Radicaloid-Type Oxidative Decomposition Of Beer Bittering Agents Revealed

Kevin Huvaere,[a] Mogens L. Andersen,[b] Karsten Olsen,[b] Leif H. Skibsted,[b] Arne Heyerick,[a] and Denis De Keukeleire*[a]

Abstract: *trans*-Isohumulones, dihydroisohumulones, tetrahydroisohumulones, and humulinones, which are important hop-derived flavor components of beer, were found, by using electrolysis of organic solutions, to be stable against oxidation, like weak acids; however, they are readily oxidized in their anionic forms as present in beer. Oxygen- and carbon-centered radicals were formed by oxidation and identified by using spin trapping under aerobic and anaerobic conditions, followed by EPR (electron paramagnetic resonance) spectroscopy. Generated radicals were reactive, most likely degrading into products lacking the tricarbonyl chromophore; this is typical of five-membered-ring hop derivatives. Thus, flavor-active beer constituents may degrade oxidatively in the absence of oxygen, thereby leading to reaction products that escape UV detection.

Keywords: beer • cyclic voltammetry • EPR spectroscopy • isohumulones • oxidation

Introduction

Flavor stability of beer remains a key concern in the brewing world and the scientific community alike. Hundreds of constituents form the complex beer matrix and, inevitably, chemical reactions lead to changes both in the composition of beer, and in features related to taste and flavor. Thus, beer quality alters constantly during storage. It seems impossible to exert rigid control on this phenomenon, since not all factors that affect flavor changes can be excluded. Although the concentration of oxygen or reactive oxygen species can be limited by applying good brewing practices, and absorption of light can be prevented to a great extent by using appropriate packaging, thermal energy, as determined by the temperature to which beer is exposed, inherently provokes reactivity. Furthermore, oxidation reactions may occur if compounds with suitable redox potentials are present.

Hop-derived compounds are exceptionally important for beer flavor. Many of these are characterized by potent organoleptic features, while their instability renders the aim to keep beers of consistent quality difficult to meet.[1] Numerous studies have addressed this issue; however, since detailed mechanistic insights are lacking, measures to efficiently protect beers against flavor damage have not led to adequate results. Among the many pathways available for decomposition, in particular, the instability of the isohumulones, the main hop-derived bittering agents in beer, is deleterious to beer quality. Thus, it was found that *trans*-isohumulones degrade much faster than *cis*-isohumulones, and the decomposition rate can be measured reliably.[2] However, information on the reaction pathway(s) is not available, since decomposition products could not, for unclear reasons, be detected. Reduced isohumulones are increasingly being used in modern brewing, mainly dihydroisohumulones (also known as rho-isohumulones) and tetrahydroisohumulones, as these derivatives are thought to be more resistant to decomposition than isohumulones.[3] It must, however, be emphasized that confusion exists as to whether or not this statement is generally true.

It was our purpose to reveal the mechanism by which isohumulones and their derivatives, collectively called five-membered-ring hop derivatives, undergo oxidative decomposition by electron loss. In order to probe the fate of the flavor compounds following oxidation, electrolysis was used to generate radicals, and electron spin resonance was applied to identify these radicals by suitable spin trapping. The results are reported herein.

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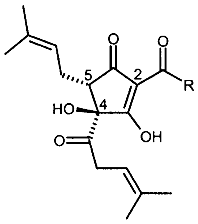
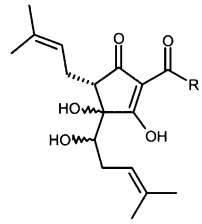
Results

Electrochemistry of *trans*-isohumulones and their derivatives: To the best of our knowledge, properties typical of five-membered-ring hop derivatives under electrochemical conditions have, up to now, not been studied in any detail, notwithstanding the fact that the β-tricarbonyl group, common to all these compounds, presents an intriguing target for oxidation, since it has a number of π and p electrons available within a conjugated system that incorporates three oxygen and four carbon atoms.[8, 9] The interest in the properties of this moiety is, furthermore, highlighted by the fact that it is the main chromophore in all molecules of interest in this study (**1**–**4**), with maximum absorption around 250–255 nm (shoulder around 270–280 nm), and molar absorptivities between 16000 and 19000 $\mathrm{l\,mol^{-1}\,cm^{-1}}$.[10] Thus, on direct irradiation of *trans*-isohumulones (**1**), excitation energy initially located in the β-tricarbonyl moiety is transferred to the α-hydroxycarbonyl group at C(4), eventually leading to the formation of the lightstruck flavor in beer.[11]



