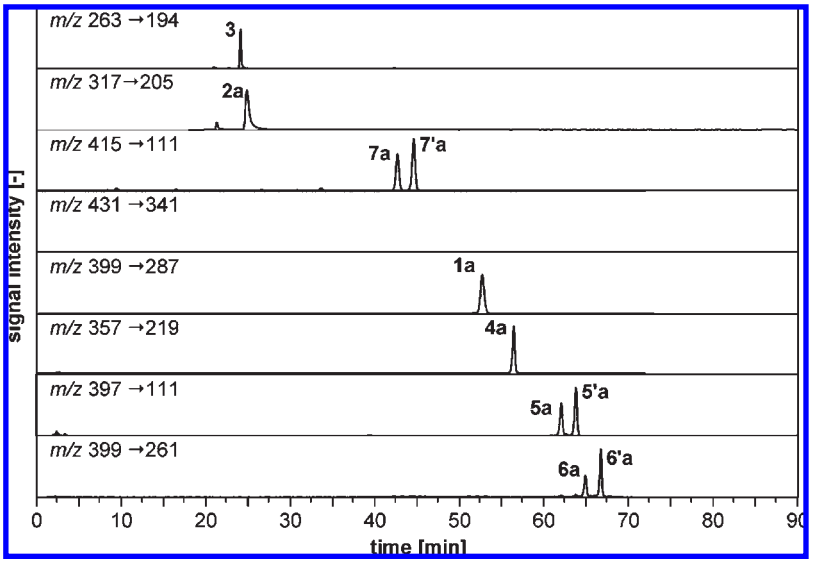

Figure 4. HPLC-MS/MS (MRM) chromatogram of a beer sample. Peak numbering refers to the chemical structures given in **Figures 1** and **3**.

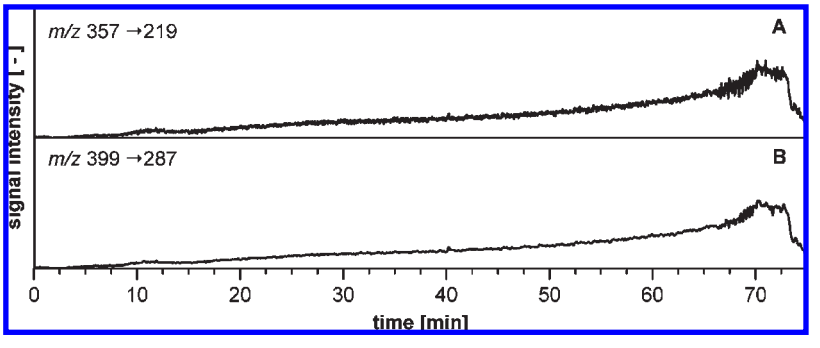

Figure 5. HPLC-MS/MS (MRM) chromatograms recorded for a beer sample while a continuous flow of nortricyclocolupone (**A**) and colupulone (**B**) was introduced into the LC-MS/MS system by means of a syringe pump.

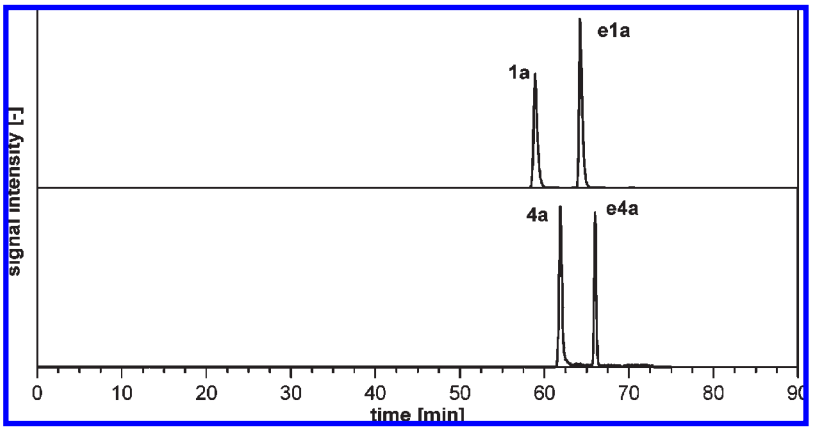

Figure 6. HPLC-MS/MS chromatogram showing the quantitative analysis of selected bitter compounds in beer using the ECHO technique. The peak of the ECHO standard is labeled with an "e" prior to the substance number referring to the chemical structures given in **Figure 1**.

