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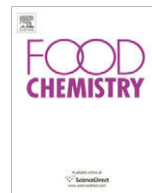

Structure determination and sensory evaluation of novel bitter compounds formed from β-acids of hop (*Humulus lupulus* L.) upon wort boiling

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ABSTRACT

In order to screen for the bitter compounds generated from hop-derived precursors during the wort boiling process, an ethanolic hop extract was fractionated; the fractions obtained were thermally treated under model wort boiling conditions and, then, sensorially evaluated for their bitterness. Besides the isomerisation of the α-acids into the intensely bitter iso-α-acids, the bitterness of the fraction containing the β-acids was also found to be enhanced after wort boiling. To gain first insights into the β-acid-derived bitter compounds, the β-acid colupulone was isolated, thermally treated under wort boiling conditions and, then, investigated for bitter tasting degradation products by means of a taste dilution analysis (TDA). Besides the cohulupone, five previously unreported bitter-tasting colupulone degradation products, all of which exhibited a lingering, β-acid-like bitter taste with low recognition thresholds between 37.9 and 90.3 μmol/l, were isolated and their structures determined as two tricyclocolupone epimers, two dehydrotricyclocolupone epimers, and nortricyclocolupone, respectively, by means of LC–TOF–MS and 1D/2D-NMR spectroscopy.

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1. Introduction

Over centuries, hop (*Humulus lupulus* L.) is used as an essential ingredient in the manufacturing of beer since its components add the typical bitter taste and contribute to the attractive aroma of the final beverage. The bitter principles of hops can be divided into the α- and β-acids **1**–**6**, varying in the second isoprenyl side chain at position C(10), as well as some prenylflavonoids, such as the xanthohumol (**7**) present in the resin fraction of hop (Fig. 1). Besides their bitter taste activity, some of these hop ingredients are well accepted to favour foam stability and, due to their antimicrobial activity, are known to increase the shelf-life of the final beer (De Keukeleire, 2000).

Multiple research studies have been focussed, in the past, on the volatile aroma components in hops and beers (Fritsch & Schieberle, 2005; Kaltner, Steinhaus, Mitter, Biendl, & Schieberle, 2003; Palamand & Aldenhoff, 1973; Schieberle, 1991), as well as on the transformation of the hop α-acids into the corresponding *cis*- and *trans*-iso-α-acids during the wort boiling process (Jascula, Kafarski, Aerts, & De Cooman, 2008; Verzele & De Keukeleire, 1991). These *cis*- and *trans*-iso-α-acids have been reported as important bitter compounds in beer (Kowaka & Kokubo, 1976; Verzele, Jansen, & Ferdinandus, 1970), whereas their progenitors, the α-acids, seem not to contribute to the beer bitterness (Fritsch & Shellhammer, 2007). Contrary to the well understood re-arrangement of the α-acids into the *cis*- and *trans*-iso-α-acids, the information available on bitter compounds generated from the β-acids upon wort boiling is rather scarce. Although some oxidative degradation products of β-acids, such as, e.g., so-called lupoxes, lupdoxes, lupdeps, and lupdols (Kokubo, Kowaka, & Kuroiwa, 1971; Kowaka, Kokubo, & Kuroiwa, 1972, 1973), as well as tricyclo-oxycolupulones (Verzele, Van de Velde, & Dewaele, 1983), are reported in literature, the sensory impact of these compounds on beer bitterness is unclear.

As the concentrations of such reaction products liberated upon wort boiling from hop-derived precursor molecules are too low in beer samples to enable their unequivocal structure determination, laboratory scale model systems have been developed to mimic the reactions occurring during the wort boiling (Malowicki & Shellhammer, 2005, 2006; Spetsig, 1955). Such model investigations revealed that the isomerisation of the α-acids is catalyzed by divalent cations and, in particular, by magnesium ions, as well as by increasing the solubility of the α-acids, e.g., by performing the laboratory wort boiling in water/methanol mixtures (Jaskula et al., 2007). Such model systems seem to be a suitable tool for

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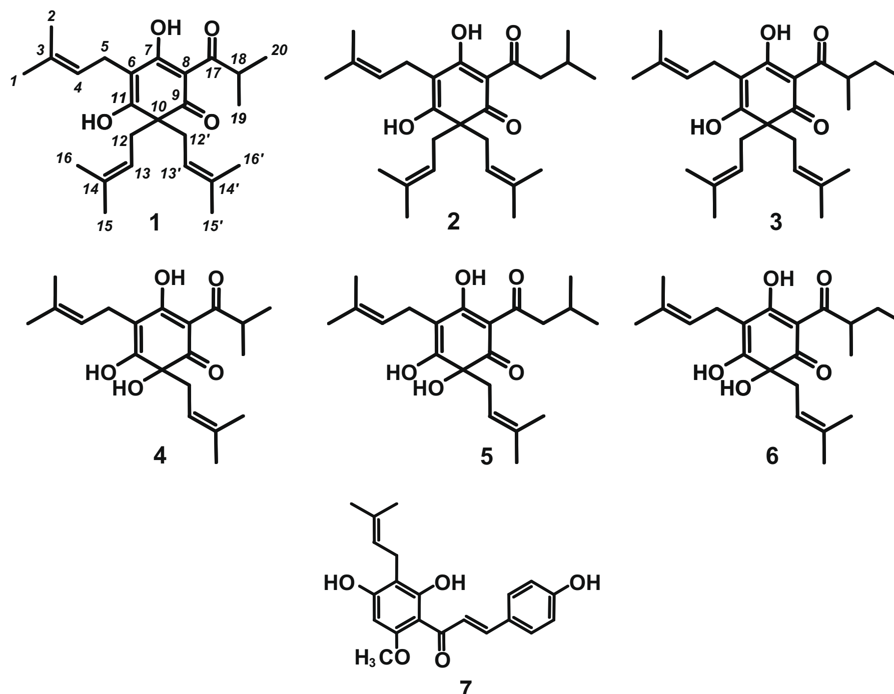

Fig. 1. Chemical structures of the hop β-acids colupulone (**1**), lupulone (**2**), and adlupulone (**3**), the hop α-acids cohumulone (**4**), humulone (**5**), and adhumulone (**6**), as well as the prenylflavonoid xanthohumol (**7**).

investigating the formation of previously unknown bitter compounds generated from hop precursors upon wort boiling.

The objective of the present investigation was to fractionate a hop extract on a preparative scale, to thermally treat each hop fraction under model wort boiling conditions, and to locate bitter taste compounds by means of sensory-directed fractionation. The bitter compounds detected were then identified by means of LC–MS and NMR spectroscopy and their bitter recognition thresholds determined by means of human sensory evaluation.

2. Materials and methods

2.1. Chemicals and materials

The following chemicals were obtained commercially: formic acid, ethanol, magnesium chloride (Merck, Darmstadt, Germany); hydrochloric acid, sodium hydroxide (Riedel-de-Haen, Seelze, Germany); solvents were of HPLC grade (Merck, Darmstadt, Germany); deuterated solvents were from Euriso-top (Saarbrücken, Germany). Deionised water used for chromatography was purified by means of a Milli-Q Gradient A10 system (Millipore, Billerica, USA). For sensory analysis, bottled water (Evian, low mineralisation: 405 mg/l) was adjusted to pH 4.4 with trace amounts of formic acid prior to use. An ethanolic hop extract (Hopsteiner ethanolic extract, Hallertauer Taurus) was obtained from the German hop industry.

2.2. Preparative fractionation of the hop extract

An aliquot (2.0 g) of the hop extract was dissolved in methanol (5.0 ml) and, after filtration, was placed onto the top of a glass column filled with LiChroprep, 25–40 μm, RP-18 material (Merck, Darmstadt, Germany). Chromatography was performed by flushing the column with mixtures (150 ml each) of acetonitrile/water 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. Each fraction was collected and the solvent removed under vacuum. The residue was taken up in water, freeze-dried twice, and kept at −18 °C prior to use.

2.3. Model wort boiling experiments

An aliquot of each individual RP-18 fraction, namely fraction I (20.5 mg), II (2.1 mg), III (3.2 mg), IV (3.2 mg), V (33.2 mg), VI (142.4 mg), VII (58.3 mg), VIII (38.0 mg), and IX (39.7 mg), respectively, was taken up in methanol (5 ml) and divided into two equal aliquots. After removing the solvent under vacuum, one aliquot was freeze-dried twice, the other aliquot was mixed with an aqueous solution of $MgCl_2$ (1.55 mol $MgCl_2$/l; 2.5 ml) and the pH value was adjusted to 5.8 with an aqueous NaOH solution (0.1 mol/l in water). This solution obtained from each RP-18 fraction was thermally treated in a closed vial (10 ml) in a laboratory oven for 1 h at 110 °C. After cooling, the reaction mixtures were placed onto the top of a Strata C18-E SPE cartridge (10 g/60 ml, Phenomenex) preconditioned with water and were first eluted with water to remove the salts, and then eluted with methanol to elute the hop-derived components. After removing the solvent under vacuum, the methanol fractions obtained were freeze-dried, and, then, sensorially evaluated for their bitter intensities and compared to the bitter intensity of the corresponding aliquot of the non-heated RP-18 fraction (Fig. 2).