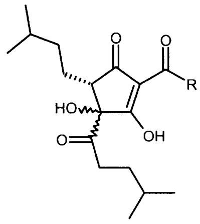
***trans*-Isohumulones**

1



Dihydroisohumulones

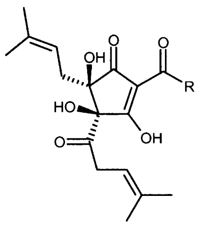
2



Tetrahydroisohumulones

3

R: $CH_2CH(CH_3)_2$
$CH(CH_3)_2$
$CH(CH_3)CH_2CH_3$



Humulinones

4

It was concluded that it was not necessary to study individual constituents, since it can be safely assumed that the nature of the R group in the side chain at C(2) should have a negligible effect on the features relevant for the present investigation, "R" being a hydrocarbon residue, representing isobutyl, isopropyl, or secondary butyl. Also, the stereochemistry both at C(4) and C(5) should not significantly interfere with the reactivity of the β-tricarbonyl group. Thus, the substrates were used as described in the Experimental Section. First, direct electrochemical oxidation of *trans*-isohumulones (**1**), and derivatives (**2**–**4**) in acetonitrile was attempted; however, to our surprise, within the range of +200 mV to +1800 mV versus SHE (standard hydrogen electrode), none of the compounds were electroactive. This supports the conclusion that five-membered-ring hop derivatives, in their undissociated (acid) forms, withstand oxidation by electron abstraction. In contrast, the corresponding anions, as salts (see Experimental Section), proved to be very labile, and prominent oxidation waves around +1400 mV versus SHE are evident (Figure 1). Moreover, the resulting radicals of all compounds immediately disappeared from the reaction medium, since all cyclic voltammetric oxidation waves were irreversible (absence of a cathodic current). The experiments were repeated with ultramicro electrodes at high sweep rates (up to 50 $\mathrm{V\,s^{-1}}$), but no reversibility was detected; this confirms the high reactivity of the one-electron oxidized species produced. As a control, ferrocene was oxidized to its ferrocenium ion, and reversibly reduced (Figure 2). The similar heights of the anodic waves indicate that isohumulones and their derivatives are oxidized in a one-electron process.

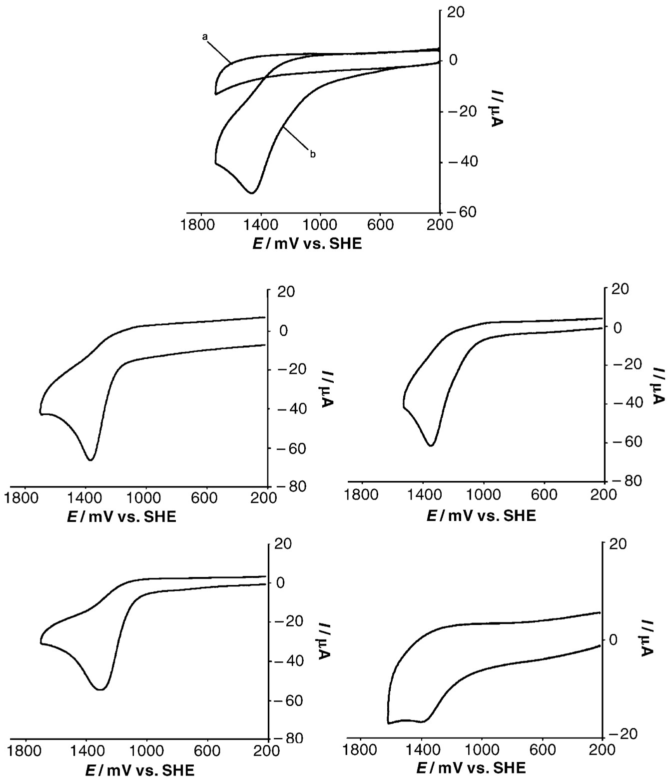


Figure 1. Top: cyclic voltammograms of *trans*-isohumulones (**1**) in a) their acidic forms and b) their anionic forms. Middle and bottom: cyclic voltammograms of the anionic forms of *trans*-isohumulones (**1**; upper left), dihydroisohumulones (**2**; bottom left), tetrahydroisohumulones (**3**; upper right), and humulinones (**4**; bottom right) (for conditions, see Experimental Section).

