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Fate of flavins in sensitized photodegradation of isohumulones and reduced derivatives: studies on formation of radicals *via* EPR combined with detailed product analyses

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Photodegradation of isohumulones accounts for formation of the lightstruck flavor in beer. The reactions involved are mediated by riboflavin, a natural photosensitizer present in beer in ppb quantities. The results of an investigation of this sensitized degradation process are presented herein. Product analyses and electron paramagnetic resonance spectroscopy, in steady-state as well as in time-resolved mode, offer extensive insight into the photophysical and photochemical details of the degradation mechanism. In contrast to energy transfer and Norrish type I α-cleavage reactions that take place on direct irradiation of isohumulones, the sensitization pathway proceeds *via* one-electron redox chemistry involving the excited triplet state of riboflavin and derivatives. The flavin semiquinone radical thus formed could be readily detected, either by steady state or by time-resolved electron paramagnetic resonance spectroscopy. Superimposed signals in the spectra revealed the presence of radical fragments derived from isohumulones or tetrahydroisohumulones, which, on recombination with riboflavin semiquinone radicals, produced stable reaction products that were identified by HPLC-MS. However, no superimposed signals were observed on sensitized irradiation of dihydroisohumulones.

Introduction

The pronounced sensitivity of beer to light is well known and leads irreversibly to the formation of lightstruck flavor (LSF), the so-called "skunking" of beer. It is the cause of a significant shelf-life problem for the brewing industry and is the primary reason for the storage of beer in dark-colored containers. The light-sensitivity of beers was first recognized as early as in 1875,[1] however it was not until the early sixties that Kuroiwa *et al.* established the basic science underlying the formation of LSF.[2] It was found that unhopped beer did not produce the typical skunky flavor, while addition of hops (*Humulus lupulus* L.) to wort during boiling in the brewing kettle reinstalled the potential for formation of LSF. Studies using model systems showed that LSF was produced in a non-enzymic light-induced reaction involving riboflavin (**1**) (as a sensitizer), a suitable sulfur-containing compound, and isohumulones (**2a–c**), the main beer-bittering principles which are derived from humulones (important secondary metabolites in hops). The typical skunky flavor was attributed to the formation of 3-methylbut-2-ene-1-thiol (MBT), a pungent off-flavor in beer with a flavor threshold of few ng per litre.[3] The effective wavelength range for formation of LSF was demonstrated to be 350–500 nm, as was deduced from sensory analysis.[4]

However, exposure of isohumulones to UV-B light (300 nm) also induces photodegradation, thereby generating radical precursors of MBT.[5] The primary photophysics were investigated using time-resolved electron paramagnetic resonance (TREPR) spectroscopy after a laser flash at 308 nm.[6] Not only were the free radical intermediates characterized, careful analysis of the chemically induced electron spin polarization (CIDEP) patterns observed in the TREPR spectra revealed that energy, initially absorbed by the enolized β-tricarbonyl chromophore (λ_{max} = 255 nm, shoulder at 275 nm), was transferred to the isolated side-chain carbonyl function. Following Norrish type I α-cleavage furnished a 3-methylbut-2-enyl radical (directly or after fast decarbonylation), which is a key intermediate on the route to formation of LSF. Under identical conditions, a 4-methylpentanoyl radical was most prominent on photolysis of tetrahydroisohumulones (**3a–c**), whereas no radicals could be detected on photolysis of dihydroisohumulones (**4a–c**), which have the α-hydroxyketone moiety reduced to a vicinal diol. Detailed product analyses upon stationary photolysis of isohumulones and reduced derivatives nicely corroborated the findings by TREPR.[7]