2.4. Isolation of colupulone (*1*) from the hop β-acid fraction

An aliquot of the RP-18 fraction VIII was separated by semipreparative RP-HPLC, using a 250 mm × 10 mm i.d., 5 μm, ODS-Hypersil C18 column (ThermoHypersil, Kleinostheim, Germany). Monitoring the effluent flow at 272 nm, the chromatography was performed, starting with a mixture (40/60, v/v) of aqueous formic

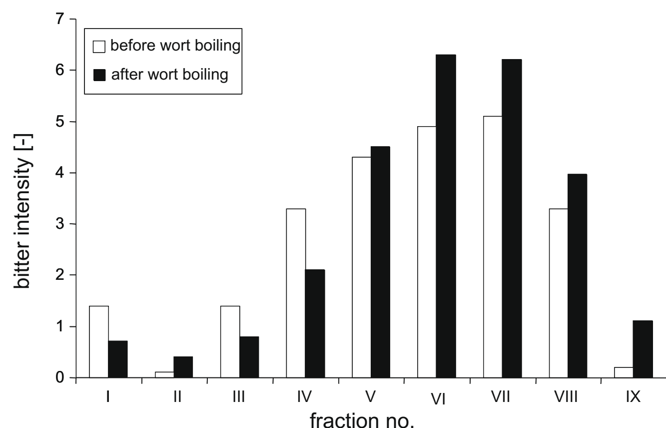

Fig. 2. Bitter intensity of the hop fractions I–IX before and after model wort boiling.

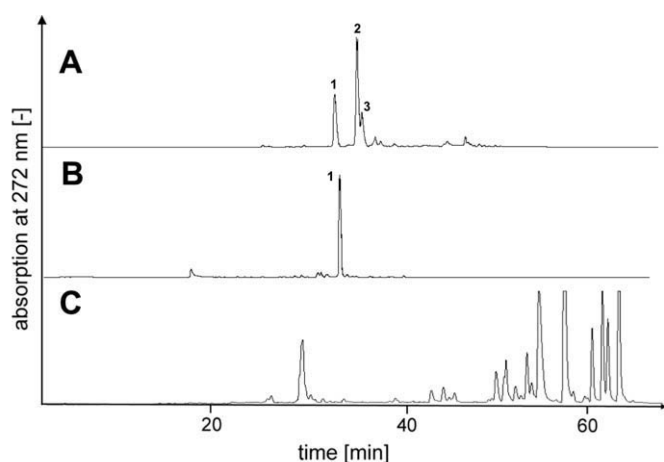

Fig. 3. RP-HPLC chromatogram (λ = 272 nm) of (A) hop fraction VIII, showing the β-acids colupulone (**1**), lupulone (**2**), and adlupulone (**3**), and of purified colupulone (**1**) before (B) and after wort boiling (C).

acid (1% in water) and acetonitrile. Thereafter the acetonitrile concentration was increased to 85% within 5 min, then maintaining the acetonitrile content for 10 min, increasing it thereafter within 5 min to 100% acetonitrile and, finally, maintaining it at this acetonitrile content for an additional 2 min. The effluents of the three peaks, shown in Fig. 3, were collected and, after removing the solvent under vacuum, were freeze-dried twice. By means of UV/Vis, LC–MS/MS, and 1D/2D-NMR experiments, the structure of colupulone (**1**) was unequivocally confirmed in the first eluting fraction. The spectroscopic data of colupulone (**1**) are summarised in Tables 1–5.

2.5. Fractionation of wort-boiled colupulone and isolation of bitter compounds 8–11

Pure colupulone (720 mg) was taken up in methanol (65.0 ml), mixed with an aqueous solution of $MgCl_2$ (1.55 mol $MgCl_2$/l; 65.0 ml), and, after adjusting the pH value to 5.8 with an aqueous NaOH solution (0.1 mol/l in water), was thermally treated in a closed vial (250 ml) in a laboratory oven for 1 h at 110 °C. After removing the solvent in a vacuum, the residue was separated by preparative RP-HPLC using a 250 mm × 10 mm i.d., 5 μm, ODS-Hypersil C18 column (ThermoHypersil, Kleinostheim, Germany). Monitoring the effluent at 272 nm, the chromatography was performed by starting with a mixture (20/80, v/v) of aqueous formic acid (1% in water) and acetonitrile, then increasing the content of organic solvent to 100% within 90 min and, finally, maintaining the acetonitrile content for an additional 5 min. The HPLC run was divided into 25 fractions (Fig. 4) and the corresponding fractions obtained from 30 runs were combined. After removing the solvent under vacuum, the residue of each fraction was taken up in water and freeze-dried twice. The individual fractions were dissolved in their "natural" ratios in 5% aqueous ethanol and were then used for the taste dilution analysis. The HPLC fractions no. 5, and 13–24 were used for purification of the bitter substances by means of semipreparative RP-HPLC, using a 250 mm × 10 mm i.d., 5 μm, ODS-Hypersil C18 column (ThermoHypersil, Kleinostheim, Germany) with a solvent gradient of aqueous formic acid (1% in water) and acetonitrile. The bitter compounds **8**, **9**, **10a**, **10b**, **11a**, and **11b** could be successfully isolated in a purity of more than 98% from HPLC fractions no. 5, 19, 20/21, 22, 23, and 24, respectively, and their structures determined by means of UV/Vis, LC–MS/MS, LC–TOF–MS, and 1D/2D-NMR experiments. The spectroscopic data of compounds **9**, **10a**, **10b**, **11a**, and **11b** are given in Tables 1–5 and Fig. 5.

Cohulupone, **8**, Fig. 6: UV/Vis (0.1% aqueous formic acid/acetonitrile; 20/80, v/v): λ_{max} = 255, 327 nm; LC–TOF–MS: *m/z* 317.1761 ([M−H]$^-$, measured), *m/z* 317.1758 ([M−H]$^-$, calcd. 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

Table 1
UV/Vis and MS data of compounds **1** and **9–11a/b**.[a]

Compound (no).	UV/Vis data[b] λmax (nm)	LC–MS (ESI$^-$) data[c] *m/z* of [M−H]$^-$	LC–MS/MS (ESI$^-$) data[d] *m/z* (%)	LC–TOF–MS data[e] *m/z*
1	275, 332	399	287 (100), 399 (75), 330 (30)	399.2547 ([M−H]$^-$ measured) 399.2547 ([M−H]$^-$ calcd. for [$C_{25}H_{36}O_4$-H]$^-$)
9	283	357	357 (100), 219 (93), 111 (50), 287 (30)	359.2218 ([M+H]$^+$ measured) 359.2216 ([M+H]$^+$ calcd. for [$C_{22}H_{30}O_4$+H]$^+$)
10a	283	397	397 (100), 259 (46), 161 (38), 232 (30)	397.2395 ([M−H]$^-$ measured) 397.2384 ([M−H]$^-$ calcd. for [$C_{25}H_{34}O_4$-H]$^-$)
10b	283	397	397 (100), 259 (60), 232 (26)	397.2367 ([M−H]$^-$ measured) 397.2384 ([M−H]$^-$ calcd. for [$C_{25}H_{34}O_4$-H]$^-$)
11a	283	399	399 (100), 261 (84), 287 (50)	399.2525 ([M−H]$^-$ measured) 399.2540 ([M−H]$^-$ calcd. for [$C_{25}H_{36}O_4$-H]$^-$)
11b	283	399	399 (100), 261 (40), 287 (28), 329 (28)	399.2552 ([M−H]$^-$ measured) 399.2540 ([M−H]$^-$ calcd. for [$C_{25}H_{36}O_4$-H]$^-$)

[a] Arbitrary numbering according to structures **1** and **9**–**11a**/**b** in Figs. 1 and 6.
[b] UV/Vis spectrum was recorded in a mixture (20/80, v/v) of 0.1% aqueous formic acid and acetonitrile.
[c] Pseudomolecular ion recorded by ESI-negative ionisation.
[d] Fragment ions detected by MS/MS experiment (−54 V).
[e] High resolution MS data measured or calculated for the given pseudomolecular ion.

Table 2
Assignment of ^{1}H NMR signals (500 MHz, CD_3OD) of compounds **1** and **9–11a/b**[a].