The similarity of the cyclic voltammograms of **1**–**4** strongly suggest oxidation of a common species. Indeed, the anions are formed from an enolized β-tricarbonyl group, which is typical of all compounds studied. The p*K*a values vary slightly for each of the five-membered-ring hop derivatives, but a value of around three can be considered a good first-order approximation.[12] Since lager beers commonly have a pH between 4.2 and 4.4, compounds **1**–**4** prevail predominantly in their anionic forms. Evidently, this may not be true for more acidic beers. On oxidation, an electron is withdrawn from the anion, thereby resulting in a radical, which may either be

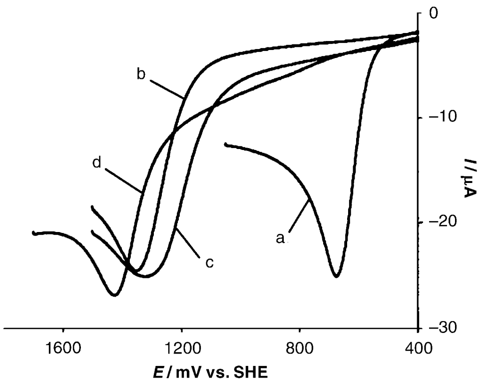


Figure 2. Cyclic voltammograms of a) ferrocene, b) *trans*-isohumulones (**1**) c) dihydroisohumulones (**2**), and d) tetrahydroisohumulones (**3**) in their respective anionic forms (0,5 mM in MeCN; for conditions, see Experimental Section).

oxygen- or carbon-centered. Apparently, the high reactivity of the radical(s) prevents direct isolation, and bulk electrolysis in the cavity of the EPR spectrometer failed to show the presence of radicals in detectable concentrations.

Kinetic analysis of the electrode reaction based on the behavior of the anodic cyclic voltammetric peaks of the isohumulones was attempted. However, the peak potentials (E_p) increased with the sweep rate, and showed a linear relationship with the logarithm of the sweep rate (Figure 3).

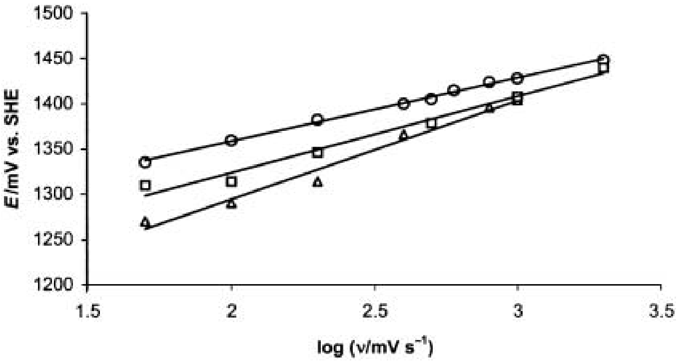


Figure 3. Dependence of the experimental E_p values of anions of *trans*-isohumulones (**1**) (○), dihydroisohumulones (**2**) (△), and tetrahydroisohumulones (**3**) (□) on the logarithm of the sweep rate.

From the value of the slope (70.3 mV for *trans*-isohumulones; 109.2 mV for dihydroisohumulones; 84.7 mV for tetrahydroisohumulones), it appears that electrochemical oxidation is kinetically controlled by the heterogeneous electron transfer from the substrates to the electrode.[13]

EPR spectroscopy of radicals resulting from electrolysis of *trans*-isohumulones and their derivatives: On bulk electrolysis of anions (salts) of **1**–**4**, the formation of radicals was evident. It was, however, not possible to generate a steady-state concentration inside the EPR cavity, hence, immediate detection was not feasible. It is probable that these radicals are too reactive to be formed in detectable concentrations. Addition of spin traps, prior to electrolysis, resulted in EPR-active adducts, which provide indirect structural information from their spectral features. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) and 2-methyl-2-nitrosopropane (MNP) were used to trap electrochemically generated radicals. The electrolysis was stopped after 0.1 F mol^{-1} had been transferred in order to avoid potential interference from electrolysis of the products. Bulk electrolysis of solutions containing only DMPO or MNP did not give any detectable EPR signal.