Ambient conditions during transport and storage allow LSF formation in beers bottled in clear or green glass due to exposure to visible light. Since isohumulones and reduced derivatives are transparent at wavelengths transmitted by these bottles,[8] the intermediacy of a sensitizer is required. As several hundreds of ppb of riboflavin (RF) are present in beer,[9] it was suggested that RF is, most likely, a key element although possibly not the unique factor in the "skunking" of beer. Most previous reports on photodegradation reactions in beer have focused on detection and quantification of MBT,[8,10–12] while the fate of RF (as a sensitizer) or of isohumulones (as photosubstrates) on exposure to visible light remained elusive. However, it was recently demonstrated that, by observing transients after laser flash photolysis at 355 nm and 440 nm, the initial interaction is a light-induced electron transfer from isohumulones to the triplet state of RF (^{3}RF*).[13] Electrolysis and indirect photolysis, followed by spin trapping of incipient radicals with subsequent electron paramagnetic resonance spectroscopy, revealed that an electron is released from the ionized β-tricarbonyl chromophore on interaction with a suitable oxidant (*e.g.*, ^{3}RF* ($E = 1.7$ V)).[14,15] *Via* radical stabilization pathways, the ensuing triacylmethyl radicals give rise to a mixture of photoreaction products, both volatile and non-volatile in nature. Therefore, products derived from one-electron oxidation of isohumulones upon illumination in the presence of a flavin, were analyzed by

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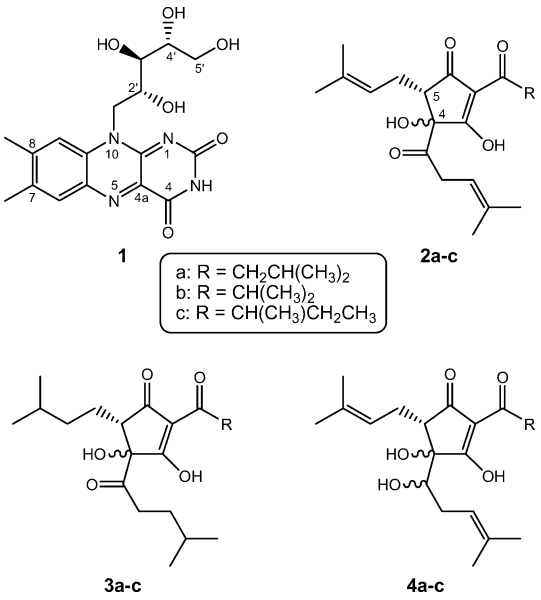
headspace gas chromatography and continuous flow injection in an electrospray ionization source, respectively, coupled to a mass spectrometer.[16] The main non-volatile photoreaction product was identified as dehydrohumulinic acid, which could be attributed to a formal α-cleavage of the α-hydroxyketone function after inter- or intramolecular hydrogen abstraction. Furthermore, the resulting 4-methylpent-3-enoyl radical underwent fast decarbonylation to a 3-methylbut-2-enyl radical, yielding 2-methylbuta-1,3-diene and 2-methylbut-2-ene after hydrogen release or hydrogen addition, respectively. Analogous photoreaction products were found on photolysis of reduced derivatives of isohumulones.



Scheme 1 Structural formulae of riboflavin (**1**), isohumulones (**2a–c**), tetrahydroisohumulones (**3a–c**), and dihydroisohumulones (**4a–c**).

This study was intended to gain further mechanistic information considering the early photochemical events and radical formation on sensitized irradiation of model systems containing a flavin and isohumulones or reduced derivatives. In particular, the fate of the interacting flavin molecules was examined. Incipient radicals on sensitized irradiation were characterized by a combination of time-resolved electron paramagnetic resonance spectroscopy on laser flash irradiation and steady-state electron paramagnetic resonance. Furthermore, comprehensive product analyses were carried out using HPLC-MS on mixtures of photoreaction products, derived from sensitized irradiation of solutions containing RF (**1**) and isohumulones (**2a–c**), tetrahydroisohumulones (**3a–c**) or dihydroisohumulones (**4a–c**).

Experimental

Materials

All solvents were of spectrophotometric grade (Biosolve, Valkenswaard, The Netherlands). RF, flavin mononucleotide (FMN), cysteine and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma-Aldrich (Bornem, Belgium). Isohumulones, tetrahydroisohumulones, and dihydroisohumulones were a generous gift from Botanix (Eardiston, near Tenbury Wells, Worcestershire, England). Pure (>99% by HPLC) *trans*-isohumulone (*trans*-**2a**; the denotation *trans* refers to the relative configuration of the tertiary hydroxyl at C(4) and the prenyl substituent at C(5)) was prepared by irradiation at 350 nm of a solution of humulone in methanol.[17] *Trans*-tetrahydroisohumulones (>99% pure by HPLC) (*trans*-**3a–c**) were separated from the *cis*-isomers by treatment of the mixture with dicyclohexylamine, which is, however, a very slow process (yield: 0.4 g, 1.5%). Thornton *et al.*[18] did not consider this separation, because, obviously, the method is not applicable in practice to prepare standard material from tetrahydroisohumulones at any reasonable scale.