Proton at carbon	Compound (δ [ppm][b]; multiplicity and intensity in brackets[c])											
	1		**9**		**10a**		**10b**		**11a**		**11b**	
C(1)	1.73	[s, 3H]	–		1.78	[m, 3H]	1.80	[s, 3H]	0.97	[dd, 3H]	1.00	[s, 3H]
C(2)	1.67	[s, 3H]	–		4.79/4.92	[m, 2H]	4.80/4.98	[m, 2H]	0.97	[dd, 3H]	0.99	[s, 3H]
C(3)	–		–		–		–		1.72	[d, 1H]	1.63	[m, 1H]
C(4)	5.02	[t, 1H]	1.64	[m, 2H]	2.07	[dd, 1H]	2.37	[m, 1H]	1.23	[dd, 1H]	1.46	[ddd, 1H]
C(5α)	3.10	[m, 1H]	1.96	[m, 1H]	2.04	[d, 1H]	2.12	[m, 1H]	2.04	[t, 1H]	1.64	[m, 1H]
C(5β)	3.10	[m, 1H]	2.41	[m, 1H]	2.47	[dd, 1H]	2.38	[m, 1H]	2.21	[dd, 1H]	2.61	[ddd, 1H]
C(12α)	2.59	[m, 1H]	1.99	[m, 1H]	1.89	[dd, 1H]	1.96	[m, 1H]	1.81	[m, 1H]	1.90	[dd, 1H]
C(12β)	2.59	[m, 1H]	1.99	[m, 1H]	1.99	[dd, 1H]	2.09	[m, 1H]	1.91	[m, 1H]	2.05	[dd, 1H]
C(13)	4.77	[m, 1H]	2.28	[dd, 1H]	2.40	[dd, 1H]	2.44	[m, 1H]	2.30	[t, 1H]	2.28	[dd, 1H]
C(15)	1.55	[s, 3H]	0.74	[s, 3H]	0.83	[s, 3H]	0.53	[s, 3H]	0.90	[s, 3H]	0.59	[s, 3H]
C(16)	1.57	[s, 3H]	0.97	[s, 3H]	0.96	[s, 3H]	0.97	[s, 3H]	0.98	[s, 3H]	0.99	[s, 3H]
C(12′α)	2.59	[m, 1H]	2.42	[m, 1H]	2.53	[d, 1H]	2.45	[m, 1H]	2.58	[dd, 1H]	2.39	[dd, 1H]
C(12′β)	2.59	[m, 1H]	2.66	[dd, 1H]	2.60	[dd, 1H]	2.68	[dd, 1H]	2.47	[dd, 1H]	2.68	[dd, 1H]
C(13′)	4.77	[m, 1H]	5.21	[m, 1H]	5.22	[m, 1H]	5.21	[m, 1H]	5.20	[m, 1H]	5.21	[m, 1H]
C(15′)	1.55	[s, 3H]	1.68	[s, 3H]	1.69	[m, 3H]	1.69	[s, 3H]	1.68	[d, 3H]	1.69	[s, 3H]
C(16′)	1.57	[s, 3H]	1.70	[s, 3H]	1.69	[m, 3H]	1.70	[s, 3H]	1.69	[d, 3H]	1.71	[s, 3H]
C(18)	3.98	[m, 1H]	3.97	[ddd, 1H]	3.82	[m, 1H]	3.95	[ddd, 1H]	3.74	[m, 1H]	3.92	[m, 1H]
C(19)	1.09	[d, 3H]	1.13	[d, 3H]	1.08	[d, 3H]	1.13	[d, 3H]	1.09	[d, 3H]	1.12	[d, 3H]
C(20)	1.09	[d, 3H]	1.14	[d, 3H]	1.12	[d, 3H]	1.14	[d, 3H]	1.08	[d, 3H]	1.12	[d, 3H]

[a] Arbitrary numbering according to structures **1** and **9–11a/b** in Figs. 1 and 6.
[b] Chemical shift of proton in relation to CD_3OD.
[c] Determined from 1D NMR spectrum.

Table 3
Assignment of homonuclear ^{1}H, ^{1}H connectivities (500 MHz, CD_3OD) of **1** and **9–11a/b**[a].

Proton at carbon	Compound (connectivity[b] with proton no.)					
	1	**9**	**10a**	**10b**	**11a**	**11b**
C(1)			H–C(2α; β)	H–C(2α; β)	H–C(3)	H–C(3)
C(2α)			H–C(1)	H–C(1, 2β, 3, 4)	H–C(3)	H–C(3)
C(2β)			H–C(1)	H–C(1, 2α)		
C(3)					H–C(1, 2, 15, 16)	H–C(1, 2)
C(4)	H–C(5)	H–C(5β)	H–C(5α; β)	H–C(2α, 5α)	H–C(5α; β)	H–C(5α; β)
C(5α)	H–C(6)	H–C(5β)	H–C(4, 5β)	H–C(4, 5β)	H–C(4)	H–C(4, 5β)
C(5β)	H–C(6)	H–C(4, 5β)	H–C(4, 5α)	H–C(5α)	H–C(4)	H–C(4, 5α)
C(12α)	H–C(13)	H–C(13)	H–C(13)	H–C(12β, 13)	H–C(12β, 13)	H–C(12β, 13)
C(12β)	H–C(13)	H–C(13)	H–C(13)	H–C(13)	H–C(12α, 13)	H–C(12α, 13)
C(13)	H–C(12α; β)	H–C(12α; β)	H–C(12α; β)	H–C(12α; β, 15, 16)	H–C(12α; β)	H–C(12α; β)
C(15)		H–C(16)		H–C(13, 16)	H–C(3)	
C(16)		H–C(15)		H–C(13, 15)	H–C(3)	
C(12′α)	H–C(13′)	H–C(12′β, 13′)	H–C(12′β, 13′, 15′, 16′)	H–C(13′, 15′, 16′)	H–C(12′β, 13′)	H–C(12′β, 13′, 15′, 16′)
C(12′β)	H–C(13′)	H–C(12′α, 13′, 15′, 16′)	H–C(12′α, 13′, 15′, 16′)	H–C(13′, 15′, 16′)	H–C(12′α, 13′)	H–C(12′α, 13′)
C(13′)	H–C(12′α; β)	H–C(12′α; β, 15′, 16′)	H–C(12′α; β, 15′, 16′)	H–C(12′α; β, 15′, 16′)	H–C(12′α; β, 15′, 16′)	H–C(12′α; β, 15′, 16′)
C(15′)		H–C(13′)	H–C(12′α; β, 13′, 16′)	H–C(12′α; β, 13′)	H–C(13′)	H–C(12′α, 13′)
C(16′)		H–C(13′)	H–C(12′α; β, 13′, 15′)	H–C(12′α; β, 13′)	H–C(13′)	H–C(12′α, 13′)
C(18)	H–C(19, 20)	H–C(19, 20)	H–C(19, 20)	H–C(19, 20)	H–C(19, 20)	H–C(19, 20)
C(19)	H–C(18)	H–C(18)	H–C(18)	H–C(18)	H–C(18)	H–C(18)
C(20)	H–C(18)	H–C(18)	H–C(18)	H–C(18)	H–C(18)	H–C(18)

[a] Arbitrary numbering according to structures **1** and **9–11a/b** in Figs. 1 and 6.
[b] Homonuclear ^{1}H,^{1}H connectivities obtained by means of a DQF–COSY experiment.

[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)].

2.6. Analytical sensory experiments

2.6.1. Panel training

In order to familiarise the subjects with the taste language used by the sensory group and to get them trained in recognising and distinguishing qualities of oral sensations, twelve assessors with no history of known taste disorders (five women and seven men, aged 25–40 years) participated in weekly training sessions. For training and classification of bitter taste, solutions of $MgSO_4$ (166 mmol/l) representing a short-lasting, metallic bitter taste quality, salicin (1.4 mmol/l), imparting a long-lasting bitter taste sensation, perceived mainly at the back of the tongue as well as the throat, and caffeine (8.0 mmol/l), providing a long-lasting bitterness perceived all over the oral cavity, were used as references. Sensory analyses were performed in a sensory panel room at 22–25 °C in three independent sessions. To prevent cross-modal interactions with odorants, the panellists used nose-clips.

2.6.2. Precautions taken for sensory analysis of fractions

Prior to sensory analysis, the fractions isolated were suspended in water, and, after removing the volatiles in a high vacuum (<5 m Pa), were freeze-dried twice. GC/MS and ion chromatographic analysis revealed that the fractions treated by that

Table 4
Assignment of ^{13}C NMR signals (125 MHz, CD_3OD; DEPT-135) of **1** and **9–11a/b**[a].