Spin trapping with DMPO: A tenfold molar excess relative to the concentrations of **1**–**4** led to readily detectable spin adducts. The EPR spectrum of **1** subjected to electrolysis in a nitrogen atmosphere consisted of a triplet of doublets with hyperfine coupling constants $a^N \sim 13.2$ G and $a^H \sim 8.9$ G (line width ~ 1.6 G) (Figure 4). A similar spectrum was observed with electrolysis under atmospheric oxygen, however, the lines were broadened ($a^N \sim 13.3$ G, $a^H \sim 8.8$ G, line width ~ 2.8 G).

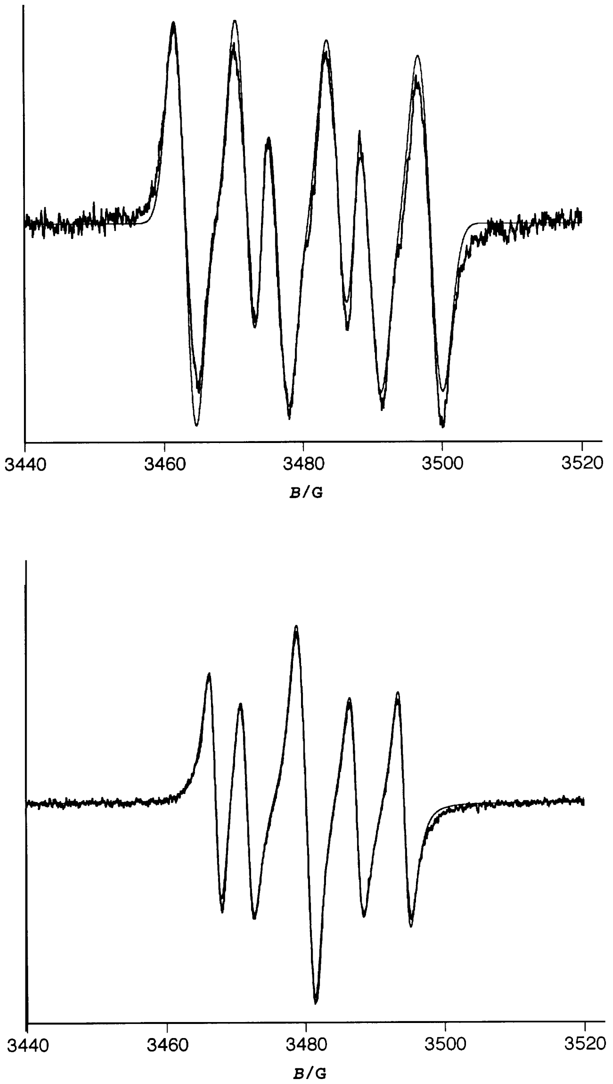


Figure 4. Experimental (——) and simulated (——) spin patterns of radicals derived from *trans*-isohumulones (**1**) after electrolysis and trapping by DMPO (top panel) and MNP (bottom panel), both under nitrogen (for conditions, see Experimental Section).

Dihydroisohumulones (**2**) gave, under oxygen, an EPR spectrum (Figure 5) with $a^{N} \sim 13.4$ G and $a^{H} \sim 9.2$ G (line width ~2.6 G), while a complex spectrum was found by using a nitrogen atmosphere. By simulation, the lines could be assigned to three nitroxyl species (Table 1).

Table 1. Relevant data from simulation of the EPR spectrum of dihydrosohumulones (**2**) trapped with DMPO under nitrogen (for conditions, see Experimental Section).

relative abundance[%]	47	29	24
a^{N}[G]	13.1	14.5	14.7
a^{H}[G]	9.5	20.2	25.7
line width[G]			
−1	3.0	1.8	1.2
0	3.0	1.8	1.2
+1	3.0	2.0	1.4