Steady-state electron paramagnetic resonance

Experiments were carried out on a RE-1X instrument (JEOL, Peabody, MA, USA) operating at the X-band frequency (9.5 GHz) interfaced with a personal computer and in-house software for data acquisition. A cylindrical sample cell (id: 1 mm) was used to accommodate the polar solvents using a cylindrical TE_{011} microwave resonator.

Time-resolved electron paramagnetic resonance

The experimental set-up for time-resolved electron paramagnetic resonance was already described in detail,[19] although particular modifications were made for the experiments under sensitized irradiation conditions. A microwave amplifier (GaAs FET (noise figure 0.9 dB)) was positioned in front of the detector to increase the sensitivity and a wideband preamplifier (0.5–50 MHz) was arranged at the back of the detector to increase time resolution. Furthermore, a double balanced mixer (DBM) was substituted for the Magic Tee with crystal microwave detector. The Nd:YAG laser (Continuum Laser Powerlite Series) produced *ca.* 30 mJ $pulse^{-1}$ at 355 nm. TREPR experiments were carried out on solutions containing 5 mM FMN (72 mg) and 10 mole equivalents of isohumulones (**2a–c**) (525 mg), tetrahydroisohumulones (**3a–c**) (539 mg), or dihydroisohumulones (**4a–c**) (530 mg), respectively, in 30 mL acetonitrile/water (1 : 1, v : v). Afterwards, another 8 mL of acetonitrile was added to the above mentioned samples to improve solubility.

Product analyses

A deaerated mixture of methanol/water (1 : 1, v : v, 10 mL) containing *trans*-isohumulone (*trans*-**2a**; 1.4 mM), *trans*-tetrahydroisohumulones (*trans*-**3a–c**; 2.7 mM) or dihydroisohumulones (**4a–c**; 2.7 mM) in the presence of 0.05 mole equivalents of riboflavin was irradiated with visible light (photoreactor containing 4 Philips cool white lamps of 8 W) at varying exposure times (up to 8 h). Deaeration of the samples was performed by flushing with solvent-saturated N_2 for *ca.* 20 min. A detailed description of sample preparation, chromatographic conditions, and mass spectral settings is described in the experimental part of ref. 7.

Results and discussion

Electron paramagnetic resonance (EPR) spectroscopy

For this study, two types of EPR experiments were carried out. The first is a standard method where equilibrium concentrations and Boltzmann spin-state populations are observed using 100 kHz field modulation and phase-sensitive detection. The method is referred to as steady-state EPR (SSEPR) and it allows detection of stable radicals such as nitroxide spin labels at moderate to low concentrations. The second experiment is called time-resolved EPR (TREPR), where field modulation is omitted and signals are detected directly from the preamplifier of the microwave bridge of the spectrometer using a gated integrator such as a boxcar or a transient digitizer if kinetic information is desired. The typical time response of the TREPR experiment at 9.5 GHz is about 50 ns.

In TREPR experiments, it is typical to observe non-equilibrium concentrations of free radicals that are produced with high-intensity short laser pulses.[19] The sensitivity that is lost by dispensing with the phase-sensitive detection is regained by signal averaging over narrow windows in time where the concentration of radicals is high. Additionally, the spin-state populations are often observed to be non-Boltzmann in TREPR experiments. In many cases, sensitivity is favored due to the

detection of transitions in emission or enhanced absorption, a phenomenon known as chemically induced electron spin polarization (CIDEP).[20] While CIDEP polarization patterns add complexity to the observed EPR spectra, they also provide a richness of mechanistic information with regard to the photophysics, photochemistry, and free radical dynamics of the process under investigation.