Carbon no.	Compound (δ [ppm][b])											
	1		**9**		**10a**		**10b**		**11a**		**11b**	
C(1)	16.5	[CH_3]	–		23.1	[CH_3]	24.2	[CH_3]	22.8	[CH_3]	23.3	[CH_3]
C(2)	24.2	[CH_3]	–		113.7	[CH_2]	113.9	[CH_2]	23.9	[CH_3]	23.3	[CH_3]
C(3)	131.0	[C]	–		145.6	[C]	144.8	[C]	29.5	[CH]	30.9	[CH]
C(4)	121.5	[CH]	43.5	[CH_2]	56.4	[CH]	58.2	[CH]	56.6	[CH]	60.5	[CH]
C(5)	20.2	[CH_2]	21.3	[CH_2]	26.9	[CH_2]	25.4	[CH_2]	26.5	[CH_2]	27.1	[CH_2]
C(6)	110.9	[C]	75.5	[C]	72.7	[C]	72.3	[C]	71.8	[C]	71.8	[C]
C(7)	189.3	[C]	*n.s.a*	[C]	*n.s.a*	[C]	*n.s.a*	[C]	*n.s.a*	[C]	*n.s.a*	[C]
C(8)	*n.s.a*	[C]	120.8	[C]	112.3	[C]	109.9	[C]	109.8	[C]	110.0	[C]
C(9)	196.9	[C]	*n.s.a*	[C]	*n.s.a*	[C]	*n.s.a*	[C]	*n.s.a*	[C]	*n.s.a*	[C]
C(10)	57.6	[C]	69.7	[C]	68.1	[C]	69.1	[C]	68.1	[C]	69.0	[C]
C(11)	172.8	[C]	209.6	[C]	209.0	[C]	209.1	[C]	207.2	[C]	209.7	[C]
C(12)	37.3	[CH_2]	29.6	[CH_2]	31.8	[CH_2]	29.0	[CH_2]	31.8	[CH_2]	28.7	[CH_2]
C(13)	117.7	[CH]	56.9	[CH]	57.5	[CH]	59.8	[CH]	59.5	[CH]	58.6	[CH]
C(14)	134.3	[C]	44.3	[C]	44.2	[C]	46.4	[C]	43.5	[C]	46.1	[C]
C(15)	24.2	[CH_3]	22.3	[CH_3]	26.2	[CH_3]	17.3	[CH_3]	25.7	[CH_3]	16.4	[CH_3]
C(16)	16.5	[CH_3]	28.3	[CH_3]	25.1	[CH_3]	27.6	[CH_3]	26.1	[CH_3]	28.6	[CH_3]
C(12′)	37.3	[CH_2]	26.6	[CH_2]	27.2	[CH_2]	26.5	[CH_2]	27.3	[CH_2]	26.5	[CH_2]
C(13′)	117.7	[CH]	120.6	[CH]	121.0	[CH]	120.9	[CH]	121.3	[CH]	121.1	[CH]
C(14′)	134.3	[C]	135.8	[C]	135.3	[C]	135.8	[C]	135.0	[C]	135.7	[C]
C(15′)	24.2	[CH_3]	18.1	[CH_3]	18.1	[CH_3]	18.0	[CH_3]	18.1	[CH_3]	18.0	[CH_3]
C(16′)	16.5	[CH_3]	26.2	[CH_3]	26.1	[CH_3]	26.2	[CH_3]	26.1	[CH_3]	26.2	[CH_3]
C(17)	207.0	[C]	209.7	[C]	209.8	[C]	210.0	[C]	209.1	[C]	210.1	[C]
C(18)	36.0	[CH]	36.3	[CH]	37.0	[CH]	36.6	[CH]	37.3	[CH]	36.8	[CH]
C(19)	17.8	[CH_3]	19.2	[CH_3]	19.2	[CH_3]	19.2	[CH_3]	19.3	[CH_3]	19.2	[CH_3]
C(20)	17.8	[CH_3]	19.3	[CH_3]	19.4	[CH_3]	19.3	[CH_3]	19.4	[CH_3]	19.3	[CH_3]

n.s.a.: no signal assignment, an unequivocal assignment of this carbon atom could not be achieved due to signal overlapping.
[a] Arbitrary numbering according to structures **1** and **9–11a/b** in Figs. 1 and 6.
[b] Chemical shift of carbon atoms in relation to CD_3OD.

procedure were essentially free of the solvents and buffer compounds used. Formic acid, which is considered as "Generally Recognised as Safe" (GRAS) as a flavouring agent for food and feed applications, was used to adjust the pH value of solutions which should be sensorially analyzed, because trace amounts of this acid do not influence the sensory profile of the test solution.

2.6.3. Taste recognition threshold concentrations

Twelve panellists 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 (Frank, Ottinger, & Hofmann, 2001; Scharbert, Holzmann, & Hofmann, 2004a; Scharbert, Jezussek, & Hofmann, 2004b; Stark & Hofmann, 2005). 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.

2.6.4. Sensory analysis of isolated hop fractions

Fractions isolated from hop by means of preparative RP-18 column chromatography were evaluated for their bitter intensities, before and after performing a wort boiling experiment. To achieve this, each fraction was taken up in 5% aqueous ethanol and the pH value was adjusted to 4.4 with aqueous formic acid (1% in water). The bitter intensity of these solutions was judged on a scale from 0 (not detectable) to 10 (strongly detectable).

2.6.5. Taste dilution analysis (TDA)

Isolated HPLC fractions were dissolved in their "natural" concentration ratios, which means that the concentrations were adjusted to their amounts in the hop extract, in a 5% aqueous ethanol solution (10.0 ml; pH 4.4) and, then, sequentially diluted 1:2 with bottled water. The serial dilutions of each of these fractions were presented to the sensory panel in order of ascending concentrations, and each dilution was evaluated for bitterness. The dilution revealing a taste difference between the diluted extract and the blank (control) in a triangle test was defined as the taste dilution (TD) factor (Frank et al., 2001). The TD factors evaluated by ten different assessors in three different sessions were averaged. The TD factors between individuals and separate sessions did not differ by more than one dilution step.

2.7. High performance liquid chromatography (HPLC)

For analytical HPLC, an HPLC apparatus (Varian, Middelburg, Netherlands) consisting of two pumps (ProStar 210), an autosampler (Midas 830, Spark, Aj Emmen, Netherlands), and a ProStar 330 type diode array detector (Varian, Netherlands), monitoring the effluent at a wavelength of 272 nm, were used. Data acquisition was performed with the software Star 6.2 Chromatography Workstation. For analysis of the isolated fractions, an aliquot was dissolved in methanol/water and analyzed on a 250 mm × 4.6 mm i.d., 5 μm, RP-18, ODS-Hypersil column (ThermoHypersil, Kleinostheim, Germany) equipped with a guard column of the same type. Using a 1% aqueous formic acid solution as solvent A and acetonitrile as solvent B, chromatography was started at a flow rate of 1 ml/min with 50% solvent B for 5 min, then increasing solvent B to 100% within 55 min, and finally, maintaining for 5 min at 100% solvent B.

For semi-preparative and preparative HPLC, the system consisted of two PU-2087 type pumps (Jasco, Groß-Umstadt, Germany), a Rh 7725i type Rheodyne injecton valve (Bensheim, Germany), a DG-2080-53 type solvent de-gasser (Uniflows Co., Tokio, Japan), a gradient mixer (Knauer ·ASI, Berlin, Germany), and a UV-detector (Jasco UV PN-2075, Groß-Umstadt, Germany), monitoring the effluent flow at 272 nm. Data acquisition was con-

Table 5
Assignment of heteronuclear ^{1}H, ^{13}C connectivities (500 MHz/125 MHz, CD_3OD) of **1** and **11a**/**b**[a].