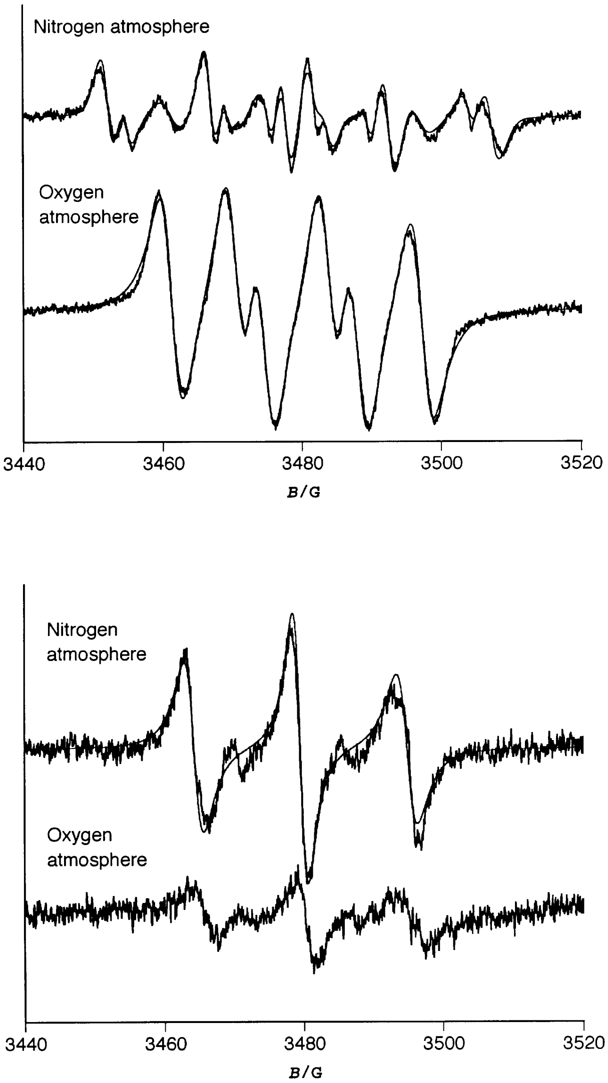


Figure 5. Experimental (——) and simulated (——) spin patterns of radicals derived from dihydroisohumulones (**2**) after electrolysis and trapping by DMPO under oxygen and nitrogen (top panel), and MNP both under nitrogen and oxygen (bottom panel) (for conditions, see Experimental Section).

Relevant data from the EPR spectrum (Figure 6) for the electrolysis of humulinones (**4**) under nitrogen, followed by DMPO trapping, are given in Table 2.

Spin trapping with MNP: Electrolysis of **1** under nitrogen gave a mixture of two spin adducts (Figure 4). Both are triplets with hyperfine coupling constants $a^{N} \sim 7.9$ G (58 %, line width ~1.7 G), and 13.6 G (42 %, line width ~1.4 G), respectively. In contrast, the spectra of **2** showed only a single spin adduct as a triplet with varying line widths (−1: 2.4 G; 0: 1.9 G; +1: 2.6 G), and with a hyperfine coupling constant $a^{N} \sim 15.3$ G (Figure 5). Line heights decreased significantly when the electrolysis was carried out under an oxygen atmosphere. Also, for **4**, a single spin adduct (triplet) was observed with a hyperfine coupling constant, $a^{N} \sim 7.9$ G, and line widths around 1.1 G (Figure 6).