the HPLC-MS system before and/or after the target sample to target the elution time of this ECHO standard into the retention time window of the analytes under investigation. Before the analysis could be performed, it was necessary to confirm that coeluting matrix components do not affect differently the ionization of the analytes and the corresponding ECHO standards. To visualize such potential matrix phenomena, a constant flow of a solution of either colupulone (**1a**) or nortricyclocolupone (**4a**) was introduced into the LC-MS/MS system via a syringe pump during the analysis of a hop-free zero beer sample. The ionization rates of nortricyclocolupone (**4a**) and colupulone (**1a**) increased slightly with increasing retention time (**Figure 5**). Taking this observation into account, colupulone (**1a**) and nortricyclocolupone (**4a**) were used as suitable ECHO standards to elute shortly after the corresponding analytes (**Figure 6**). As a consequence, ECHO standard and the corresponding analyte should be affected by the matrix effects to similar extents, thus making their quantitative analysis possible.

To check the accuracy of the analytical method, recovery experiments were performed (**Table 2**). To achieve this, hop-free zero beer was spiked with purified colupulone (**1a**) as well as its degradation products **2a**, **3**, and **4a**−**8a**, respectively, in three different concentrations prior to quantitative analysis, and the amounts determined after spiking were compared with those found in the zero beer (control). The averaged recovery rates, calculated on the basis of the content of each compound added to the zero beer prior to analysis, were found to be 94% for colupulone (**1a**), 95% for cohulupone (**2a**), 105% for hulupinic acid (**3**), 96% for nortricyclocolupone (**4a**) and dehydrotricyclocolupone (**5a**), 105% for tricyclocolupone (**6a**), 104% for hydroxytricyclocolupone (**7a**), and 108% for hydroperoxytricyclocolupone (**8a**), respectively (**Table 2**). These data clearly demonstrate that the developed ECHO technique is a reliable method enabling an accurate quantitative determination of colupulone and its degradation products in beer samples without the need of any sample cleanup.

In addition, detection limits and quantitation limits of the individual bitter taste compounds were determined in hop-free zero beer by analyzing the prevailing signal-to-noise ratio (1:3 and 1:5) for the individual MRM chromatograms (**Table 2**). Using the HPLC-MS/MS-ECHO technique developed, very low detection limits of 0.8−3.0 nmol/L as well as quantitation limits of 1.3−5.0 nmol/L were found for all of the taste compounds with the exception of cohulupone, for which a comparatively high detection limit of 121.1 nmol/L and a high quantitation limit of 201.8 nmol/L were determined.

Table 2. Determination of the Recovery Rates for the Quantitative Analysis of Colupulone and Its Transformation Products in Beer Samples

compound no.	amount added[a] (μmol/L)	concentration (μmol/L)	recovery (%)	mean value (%)
1a	0.45	0.45	99	94
	0.11	0.09	83	
	0.02	0.02	99	
2a	2.65	2.56	96	95
	0.17	0.17	95	
	0.02	0.02	94	
3a	3.06	3.25	106	105
	0.15	0.13	92	
	0.03	0.03	118	
4a	0.58	0.57	96	96
	0.15	0.15	98	
	0.07	0.07	95	
5a	0.79	0.76	96	96
	0.16	0.16	98	
	0.05	0.04	95	
6a	0.77	0.79	102	105
	0.18	0.20	110	
	0.06	0.06	102	
7a	0.67	0.63	95	104
	0.04	0.05	109	
	0.02	0.02	107	
8a	2.51	2.93	117	108
	0.10	0.09	89	
	0.01	0.01	118	

[a] Hop-free "zero beer" was spiked with defined amounts of colupulone (**1a**), cohulupone (**2a**), hulupinic acid (**3**), nortricyclocolupone (**4a**), dehydrotricyclocolupone (**5a**), tricyclocolupone (**6a**), hydroxytricyclocolupone (**7a**), and hydroperoxytricyclocolupone (**8a**) in three different concentrations prior to quantitative analysis, and the amounts determined after spiking were compared with those found in the zero beer (control), [b] Structures of compounds are given in **Figures 1** and **3**.