Proton at carbon	Compound (connectivity[b] with carbon no.)											
	1		**9**		**10a**		**10b**		**11a**		**11b**	
	^{1}J	$^{2,3,4}J$	^{1}J	$^{2,3,4}J$	^{1}J	$^{2,3,4}J$	^{1}J	$^{2,3,4}J$	^{1}J	$^{2,3,4}J$	^{1}J	$^{2,3,4}J$
C(1)	C(1)	C(3, 4, 6)	–	–	C(1)	C(2, 3, 4, 14)	C(1)	C(2α; β, 3, 13)	C(1)	C(3, 4, 14)	C(1)	C(2, 3, 4, 16)
C(2α)	C(2)	C(3, 4, 6)	–	–	C(2)	C(1, 3, 4, 14, 16)	C(2)	C(1, 13)	C(2)	C(3, 4, 14)	C(2)	C(1, 3, 4, 16)
C(2β)						C(1, 3, 4, 14, 16)		C(1, 13)				
C(3)	–	–	–	–	–	–	–	–	C(3)	C(2, 4)	C(3)	C(1, 2, 4, 5α, β, 14)
C(4)	C(4)	C(1, 2, 5)	C(4)	C(5α; β, 6, 14, 15)	C(4)	C(1, 2, 3, 5α; β, 6, 11, 13, 14, 15, 16)	C(4)	C(2, 3, 5α; β)	C(4)	C(1)	C(4)	C(1, 3, 5β, 14, 15)
C(5α)	C(5)	C(3, 4, 6, 7, 11)	C(5α)	C(4, 6)	C(5α)	C(2, 4, 6, 11)	C(5α)	C(2, 6, 11)	C(5α)	C(3, 4, 6, 11, 14)	C(5α)	C(6, 11)
C(5β)			C(5β)	C(6, 14)	C(5β)	C(6, 11, 14)	C(5β)	C(6)	C(5β)	C(3, 4, 6, 11, 13, 14)	C(5β)	C(6, 11, 14)
C(12α)	C(12)	C(9, 10, 11, 13, 14)	C(12α)	C(10, 11, 12′α; β, 13)	C(12α)	C(10, 12′α; β, 14, 15, 16)	C(12α)	C(11)	C(12α)	C(10, 13, 14)	C(12α)	C(11, , 12′α; β, 13, 14, 15)
C(12β)			C(12β)	C(10, 11, 12′α; β, 13)	C(12β)	C(10, 11, 12′α; β, 13, 14, 15, 16)	C(12β)	C(10, 11, 13, 14)	C(12β)	C(14)	C(12β)	C(10, 11, 12′α; β, 13, 14, 15)
C(13)	C(13)		C(13)	C(6, 11, 14, 16)	C(13)	C(3, 6, 10, 11, 12α; β, 14)	C(13)	C(3, 11, 12α; β, 14)	C(13)	C(6, 11, 12α; β, 14, 15)	C(13)	C(5β, 11, 12α; β, 12′α; β, 14, 15, 16)
C(15)	C(15)	C(10, 12, 13, 14, 16)	C(15)	C(4, 13, 14, 16)	C15)	C(4)	C(15)	C(4, 10, 13, 14, 16)	C15)	C(4, 14, 16)	C15)	C(12′α; β, 13,14)
C(16)	C(16)	C(10, 12, 13, 14, 15)	C(16)	C(4, 13, 14, 15)	C(16)	C(4, 12α; β)	C(16)	C(4, 10, 13, 14, 15)	C(16)	C(4, 14, 15)	C(16)	C(4, 13, 14)
C(12′α)	C(12′)	C(9, 10, 11, 13′, 14′)	C(12′α)	C(10, 11, 12α; β, 13, 13′, 14′)	C(12′α)	C(10, 12α; β, 13′, 14′)	C(12′α)	C(10, 11, 13′, 14′)	C(12′α)	C(10, 11, 12α; β, 13, 13′, 14′)	C(12′α)	C(11, 12β, 14′)
C(12′β)			C(12′β)	C(10, 11, 12α; β, 13′, 14′)	C(12′β)	C(10, 11, 13′, 14′)	C(12′β)	C(10, 11, 13′, 14′)	C(12′β)	C(10, 12α; β, 13′, 14′)	C(12′β)	C(10, 11, 12β, 14′)
C(13′)	C(13′)		C(13′)	C(10, 12′α; β, 15′)	C(13′)	C(12′α; β)	C(13′)	C(12′α; β)	C(13′)		C(13′)	C(15′, 16′)
C(15′)	C(15′)	C(10, 12′, 13′, 14′, 16′)	C(15′)	C(10, 13′, 14′, 16′)	C(15′)	C(10, 13′, 14′, 16′)	C(15′)	C(13′, 14′, 16′)	C(15′)	C(13′, 14′, 16′)	C(15′)	C(13′, 14′)
C(16′)	C(16′)	C(10, 12′, 13′, 14′, 15′)	C(16′)	C(10, 13′, 14′, 15′)	C(16′)	C(10, 13′, 14′, 15′)	C(16′)	C(13′, 14′, 15′)	C(16′)	C(10, 13′, 14′, 15′)	C(16′)	C(10, 13′, 14′)
C(18)	C(18)	C(17, 19, 20)	C(18)	C(8, 17, 19, 20)	C(18)	C(17, 19, 20)	C(18)	C(17)	C(18)		C(18)	C(17, 19, 20)
C(19)	C(19)	C(17, 18)	C(19)	C(17, 18, 20)	C(19)	C(17, 18, 20)	C(19)	C(17, 18)	C(19)	C(17, 18, 20)	C(19)	C(17, 18, 20)
C(20)	C(20)	C(17, 18)	C(20)	C(17, 18, 19)	C(20)	C(17, 18, 19)	C(20)	C(17, 18)	C(20)	C(17, 18, 19)	C(20)	C(17, 18, 19)

[a] Arbitrary numbering according to structures **1** and **9–11a**/**b** in Figs. 1 and 6.
[b] Homonuclear ^{1}H, ^{13}C connectivities obtained by means of a HMQC (^{1}J) and a HMBC experiment ($^{2,3,4}J$), respectively.

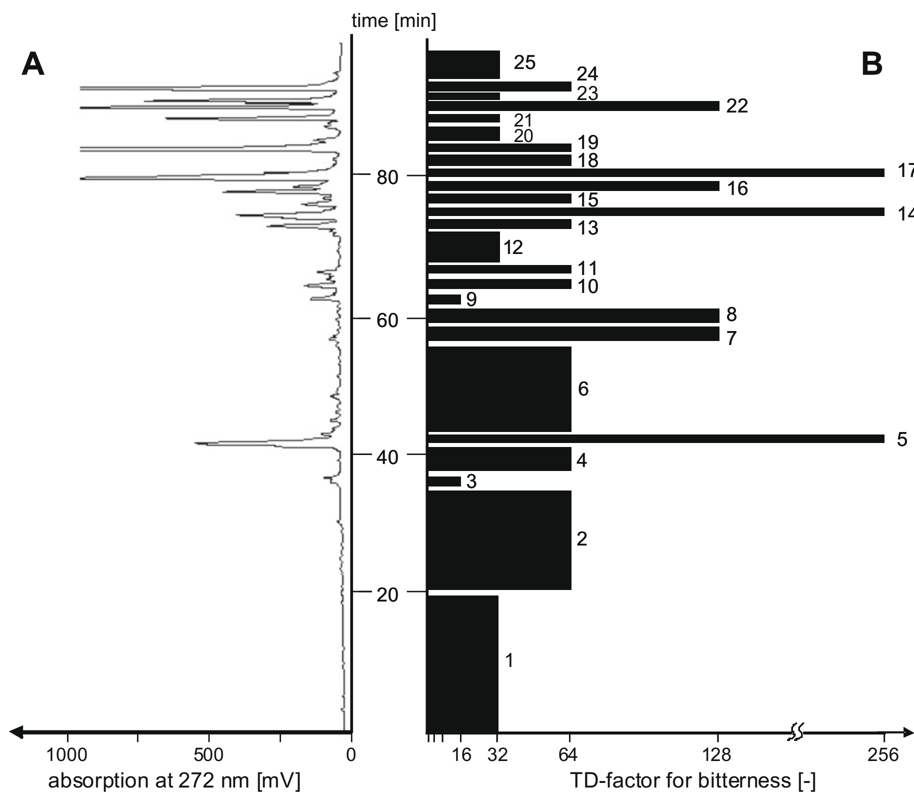

Fig. 4. RP-HPLC chromatogram (λ = 272 nm) (A) and taste dilution (TD)-chromatogram (B) of the wort-boiled colupulone.

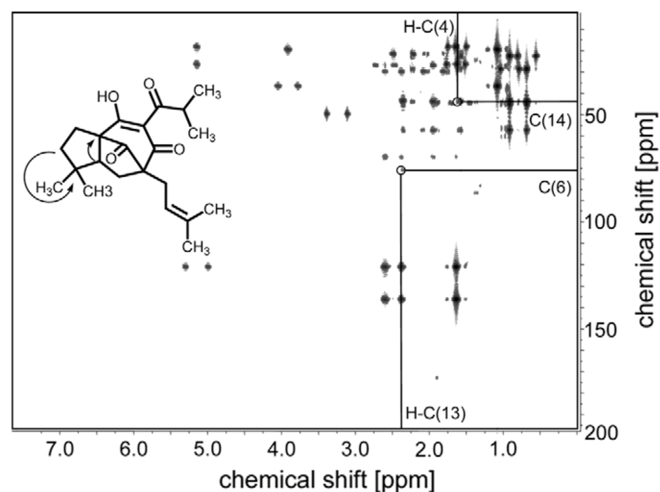

Fig. 5. HMBC spectrum (500 MHz, CD_3OD) and chemical structure of nortricyclocolupone (**9**).