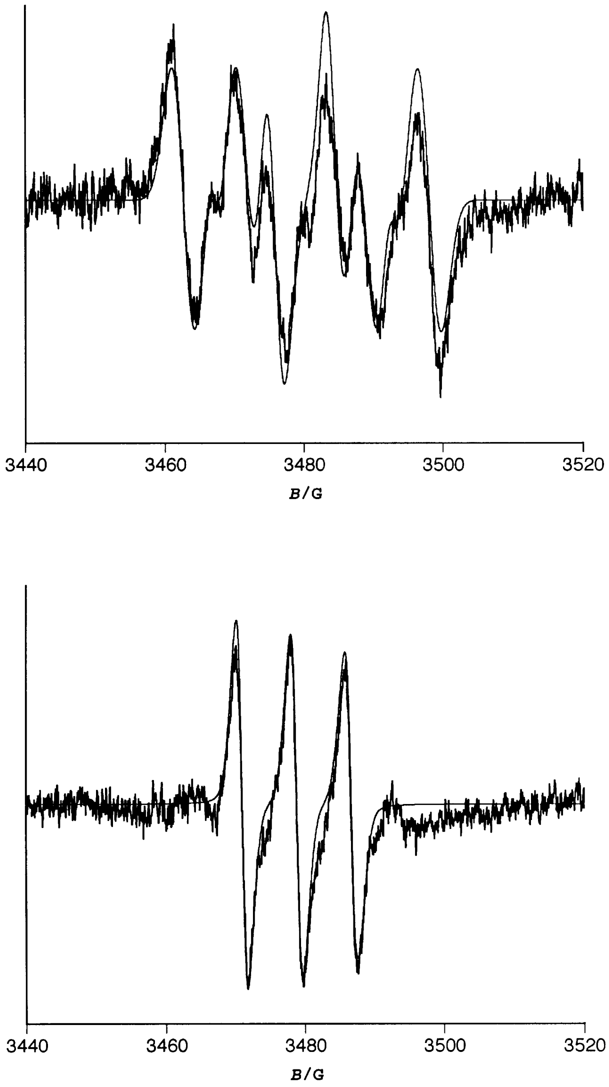


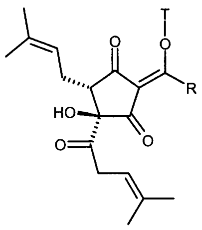
Figure 6. Experimental (——) and simulated (——) spin patterns of radicals derived from humulinones (**4**) after electrolysis and trapping by DMPO (top panel) and MNP (bottom panel) both under nitrogen (for conditions, see Experimental Section).

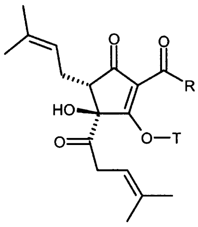
Table 2. Relevant data from simulation of the EPR spectrum of humulinones (**4**) trapped with DMPO under nitrogen (for conditions, see: Experimental Section).

relative abundance[%]	92	8
a^N [G]	13.1	8.5
a^H [G]	9.2	9.0
line width [G]	1.7	1.1

Discussion

Five-membered-ring hop derivatives have been judiciously chosen for the EPR study. Isohumulones (**1**) are the main bittering compounds in beers and the mechanism of their decomposition, which affects beer flavor stability most significantly, has been elusive until now. A number of oxidation products have been identified, mostly arising from simulated autoxidation conditions. Best known are the so-called abeo-isohumulones, which are formed by initial oxidation of the side-chain double bonds followed by various secondary reactions.[14] It is remarkable that the oxidized derivatives from isohumulones which have been identified until now, all retain the β-tricarbonyl chromophore. Moreover, yields are invariably low even under forced conditions, and it appears that these compounds including abeo-isohumulones represent very minor oxidation products. In this regard, abeo-isohumulones have not been unambiguously detected in beer. Also, during our studies on the flavor stability of beers, we have never been able to find well-defined reaction products concomitant with the disappearance of isohumulones, although a small and relatively unimportant increase in the so-called S-fraction has occasionally been observed.[15] *trans*-Isohumulones can be readily separated from the *cis*-isomers,[4] and are much more liable to undergo decomposition; hence they are most relevant in this study.[2]


1i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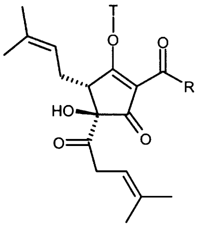
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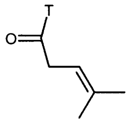
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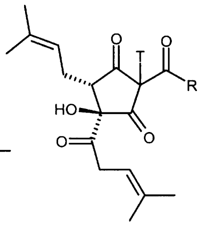
1v

T: spin trap

Dihydroisohumulones (**2**) are supposed to be light-stable, since they do not possess the α-hydroxycarbonyl group, which is pivotal in the light-induced decomposition.[11] By including these compounds in our study, we ascertained that radical formation at C(4) was abolished. Similarly, in humulinones (**4**), which are fully substituted at C(5), radical formation is prevented. In this respect, it should be noted that previous studies in our laboratory have indicated that some decomposition products could have arisen from intermediates exhibiting a radical character at C(5).[16] Finally, tetrahydro-isohumulones (**3**) should resemble isohumulones in all aspects regarding reactivity except for oxidation of the double bonds in the side chains at C(4) and C(5), this allows comparison of alkene-type oxidation reactions and oxidations at other sites in the five-membered-ring hop derivatives.