To gain a first insight into the concentrations of colupulone and its 11 degradation products in different beer samples, these were quantitatively determined in 12 commercially available beer samples, representing a large variety of different types of beer such as dark beer (I−V), country lager (VI), Pilsner-type beer (VII, VIII), lager (IX), bitter beer (X, XI), and wheat beer (XII) (**Table 3**). The developed RP-HPLC-ESI-MS/MS method using

Table 3. Limits of Detection and Quantitation as well as Concentrations of Colupulone and Its Transformation Products in Beer

	concentration (nmol/L) and standard deviation in (nmol/L) in beer of compound no.											
sample	**1a**	**2a**	**3a**	**4a**	**5a**	**5′a**	**6a**	**6′a**	**7a**	**7′a**	**8a**	**8′a**
DL[b] (nmol/L)	0.8	121.1	3.0	2.5	3.0	1.5	1.3	2.3	2.5	2.1	1.0	0.9
QL[b] (nmol/L)	1.3	201.8	5.0	4.2	5.0	2.5	1.7	3.0	4.2	3.6	1.7	1.4
dark beer												
I	469.5 ± 0.6	310.1 ± 4.9	≤3.0	206.4 ± 1.6	168.5 ± 1.5	265.7 ± 4.3	82.6 ± 1.4	120.6 ± 4.4	663.5 ± 1.5	1359.6 ± 15.4	≤1.0	≤0.9
II	208.5 ± 1.2	236.3 ± 5.7	22.8 ± 0.2	7.4 ± 0.6	14.7 ± 0.3	6.5 ± 0.5	28.3 ± 1.7	21.5 ± 1.5	49.6 ± 0.4	118.2 ± 0.8	≤1.0	≤0.9
III	1209.4 ± 1.6	246.7 ± 5.3	≤3.0	6.5 ± 0.5	13.7 ± 0.3	7.7 ± 0.3	15.6 ± 0.4	28.8 ± 1.2	69.6 ± 0.4	148.6 ± 1.4	≤1.0	≤0.9
IV	284.8 ± 3.4	230.7 ± 7.3	≤3.0	938.4 ± 6.6	15.2 ± 0.8	8.8 ± 0.2	12.5 ± 1.5	23.3 ± 0.7	89.7 ± 1.3	279.9 ± 2.1	≤1.0	≤0.9
V	32.5 ± 2.5	317.6 ± 6.4	80.3 ± 1.7	4.4 ± 0.6	27.1 ± 0.9	14.7 ± 0.3	17.9 ± 0.4	26.7 ± 0.3	44.7 ± 0.4	114.4 ± 1.6	≤1.0	≤0.9
country lager												
VI	356.0 ± 5.3	372.2 ± 7.8	224.1 ± 6.7	≤2.5	≤3.0	≤1.5	≤1.3	≤2.3	113.9 ± 3.4	236.3 ± 7.1	≤1.0	≤0.9
Pilsner-type												
VII	8.7 ± 2.7	256.2 ± 3.8	5.4 ± 0.4	≤2.5	≤3.0	≤1.5	≤1.3	≤2.3	205.5 ± 1.5	477.9 ± 4.1	≤1.0	≤0.9
VIII	21.0 ± 0.6	164.9 ± 4.9	262.7 ± 7.9	≤2.5	≤3.0	≤1.5	≤1.3	≤2.3	167.5 ± 5.0	307.4 ± 9.2	≤1.0	≤0.9
lager												
IX	27.0 ± 0.8	3421.3 ± 10.3	6097.4 ± 9.1	≤2.5	≤3.0	≤1.5	≤1.3	≤2.3	427.3 ± 12.8	812.2 ± 8.1	≤1.0	≤0.9
bitter beer												
X	126.2 ± 3.4	1029.4 ± 27.8	39.8 ± 1.1	≤2.5	≤3.0	≤1.5	≤1.3	≤2.3	25.6 ± 0.7	45.8 ± 1.2	≤1.0	≤0.9
XI	188.8 ± 3.8	3998.1 ± 17.6	619.8 ± 12.4	≤2.5	≤3.0	≤1.5	≤1.3	≤2.3	28.5 ± 0.6	34.8 ± 0.7	≤1.0	≤0.9
wheat beer												
XII	280.0 ± 8.4	178.6 ± 5.4	114.5 ± 3.4	≤2.5	≤3.0	≤1.5	≤1.3	≤2.3	122.0 ± 3.7	225.7 ± 6.8	≤1.0	≤0.9