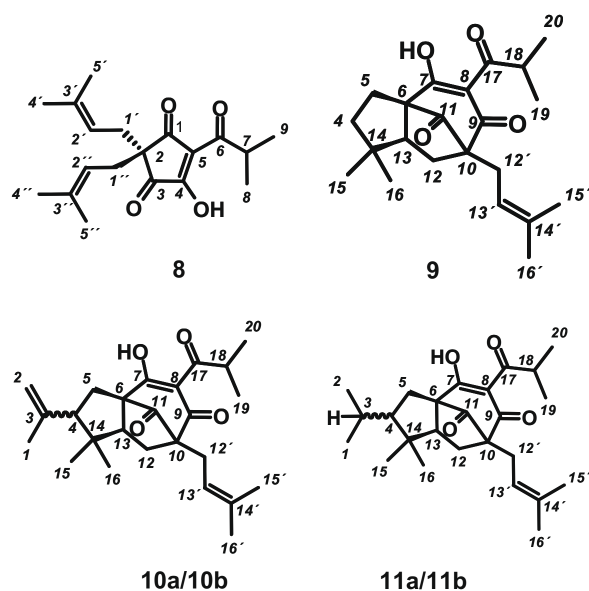

Fig. 6. Chemical structures of nortricyclocolupone (**9**), the dehydrotricyclocolupone epimers **10a** and **10b**, and the tricyclocolupone epimers **11a** and **11b** isolated from a wort-boiled colupulone solution.

ducted by means of the ChromPass V.1.8.6.1. software. HPLC runs were done using a 0.5 or 2.0 ml sample loop and a 250 × 10.0 mm i.d., 5 μm, or 250 × 21.2 mm i.d., 5 μm, RP-18, ODS-Hypersil column (ThermoHypersil, Kleinostheim, Germany) equipped with a guard column of the same type. Chromatography was performed at a flow rate of 4.5 or 20.0 ml/min, starting with a mixture (40/60 or 50/50, v/v) of aqueous formic acid (1% in water) and acetonitrile. Thereafter the acetonitrile concentration was increased to 85% within 5 min or to 90% within 15 min, then maintained for 10 min, increasing thereafter within 5 min to 100% acetonitrile and, finally, maintaining at this acetonitrile content for an additional 2 min.

2.8. Exact mass measurements

High resolution mass spectra of the purified bitter 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 less than 5 mamu.

2.9. ESI–MS/MS experiments

Electrospray ionisation (ESI) mass and the product ion spectra were acquired on a API 4000 QTRAP mass spectrometer (AB Sciex Instruments, Darmstadt, Germany) with direct flow infusion. The isolated fractions were dissolved in water/acetonitrile and directly introduced into the mass spectrometer using a syringe pump. For electrospray ionisation, the ion spray voltage was set at −4500 V in the negative mode. Nitrogen served as curtain gas (20 psi) and the declustering potential was set at −10 to −40 V in the ESI-mode. The collision energies used for the analysis of the individual compounds are given above in the spectroscopic data section.

2.10. Nuclear magnetic resonance spectroscopy (NMR)

The ^{1}H, ^{13}C and 2D-NMR experiments were performed using a 400 MHz DRX and a 500 MHz Avance III spectrometer (Bruker, Rheinstetten, Germany), respectively. Samples were dissolved in CD_3OD and placed in NMR tubes (Schott Professional 178 × 5 mm) prior to measurement. Data processing was performed using XWin-NMR software (version 3.5, Bruker, Rheinsetten, Germany), as well as MestReNova V. 5.1.0-2940 (Mestrelab Research, LA CORUNA, Spain).

3. Results and discussion

3.1. Screening for precursors of bitter compounds

Aimed at identifying the hop precursor compounds liberating bitter tastants upon the wort boiling process, an ethanolic hop extract was fractionated by means of preparative RP-18 column chromatography to give nine fractions I–IX. An aliquot of each fraction was mixed with an aqueous methanolic $MgCl_2$ solution (pH 5.8) and was then thermally treated in a closed vial for 1 h at 110 °C, using laboratory wort boiling conditions. The heated fractions were then used to screen for bitter taste compounds generated, whereas the bitter potential of their precursors was evaluated in the corresponding non-treated fractions.

In order to overcome the limited water-solubility of these fractions and to evaluate their bitter impacts in a beer-like medium, each individual fraction was dissolved in 5% aqueous ethanol (pH 4.4) and, then, rated for their bitterness on a scale from 0 (no bitterness detectable) to 10 (intensely bitter). The sensory evaluation revealed that the entire set of the non-treated hop fractions I–IX exhibited some bitterness. Among these, fractions V–VII were judged to have the highest bitter intensities, between 3.3 and 5.2 (Fig. 2). After application of the laboratory wort boiling process to these nine fractions, the fractions numbered I, III, and IV were rated with lower bitter intensities when compared to the corresponding non-heated fraction. In contrast, the wort boiling process was found to increase the bitter intensity of the other six fractions and, in particular, that of fractions numbered VI–VIII.

Comparison of chromatographic (RP-HPLC) and spectroscopic data (UV/Vis, LC–MS/MS) with those of the reference compounds, followed by co-chromatography, demonstrated that the well-known α-acids cohumulone, humulone, and adhumulone (**4**–**6**, Fig. 1) were present in fractions VI and VII, and xanthohumol (**7**) was eluting in fraction no. III. After the laboratory wort boiling process, the α-acids were found to be isomerised into the more intensely bitter-tasting iso-α-acids (data not shown) which are reported as key bitter compounds in wort and beer (Jascula et al., 2008; Verzele & De Keukeleire, 1991). Due to the relatively high bitter intensity, the following experiments were focused on the heat-treated fraction, no. VIII (Fig. 2), which were found to contain the pure β-acids **1**–**3** prior to thermal treatment (**A**, Fig. 3).

In order to determine the structures of the bitter compounds generated from precursors in fraction VIII upon wort boiling, β-acids were isolated and purified in preparative amounts. As thermal treatment of the mixture of the β-acids **1**–**3** led to an enormous multiplicity of degradation products which did not allow a straightforward isolation and identification of the bitter compounds (data not shown), colupulone (**1**) was isolated and purified by means of semipreparative RP-HPLC (**B**, Fig. 3) and, then, thermally treated by means of the wort boiling process. RP-HPLC analysis of the processed colupulone solution indicated the generation of multiple reaction products (**C**, Fig. 3).

3.2. Taste dilution analysis of wort-boiled colupulone

To focus the laborious structure determination experiments on the important bitter compounds, it was necessary to separate the strongly bitter tasting molecules from the less bitter and tasteless compounds, respectively. To achieve this, the thermally treated colupulone solution was separated into 25 fractions (**A**, Fig. 4) and the solvents were removed in high vacuum. In order to overcome the limited water-solubility of these fractions and to evaluate their bitter impacts in a beer-like medium, each individual fraction was dissolved in 5% aqueous ethanol (pH 4.4) and, then, evaluated for its bitter impact by means of the taste dilution analysis (**B**, Fig. 4). Because of their high TD factor of 256, fractions 5, 14, and 17 were evaluated with the highest impact for bitterness, followed by fractions 7, 8, 16 and 22 with a TD-factor of 128. In comparison, the remaining fractions showed somewhat lower TD-factors for bitterness. By means of semipreparative RP-HPLC, we then tried to isolate and purify the key bitter compounds from the individual bitter tasting fractions.

3.3. Structure determination of bitter-tasting colupulone degradation products

LC–MS/MS(ESI^-) analysis of the bitter tasting compound isolated from HPLC fraction no. 5, judged with a high TD factor of 256, showed *m/z* 317 to be the pseudo molecular ion $[M-H]^-$, thus suggesting a molecular mass of 318 Da. This was confirmed by LC–TOF–MS experiments which revealed an empirical formula of $C_{19}H_{26}O_4$. ^{1}H NMR, as well as homonuclear H,H chemical shift correlation experiments (COSY), showed resonances integrating for a total of 25 carbon-bound protons, among which, 18 protons, resonating between 1.09 and 1.57 ppm, could be assigned as six methyl groups by means of a DEPT-135 measurement. The coupling constant of 7.8 Hz, measured between the methine proton H–C(2′) and the methylene protons H-C(1′), as well as between H–C(2″) and H–C(1″), respectively, indicated the presence of two intact isoprenyl side chains in the molecule. In addition, the coupling between the methine proton H–C(7) and the methyl groups H–C(8) and H–C(9) confirmed that, also, the isobutyryl moiety of the β-acid remained intact in that colupulone degradation product. Based on the careful interpretation of heteronuclear correlation experiments (HMQC, HMBC) and comparison of the data obtained with those reported in the literature (Stevens & Wright, 1963; Wright, 1963), the chemical structure of the bitter compound in HPLC fraction no. 5 could be unequivocally identified as the cohulupone (**8**, Fig. 6).