While DMPO forms stable spin adducts with both oxygen- and carbon-centered radicals, stable spin adducts with MNP are only observed with carbon-centered radicals. Indeed, the number of hydrogen atoms bonded to the added carbon radical can be detected from the pattern of hyperfine splittings, while the values of these provide information on the nature of substituents on the carbon radical. DMPO trapping of electrolytically generated radicals from **1** probably indicates that an oxygen radical such as **1i**, or a tautomeric form thereof (**1ii** or **1iii**), was added to DMPO.

Observations on trapping with MNP are most revealing. The lack of hyperfine coupling to protons, in both spin adducts detected, indicates that tertiary carbon radicals had been trapped. An a^N value of about 7.9 G suggests an acyl radical adduct of MNP, that is, **1iv**, whereas an a^N value of 13.6 G would be in agreement with a carbon radical with low-electron density, such as a trichloromethyl radical.[17] Presumably, a triacylmethyl radical such as **1v** had been trapped by MNP.

Interesting results were obtained for dihydroisohumulones (**2**) that showed only an oxygen radical being trapped by DMPO under oxygen, whereas radicals such as $RO^{\bullet}$ were evident in the EPR spectrum of spin adducts obtained under a nitrogen atmosphere, in addition to spin adducts of two carbon-centered radicals. In sharp contrast is the observation of a single spin adduct on trapping with MNP (both under oxygen and nitrogen atmospheres). The varying line heights indicate that a relatively large radical was trapped, while the lack of hyperfine coupling to protons and a high value of a^N (see results for **1**) suggest a tertiary acyl radical.

The experiments with humulinones (**4**) gave somewhat puzzling results. The main species on DMPO trapping is a $RO^{\bullet}$ radical with features quite similar to those observed for the main oxygen radical arising from **2** (a^N in both cases 13.1 G, a^H ca. 9.5 G for **2** and 9.2 G for **4**). However, the a^N value of 8.5 G in the minor adduct (8%) would indicate the presence of an acyl radical, but the hyperfine coupling to a proton is not in accordance with the lack of couplings in the other molecules. The sole adduct found on MNP trapping with an a^N value of 7.8 G is, indeed, indicative of an acyl radical.

Results observed for MNP radical adducts are most readily interpreted. Strikingly, for the three series of compounds (**1**, **2**, and **4**), spin adducts with an a^N value of about 7.9 G, typical of an acyl radical, were formed, while for **1** and **2** only, a triacyl radical ($a^N = 13.6$ G and 15.3 G, respectively) was evident. This suggests that the presence of an extra hydroxyl group perturbs the reactivity of a triacyl radical formed in **4**.

Anodic one-electron oxidation of β-tricarbonylmethanes has been shown to give both acyclic and cyclic dimeric products.[8] The weak absorbances of these end products lacking enolization in the β-tricarbonyl chromophore explain why the majority of oxidation products of isohumulones escape detection by using HPLC with UV monitoring, thus resolving an issue, which, to this day, has been a matter of debate. The results in the present study would, furthermore, suggest that the double bonds in the side chains of five-membered-ring hop derivatives are far less reactive than was commonly thought until now. However, oxygen or reactive oxygen species may attack under particular reaction conditions, such as in acidic medium, the double bonds, thus providing an alternative route to oxidative decomposition of five-membered-ring hop derivatives.

Conclusion

Electrochemical oxidation of *trans*-isohumulones (**1**), dihydroisohumulones (**2**), tetrahydroisohumulones (**3**), and humulinones (**4**) has provided clear evidence for the instability of their anionic forms, as are present in beer, while the acidic forms resist oxidation. Release of an electron leads to radicals, which could only be detected by electron spin resonance (EPR) after trapping by suitable spin traps, such as DMPO and MNP. Analysis of the various spin patterns observed by EPR concurrent with adequate simulation has led to the identification of both oxygen- and carbon-centered radicals. It was also confirmed that radicals, formed on electron abstraction, are very reactive, thereby giving rise to reaction products of varying nature, but lacking the enolization pattern of a β-tricarbonyl chromophore necessary for ready detection by absorbance in the UV region of the electromagnetic spectrum. These novel findings demonstrate, for the first time, that flavor-active five-membered-ring hop derivatives degrade in the presence of suitable electron acceptors, which do not necessarily involve the intermediacy of oxygen-containing entities. Thus, the presence of powerful electron-withdrawing species could convert anions of beer bittering agents to reactive radicals thereby adversely affecting beer flavor.