[a] Chemical structures of compounds are given in **Figures 1** and **3**. [b] Limit of detection (DL) and limit of quantification (QL) were determined in hop-free zero beer according to the signal-to-noise ratio of 1:3 and 1:5, respectively.

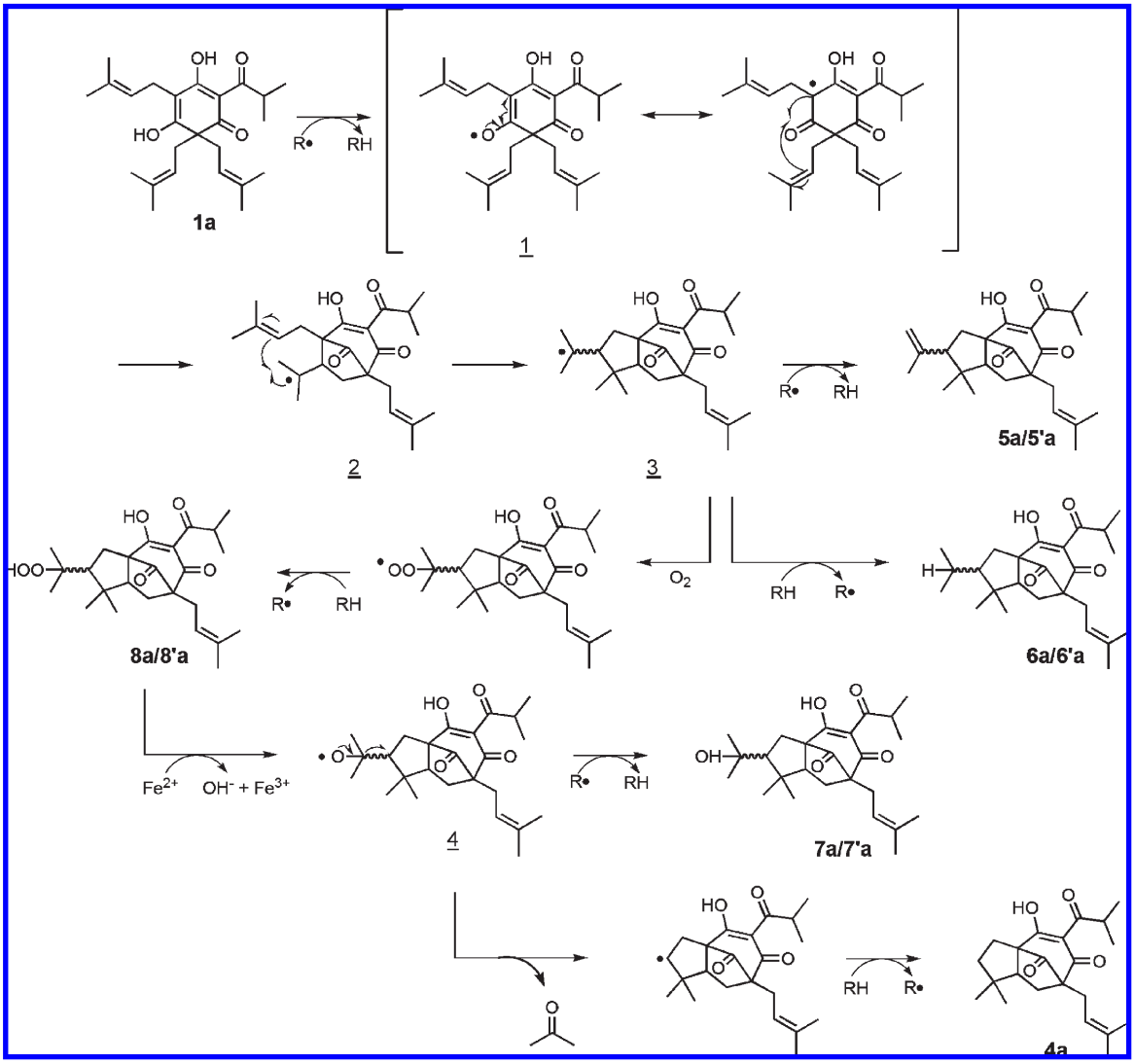

Figure 7. Postulated reaction route leading to the formation of bitter-tasting transformation products (**4a**−**8a**/**8′a**) of colupulone (**1a**) during wort boiling.