Steady-state experiments (SSEPR)

A 1 kW high-pressure mercury arc, exhibiting a broad emission spectrum in the UV and the visible-wavelength regions, was preferred over a laser as the excitation source in the SSEPR experiments. As a control, and in the absence of light, a 1 mM solution of 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO, a stable nitroxyl radical) in water resulted in a very intense three-line signal (spectrum not shown, simulated with a_N = 16.7 G). Under identical conditions, but in the presence of light, a mixture of acetonitrile/water (1 : 1, v : v) containing 0.8 mM RF and 8 mM ethylenediamine tetraacetic acid (EDTA) was investigated by SSEPR. EDTA was supposed to act as an electron donor towards ^{3}RF*, however, the system remained EPR silent. Subsequent addition of a small amount (*ca.* 2 mL) of the TEMPO solution (1 mM in water) was attempted in order to test the instrument response. Initially, a signal for TEMPO identical to that in the literature was observed, but its intensity decreased rapidly over few minutes, leaving no other signals to appear. In order to characterize the interacting species leading to quenching of the TEMPO signal, four model systems were irradiated and simultaneously evaluated with SSEPR: (a) 1 mM TEMPO, (b) 0.7 mM RF and 1 mM TEMPO, (c) 0.7 mM RF, 7 mM EDTA, and 1 mM TEMPO, (d) 7 mM EDTA and 1 mM TEMPO. The signal originating from TEMPO did not change significantly during irradiation (>1 h) in solutions (a), (b), and (d), indicating the photostability of TEMPO. However, in the presence of both RF and EDTA (c) the EPR signal originating from TEMPO was efficiently quenched. Furthermore, the rate of signal disappearance was found to be concentration-dependent. For a sample containing 0.1 mM TEMPO, 0.7 mM RF, and 7 mM EDTA the TEMPO signal showed complete disappearance within 3 min of irradiation, resulting in a marked decrease in intensity of the signal even during recording of the spectrum (Fig. 1).

Fig. 1 SSEPR spectrum of a solution containing 0.1 mM 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), 0.7 mM riboflavin, and 7 mM EDTA under steady-state irradiation (recording time: 1 min).

The major reactions of nitroxide radicals leading to loss of paramagnetism are oxidation, reduction, and free-radical recombination.[21] Most likely, free-radical recombination is the major factor contributing to the quenching of the TEMPO-derived SSEPR signal. Only when the light-absorbing RF is present, radicals can be formed on irradiation, but, in the absence of EDTA, the steady-state concentration of RF-derived radicals is low. The addition of an efficient one-electron donor results in significant amounts of the flavin semiquinone radical and the corresponding EDTA radical, formed *via* one-electron transfer to triplet-excited RF (followed by fast proton exchange). Thus, significant concentrations of free radicals are generated, provoking a rapid quenching of the TEMPO signal.

By changing the field modulation width from 0.1 G to 5 G, a weak, broad signal became apparent after the signal of TEMPO had disappeared completely (Fig. 2). The sharper signal in the center of the spectrum (marked with asterisks) is an artefact resulting from defects in the quartz sample cell. The broad signal corresponds to the highly delocalized flavin semiquinone radical and the large line width results from unresolved ^{14}N-hyperfine couplings that occur in view of appreciable spin density on N(5) and N(10) of the flavin ring.[22] Additionally, substantial couplings to the methyl protons at C(8) and, for the neutral radical, to the proton at N(5) are possible.[23] The neutral and anionic flavin radicals can be readily distinguished, since loss of a hyperfine coupling to the proton at N(5) in the anion results in a characteristic narrowing of the EPR linewidth from 18–19 G for the neutral radical to *ca.* 15 G for the anionic radical.[24] The broad linewidth of 21.0 G that is observed in this case may be caused by interference of EDTA-derived radicals.[25]

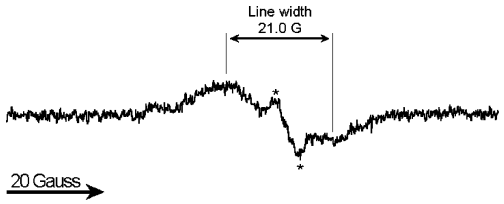


Fig. 2 SSEPR spectrum of a solution containing 0.1 mM 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), 0.7 mM riboflavin, and 7 mM EDTA after full quenching of the signal from TEMPO.