Experimental Section

trans-Isohumulones (**1**) (*trans* denotes the relative configuration of the tertiary hydroxyl at C(4) and the prenyl substituent at C(5)) were prepared from a commercially available aqueous solution (100 mL; ca. 30% w/v) of the potassium salts of isohumulones (Yakima Chief, Sunnyside, Washington, USA), which consisted of a mixture of *trans*- and *cis*-isohumulones. After acidification to pH 1 (HCl, 1M), and extraction with ethyl acetate (200 mL) the organic phase was dried over $MgSO_4$. Removal of the solvent in vacuo resulted in an oily residue (24 g), which was redissolved in ethyl acetate (200 mL), and treated with dicyclohexylamine (DCHA) (50.7 g, 0.28 mol). The DCHA salts of *trans*-isohumulones were selectively precipitated and subsequently collected.[4] Recrystallization from methanol/water (4:1 v/v; 50 mL) gave pure DCHA-salts of *trans*-isohumulones (10.1 g) as white flakes. Free acids (>99% pure by HPLC) were obtained by re-dissolution of the salts in warm ethyl acetate (200 mL), acidification to pH 1 (HCl, 1M), isolation of the organic phase, drying over $MgSO_4$, and in vacuo removal of the solvent.

Dihydroisohumulones (**2**) were commercially available as an aqueous solution (ca. 35% w/v) of the potassium salts (Botanix, Eardiston, UK), and tetrahydroisohumulones (**3**) were used as an aqueous solution (ca. 10% w/v) of the potassium salts (Kalsec, Michigan, USA).

The sodium salts of humulinones (**4**) were prepared, according to the literature procedure, by dissolving humulones (2 g) in a biphasic system of diethyl ether (150 mL) and a saturated sodium hydrogen carbonate solution (150 mL).[5] After a catalytic amount of cumenylhydroperoxide was added, the reaction mixture was left for two days under ambient light conditions. The sodium salts of humulinones were formed at the interface between the two liquid phases and collected as white crystals (1.08 g, yield: 51%) by filtration.

Cyclic voltammetry of **1**–**4** was carried out with a BAS CV-50W voltammetric analyzer (Bioanalytical Systems, Indiana, USA) by using a glassy carbon electrode (BAS MF-2012) as a working electrode, a non-aqueous reference electrode (BAS MF-2026), and a platinum wire as an auxiliary electrode (BAS MW-1032). Compounds were dissolved in concentrations varying from 0.5mM up to 2mM in Bu_4NPF_6/MeCN (0.1M). Solutions were degassed with nitrogen before measurements. The sweep rate was 200 mV s^{-1}. The potential of the reference electrode was checked daily against the standard potential of the ferrocene/ferrocenium couple, Fc/Fc$^+$. The measured potentials are reported against the standard hydrogen electrode (SHE) by using $E^\circ = +650$ mV versus SHE for the Fc/Fc$^+$ couple in MeCN.[6]

Bulk electrolysis was carried out with a reticulated vitreous carbon electrode (BAS MF-2077) as a working electrode and a platinum wire (BAS MW-1033), separated from the solution by an Al_2O_3 layer, as an auxiliary electrode. Solutions (1mM) of the respective salts and DMPO (10mM) or MNP (Aldrich, Bornem, Belgium) in Bu_4NPF_6/MeCN (0.1M) were prepared. Solutions were degassed with nitrogen prior to use. Immediately after electrolysis (+1400 mV vs SHE, 0.1 F mol^{-1}), samples were analyzed by EPR spectroscopy on a Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany) by using the following settings: center field: 3480 Gauss; sweep width: 80 G; microwave power: 10 mW; modulation frequency: 100 kHz; modulation amplitude: 1.0 Gauss; conversion time: 82 ms; sweep time: 84 s. Simulation and fitting of the EPR spectra was performed by the PEST WinSIM program.[7]

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