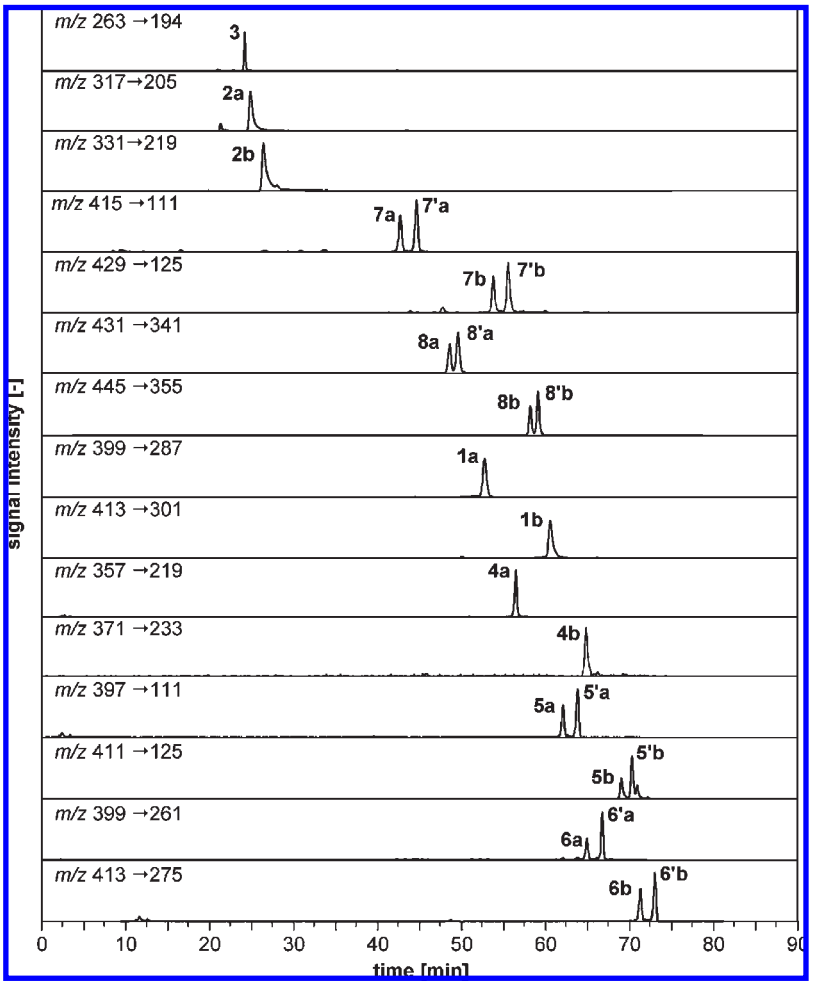

Figure 8. HPLC-MS/MS (MRM) chromatograms of bitter compounds after thermal treatment of colupulone (**1a**) and lupulone (**1b**) under wort boiling conditions. Peak numbering refers to the chemical structures given in **Figures 1** and **3**.