It is evident from these results that irradiation of RF in the presence of an efficient one-electron donor leads to an appreciable concentration of the neutral semiquinone radical of RF, together with the corresponding radical derived from the one-electron donor. Conversely, if only RF is present, bimolecular triplet–triplet or triplet–ground state reactions may lead to radical anions and radical cations of RF *via* one-electron transfer.[26] Efficient back-electron transfer, however, results in low radical concentrations, which explains why these species remain undetected in our investigations, either directly or *via* quenching of the TEMPO signal.

Time-resolved electron paramagnetic resonance (TREPR)

For TREPR investigations on sensitized irradiation of isohumulones and reduced derivatives, a similar set-up was used as described in ref. 19. However, a pulsed Nd:YAG laser (third harmonic at 355 nm) was substituted for the excimer laser at 308 nm and other modifications are listed in the experimental section. Using a maximum concentration of 0.8 mM of RF (due to solubility problems) and varying concentrations of EDTA as an efficient electron donor, the concentration of incipient paramagnetic species was too low to produce a significant TREPR signal. Therefore, flavin mononucleotide (FMN) was preferred over RF as a sensitizer, because of the significantly increased solubility due to the incorporation of an anionic phosphate group at the 5′-position of the ribityl side chain. Since the phosphate group is known to be photochemically transparent at 355 nm,[27] the photochemical characteristics of both compounds with identical chromophores are very similar.

As successfully applied in SSEPR experiments, TEMPO could also be used to indirectly observe paramagnetic species *via* the radical-triplet pair mechanism of CIDEP.[28] A mixture containing 10 mM flavin mononucleotide (FMN) and 1 mM TEMPO resulted in the expected intense three-line spectrum with a maximal intensity at a delay time of *ca.* 750 ns, originating from polarization of the triplet energy levels of TEMPO in the collision with triplet-excited FMN (Fig. 3).

On irradiation of a 10 mM FMN solution, a very weak, predominantly emissive, TREPR signal was observed, while the intensity of the signal appeared to be enhanced at longer

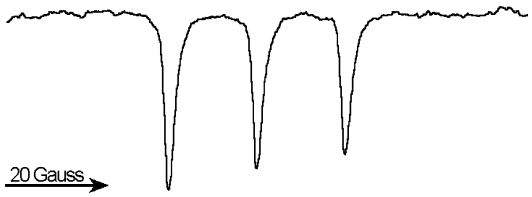


Fig. 3 TREPR spectrum of a mixture containing 1 mM 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) and 10 mM flavin mononucleotide (FMN) recorded at a delay time of 750 ns.

delay times (3–4 μs). On the other hand, irradiation of a mixture containing 5 mM FMN and 50 mM EDTA resulted in a significant emissive–absorptive signal with a predominant emissive component, while a maximum amplitude was observed after a delay time of *ca.* 400 ns (Fig. 4). The overall shape of the signal did not change over a time interval of 100 ns to 3 μs. The linewidth of 19.0 G is consistent with the formation of the neutral semiquinone radical, while the emissive–absorptive pattern stems from the radical pair mechanism (RPM) of CIDEP, typically observed with this phase for excited triplet-state precursors.[29]

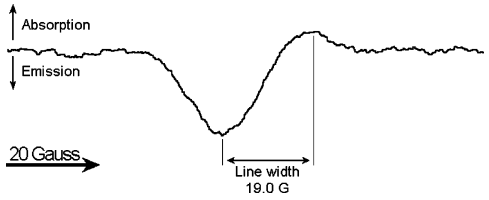


Fig. 4 TREPR spectrum of a mixture containing 5 mM flavin mononucleotide (FMN) and 50 mM EDTA recorded at a delay time of 400 ns.

In agreement with the results described above, addition of an efficient one-electron donor such as EDTA rapidly leads to a significant concentration of radicals derived from one-electron transfer (accompanied by proton transfer) towards the excited triplet state of FMN. In the absence of an efficient one-electron donor, reactions of excited flavins may also result in one-electron transfer, thereby giving rise to flavin-derived anion radicals and cation radicals.[26] The concentration of radicals is, however, much lower due to efficient back-electron transfer and to the lower concentration of possible substrates for efficient one-electron transfer.