As the very low amounts of the compounds in fractions no. 7 and 8, as well as the high reactivity and instability of the bitter compounds in fractions nos. 14 and 17, did not allow their pure isolation in amounts suitable for their spectroscopic structure determination, the following identification experiments were focused on the bitter molecules in fractions 19–24 (Fig. 4).

The UV/Vis spectrum of the bitter compound **9** in fraction 19 showed only one single absorption maximum at 283 nm (Table 1). Comparison of this UV/Vis spectrum with that obtained for the precursor colupulone (**1**), exhibiting typical maxima at 275 and 332 nm, undoubtedly indicated that the chromophoric system of **1** had changed upon wort boiling. LC–TOF–MS of the bitter compound gave *m/z* 359.2218 as the pseudo molecular ion $[M+H]^+$ and indicated $C_{22}H_{30}O_4$ as the molecular formula (Table 1). Comparison of these data with those obtained for colupulone (**1**) clearly indicated that three carbon atoms were split off from **1** to give the bitter compound **9**. The ^{1}H NMR spectrum showed 16 resonance signals integrating for a total of 29 protons. In comparison to the eight methyl groups present in colupulone (**1**), the ^{1}H NMR of the bitter compound **9** showed only six methyl groups resonating between 0.74 and 1.70 ppm (Table 2). The resonance signals of the protons H–C(18–20) observed at 3.97, 1.14, and 1.13 ppm, as well as the signals of the protons H–C(12′), H–C(13′), H–C(15′) and H–C(16′), resonating at 2.42/2.66, 5.21, 1.68, and 1.70 ppm, indicated that the isobutyryl side chain, as well as one of the isoprenyl side chains of the precursor **1,** remained unaltered in the degradation product. But the existence of the methylene group H–C(4), resonating at 1.64 ppm as a multiplet, and its connectivity to the methylene protons H–C(5) detected in compound **9,** were identified as major differences between the structure of colupulone and its degradation product (Tables 2 and 3). Also, the ^{13}C NMR spectrum, as well as the data obtained from heteronuclear correlation experiments (HMQC and HMBC) signified differences between the structures of **9** and its precursor (**1**) (Tables 4 and 5). The HMBC data demonstrated the attachment of the isoprenyl carbon skeleton C(12′–16′) at the quaternary bridge atom C(10) and the presence of novel bonds between the carbon C(6) or C(4) and the carbon C(13) or C(14) as part of the former isoprenyl carbon skeleton C(12–16) of the β-acid. This is well in line with the heteronuclear ^3J coupling between H–C(13) and the methylene carbon atom C(4), as well as between the methylene protons H–C(4) and the carbons C(14) and C(15) (Fig. 5). Careful assignment of all spectroscopic data enabled the unequivocal identification of the bitter compound in fraction 19 as the structure **9**, coined nortricyclocolupone (Fig. 6). To the best of our knowledge, this compound has not been previously reported in the literature.

The UV/Vis spectrum of the bitter compound **10a,** isolated from HPLC fractions 20 and 21, showed an absorption maximum at 283 nm, thus being identical to that of compound **9**. LC/MS analysis showed *m/z* 397 as the pseudo molecular ion $[M-H]^-$ and LC–TOF–MS analysis revealed $C_{25}H_{34}O_4$ as the sum formula of the bitter compound (Table 1). The ^{1}H NMR spectrum showed a total of 33 protons, among which 21 protons were assigned as the methyl groups: H–C(1), H–C(15), H–C(15′), H–C(16), H–C(16′), H–C(19), and H–C(20) (Table 2). As with compound **9**, COSY, DEPT-135, and HMBC experiments demonstrated that the isoprenyl side chain with the carbon skeleton C(12′–16′), as well as the isobutanoyl side chain with the carbon atoms C(17-20), remained unaltered in **10a** when compared to the precursor colupulone (**1**) (Tables 3–5). Further comparison of the chemical shifts of the ^{1}H and ^{13}C NMR resonances, as well as the homo- and heteronuclear connectivities demonstrated major similarities between the structures **9** and **10a**. But, in contrast to **9**, the HMBC experiment of compound **10a** revealed that an additional 2-isopropylene moiety, with carbon resonances at 23.1 [C(1)], 113.7 [C(2)], and 145.6 ppm [C(3)], is bound to carbon atom C(4), which was shifted from 43.5 (**9**) to 56.4 ppm (**10a**) and was unequivocally detected as a methine carbon atom in the DEPT-135 and HMQC experiments. Based on the careful consideration of all the 1D/2D-NMR, as well as LC–MS/MS data, the bitter compound **10a** isolated from fractions 20 and 21 could be unequivocally identified as the dehydrotricyclocolupone diastereomer **10a** (Fig. 6), which to the best of our knowledge has not previously been reported.

UV/Vis and LC–MS/MS analysis of the bitter compound **10b** isolated from the HPLC fraction 22 revealed data identical to those found for compound **10a** (Table 1). Also, the ^{1}H and ^{13}C NMR data, as well as the homo- and heteronuclear connectivities measured for **10b,** were very similar to those obtained for **10a** (Tables 2–5), thus indicating the presence of another dehydrotricyclocolupone diastereomer. As the largest chemical shift difference of 0.3 ppm, between the protons in **10a** and **10b,** was found for the methine proton H–C(4) and the neighbouring methyl protons H–C(15), the structure of **10b** was proposed to differ from that of **10a** by the chirality at carbon C(4). This was further strengthened by the observation that the methine carbon C(4) is downfield-shifted, by 3.8 ppm, from 56.4 (**10a**) to 58.2 ppm (**10b**) and the methyl carbon C(15) is strongly upfield-shifted, by 9 ppm, from 26.2 (**10a**) to 17.3 ppm (**10b**), whereas the resonances of the other carbon atoms in **10a** and **10b** differed just to a minor extent. Based on these considerations, the structure of compound **10b,** isolated from HPLC fraction 22, was proposed to be a dehydrotricyclocolupone epimer (Fig. 6), most likely differing from **10a** by another chirality at C(4).

UV/Vis spectroscopy of the bitter compound **11a** isolated from HPLC fraction 23 revealed an absorption spectrum identical to that obtained for the dehydrotricyclocolupone epimers **10a**/**10b**. But, LC–TOF–MS analysis of **11a** revealed *m/z* 399.2525 as the pseudo molecular ion and indicated a sum formula of $C_{25}H_{36}O_4$, thus differing by two hydrogens from the sum formula $C_{25}H_{34}O_4$ of **10a**/**10b** (Table 1). This was further strengthened by comparing the ^{1}H NMR spectra, showing 33 protons for **10a/10b** and 35 protons for **11a** (Table 2). As the NMR data of compound **11a** indicated the presence of eight, instead of seven, methyl groups and, when compared to **10a**/**10b**, the largest chemical shift differences for the protons H–C(1), H–C(2), and H–C(4), as well as an additional methine proton H–C(3) resonating at 1.72 ppm, the exocyclic double bond C(2)–C(3) in the isopropylene moiety of **10a**/**10b** was proposed to be hydrogenated to an isopropyl moiety in **11a**. This was further strengthened by heteronuclear correlation experiments (HMQC and HMBC), which showed the linkage between the 2-isopropyl moiety and the carbon C(4) of the five-membered ring in the target molecule, as well as the observation that the remaining protons and carbons in **11a** show similar chemical shifts and coupling patterns to those found for **10a**/**10b** (Tables 3–5). Based on the careful consideration of all the 1D/2D-NMR, as well as LC–MS/MS data, the bitter compound **11a** isolated from fraction 23 could be unequivocally identified as a tricyclocolupone diastereomer (Fig. 6) which, to the best of our knowledge, has not previously been reported.