the ECHO technique successfully quantitatively determined the individual bitter compounds in these beer samples. Interestingly, the dark beer samples I−V contained significantly higher amounts of the hydrophobic degradation products **4a**−**6a**/**6′a** when compared to the other types of beer, whereas the more polar cohulupone (**2a**) as well as the alcohols **7a**/**7′a** were also present in rather high concentrations in the other beer samples (**Table 3**). Again, compounds **8a**/**8′a** were below the limit of detection, most likely due to the instability of these hydroperoxides. The beer samples investigated showed huge differences in the concentration of the five-membered ring degradation products cohulupone (**2a**) and hulupinic acid (**3a**); for example, the lager beer IX and the bitter beer XI contained comparatively high amounts of 3421.3 and 3998.1 nmol/L of **2a** and very high levels of 6097.4 and 619.8 nmol/L of **3a**, respectively. Besides the dark beer sample, also the wheat beer (XII) as well as the bitter beers (IX, X) showed high colupulone levels concentrations, whereas Pilsner-type beers (VII, VIII) contained only 9 and 21 nmol/L (**Table 3**), respectively. These huge quantitative differences in the distribution of the individual β-acid transformation products in the beer samples investigated might be due to variations in the composition of the hop product used for beer manufacturing and the hop dosage, as well as other process-specific parameters applied during beer manufacturing.

Proposed Reaction Route Leading to the Formation of Bitter β-Acid Transformation Products. On the basis of the structures of the bitter taste compounds identified in the beer samples, a reaction cascade showing the formation of the compounds **4a**−**8a**/**8′a** from the β-acid colupulone (**1a**) is proposed in **Figure 7**. Upon wort boiling, a hydrogen radical is abstracted from the hydroxyl function HO−C(11) of **1a** to give its corresponding alkoxy radical (1), which, upon mesomerization and radical cyclization, generates the novel C(6)−C(12) linkage in the transient alkyl radical intermediate (2). A second cyclization of this intermediate induced by the attack of the alkyl radical at the double bond of the isoprenyl moiety at C(6) then gives rise to the intermediary isopropyl radical 3 with a novel C(4)−C(14) linkage in the structure. This transient radical (3) is the common intermediate to generate the nine tricyclic compounds **4a**−**8a**/**8′a** via three alternative reaction routes. Proton abstraction at C(2) of the isopropyl radical leads to the formation of the epimers **5a** and **5′a** with a double bond in the isopropenyl moiety (**Figure 7**). Saturation of the radical 3 by abstracting a proton from a hydrogen donor molecule such as colupulone (**1a**) gives rise to the epimers **6a** and **6′a**, respectively. Upon addition of triplet oxygen, key intermediate 3 forms a hydrohydroperoxy radical, which by abstracting a hydrogen from a donor molecule gives rise to the hydroperoxytricyclocolupone epimers **8a** and **8′a**. These hydroperoxides were found to be rather unstable and to liberate the corresponding alkoxy radical (4) in the presence of transition metals by means of a Fenton-type reaction. This intermediary alkoxyradical 4 either can be saturated by hydrogen abstraction from a donor molecule to give the alcohols **7a** and **7′a** or can split off one molecule of 2-propanone, thus leading to the nortricyclocolupone (**4a**).

As all of the degradation products formed from **1a** showed the isopropyl moiety in their chemical structure, the generation of these compounds seems to be independent of the variable alkanoyl side chain of the β-acids (**1a**−**1c**). To confirm that homologous compounds are formed from the other β-acids, a binary mixture of the major β-acids **1a** and **2b** was thermally treated under model wort boiling conditions and then analyzed by HPLC-MS/MS-MRM using the compound-specific mass transitions experimentally determined for **1a**−**8a**/**8′a** and calculated for the corresponding homologues **1b**−**8b**/**8′b**. As outlined in **Figure 8**, the thermally treated solution of the congeners **1a** and **1b** showed the expected compounds with the mass transitions of all the potential degradation products. In addition, further structure confirmation of the reaction products was performed by specific MS fragmentation experiments (data not shown). These data clearly show that the proposed reaction pathway leading to the formation of the degradation products **1**−**8**/**8′** is independent from the alkanoyl side chain of the corresponding β-acid.

In conclusion, HPLC-MS/MS operating in the MRM mode allowed for the first time a simultaneous detection and quantitation of bitter-tasting β-acid transformation products in authentic beer samples without any sample cleanup steps. These studies were able to demonstrate remarkable differences in the concentrations of the individual β-acid transformation products depending on the type of beer. Further studies are currently under investigation to answer the question as to whether these differences are due to the use of different hop types such as aroma hops and bitter hops, different hop products such as fresh cones, pellets, or ethanolic or carbon dioxide extracts, or some process-specific downstream technologies used in beer manufacturing.

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Supporting Information Available: Mass spectrometric parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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