The bitter taste molecule **11b,** isolated from HPLC fraction 24, showed identical UV/Vis and LC–MS/MS data and rather similar 1D- and 2D-NMR data to those found for compound **11a** (Table 1), thus indicating the presence of another tricyclocolupone diastereomer. As the largest chemical shift differences of 1.46 and 0.59 ppm were observed for H–C(4) and the neighbouring methyl protons H–C(15) in **11a** and **11b**, respectively, the structure of **11b** was proposed to differ from that of **11a** by the chirality at carbon C(4) (Table 2). This was further strengthened by the observation that the methine carbon C(4) is downfield-shifted, by 3.9 ppm, from 56.6 (**11a**) to 60.5 ppm (**11b**) and the methyl carbon C(15) is strongly upfield-shifted, by 9.3 ppm, from 25.7 (**11a**) to 16.4 ppm (**11b**), whereas the resonances of the other carbon atoms in **11a** and **11b** differed just to a minor extent (Table 4). Based on these considerations, the structure of compound **11b,** isolated from HPLC fraction 24, was proposed as a tricyclocolupone epimer (Fig. 6), most likely differing from **11a** by another chirality at C(4).

3.4. Proposed reaction pathways leading to the formation of bitter compounds *9–11a/b*

Based on the structures of the bitter taste compounds identified, a reaction cascade showing the formation of the compounds **9–11a/b** from the β-acid colupulone (**1**) is depicted in Fig. 7. Upon wort boiling, a hydrogen radical is abstracted from the hydroxyl function HO–C(11) of colupulone (**1**) to give its corresponding alkoxy radical (1) which, upon mesomerisation, followed by radical cyclisation, generates the novel C(6)–C(12) linkage in the transient alkyl radical intermediate (2). This intermediate undergoes a second cyclisation by the attack of the alkyl radical at the double bond of the isoprenyl moiety at C(6), thus giving rise to a novel C(4)–C(14) linkage and the intermediary isopropyl radical 3, exhibiting a chiral centre at C(4). This intermediate (3) is functioning as a switch and allows three alternative reaction routes, leading to the five bitter compounds **9–11a/b** (Fig. 7). Proton abstraction at C(2) of the isopropyl radical leads to the formation of a double bond in the isopropenyl moiety of the epimers **10a** and **10b**. Saturation of the radical 3 by abstracting a proton from a hydrogen donor molecule (RH), such as colupulone (**1**), gives rise to the epimeric bitter compounds **11a** and **11b**.

Alternatively, compound **9** might be formed from 3 via a classical lipid peroxidation mechanism (Fig. 7). Upon addition of triplet oxygen, a hydroperoxy radical (4) is formed, which is saturated by hydrogen abstraction from a donor molecule (RH) to give the hydroperoxide 5 as a key intermediate, which was reported earlier as a ß-acid oxidation product (De Potter & De Keukeleire, 1978). Cleavage of this hydroperoxide, induced by a Fenton-type reaction with transition metals or thermolytic cleavage, might liberate the alkoxyradical 6 which, upon splitting off one molecule of 2-propanone, releases the nortricyclocolupone radical 7. Saturation of this alkyl radical by hydrogen abstraction from another substrate then gives rise to the bitter compound **9**.

3.5. Sensory evaluation of bitter compounds

In order to evaluate the sensory activity of colupulone (**1**), as well as its wort-boiling products **8–11a/11b**, the purity of the isolated substances was checked by LC–MS as well as by ^{1}H NMR spectroscopy, prior to sensory analysis. In order to overcome the limited water-solubility of the bitter compounds and to evaluate their bitter thresholds in a beer-like medium, the human bitter taste threshold concentrations of these compounds were determined in 5% aqueous ethanol (pH 4.4) by means of a triangle test. The sensory analysis revealed a clear bitter taste of these compounds and, depending on their chemical structure, low recognition thresholds, between 7.9 and 90.3 μmol/l (Table 6). Whereas cohulupone induced a short-lasting, iso-α-acid like bitter sensation, the tricyclic compounds **9–11a/b** had a long-lasting, lingering bitterness, similar to the taste profile of the β-acids.

Being well in line with the observation that the iso-α-acids exhibiting a five-membered ring structure are less bitter than the six-membered ring α-acids (Gienapp & Schröder, 1975), the lowest

Table 6
Bitter recognition threshold concentrations of colupulone (**1**) and its degradation products **8–11a/11b**.

Compound (no.)[a]	Threshold concentration (μmol/l)[b]
Colupulone (**1**)	39.3
Cohulupone (**8**)	7.9
Nortricylocolupone (**9**)	90.3
Dehydrotricyclocolupone epimer 1 (**10a**)	40.5
Dehydrotricyclocolupone epimer 2 (**10b**)	40.8
Tricyclocolupone epimer 1 (**11a**)	54.4
Tricyclocolupone epimer 2 (**11b**)	37.9

[a] Structure and numbering of the compounds refer to Figs. 1 and 6.
[b] Taste threshold concentrations were determined in 5.0% aqueous ethanol (pH 4.4) by means of a triangle test.

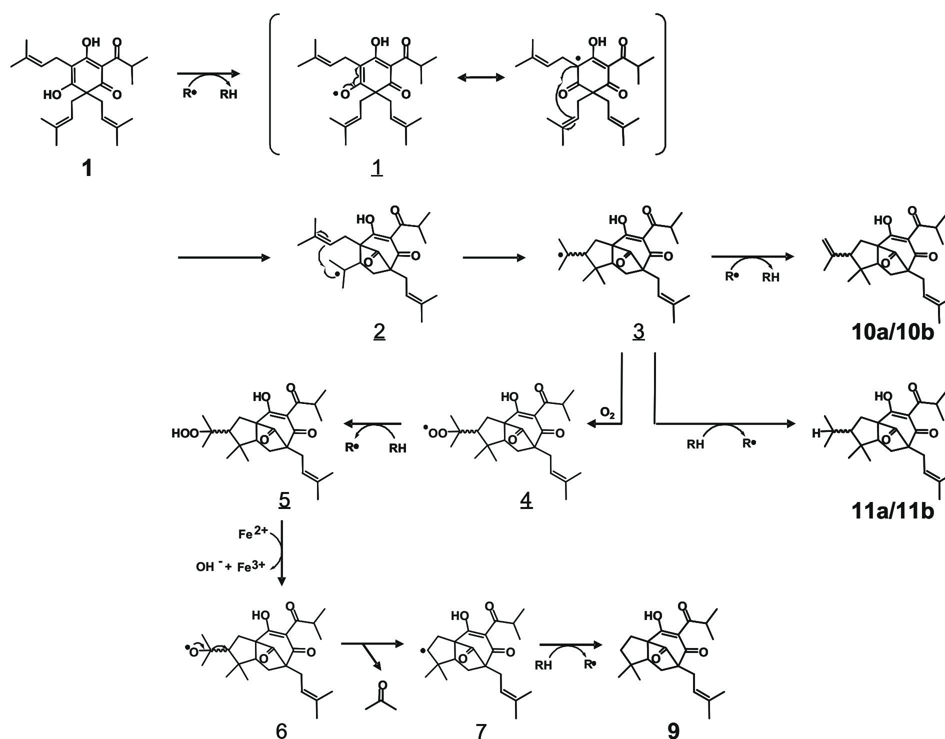

Fig. 7. Reaction scheme proposed for the formation of bitter compounds **9–11a/11b** upon wort boiling of the β-acid colupulone (**1**).

threshold concentration of 7.9 μmol/l was found for the five-membered ring compound cohulupone (**8**), whereas the corresponding β-acid colupulone (**1**), as well as the bitter compounds **9**–**11a/11b**, all of which had six-membered ring structures, showed significantly higher bitter thresholds. The range of the bitter thresholds (37.9–54.4 μmol/l) determined for the dehydrotricyclocolupone epimers **10a**/**10b,** as well as for the tricyclocolupone epimers **11a**/**11b** was observed to be identical to the threshold of 39.3 μmol/l found for the β-acid colupulone (**1**). Comparison of the relatively high threshold value of 90.3 μmol/l, determined for nortricyclocolupone (**9**), with the lower thresholds found for **10a**/**10b** and **11a**/**11b,** gives first evidence that the presence of both the isopropyl and the isopropenyl moiety, is important for a low bitter taste threshold of this class of compounds.

4. Conclusions

Activity-guided separation of a hop extract, before and after wort-boiling, revealed that, besides the α-acids, the β-acids also are potential bitter taste precursors in hop. Application of the TDA on a thermally treated solution of colupulone (**1**), followed by spectroscopic structure determination, allowed the identification of cohulupone (**8**) and the previously unreported compounds **9**–**11a/11b** as bitter degradation products. As the formation of these bitter compounds does not depend on the 2-methylpropanoyl moiety of colupulone (**1**), analogous compounds exhibiting a 3-methylbutanoyl- and a 2-methylbutanoyl moiety are expected to be formed from the corresponding β-acids lupulone (**2**) and adlupulone (**3**), respectively, upon wort boiling.

In order to investigate the importance of this novel family of hop-derived bitter taste compounds for the bitter taste of beer, quantitative LC–MS/MS studies are currently in progress and will be published elsewhere.

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