Subsequently, solutions containing 5 mM FMN and 10 mole equivalents of isohumulones, tetrahydroisohumulones or dihydroisohumulones, respectively, were prepared in a water/acetonitrile mixture (2 : 3, v : v). TREPR spectra were recorded for all three samples over a time interval of 100 ns–4 μs. Pulsed irradiation of these samples resulted in significant emissive–absorptive TREPR signals, with an overall emissive component (Fig. 5). These signals appeared very similar to those observed for the model system comprising 5 mM FMN and 50 mM EDTA, which showed a maximum amplitude after a delay time of *ca.* 300–400 ns. In addition, a superimposed emissive signal was observed for isohumulones (**2a–c**) and tetrahydroisohumulones (**3a–c**), however, no such signal was observed in the broad emissive–absorptive TREPR signal of dihydroisohumulones (**4a–c**).

The origin of the superimposed signals can be attributed to the formation of secondary radicals, resulting from radical stabilization pathways of one-electron oxidized five-membered ring hop derivatives. A broadened, less intense spectral line is expected for delocalized radicals due to multiple hyperfine interactions. Therefore, the superimposed signal derived from irradiation of isohumulones can be ascribed to the formation of a 3-methylbut-2-enyl radical, an intermediate which was also observed on direct exposure of isohumulones to 300-nm light.[6] Tetrahydroisohumulones give rise to a sharper TREPR signal,

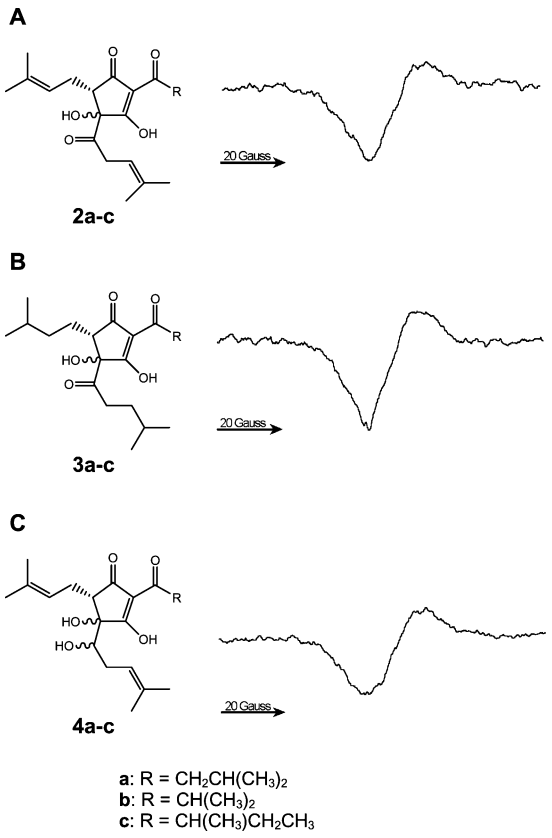


Fig. 5 TREPR spectra of mixtures containing 5 mM flavin mononucleotide (FMN) and 50 mM of isohumulones (A), tetrahydroisohumulones (B), or dihydroisohumulones (C) on irradiation at 355 nm at a delay time of 300 ns.

suggesting the presence of radicals with restricted delocalization properties, such as a 4-methylpentanoyl radical or a 3-methylbutyl radical. The occurrence of the aforementioned radicals was indirectly demonstrated by detailed product analyses of photooxidation of isohumulones and derivatives.[16] Although these results also indicated a radicaloid-type degradation of dihydroisohumulones on visible-light irradiation in the presence of flavins, no superimposed signals could be detected in the TREPR spectra. Most likely, the resulting ketyl radical is highly unstable and instantly releases a hydrogen atom to yield the observed diamagnetic reaction products.

In addition to the samples with FMN and isohumulones, tetrahydroisohumulones or dihydroisohumulones, respectively, a similar batch of samples was prepared, to which 25 mM cysteine was added in order to investigate a potential role of a sulfur source during formation of radicals on sensitized irradiation. On irradiation of a sample containing 5 mM FMN and 50 mM cysteine, an emissive–absorptive TREPR signal was observed, which was very similar to that recorded for a sample containing 5 mM FMN and 50 mM EDTA. Consequently, these TREPR spectra must originate from one-electron transfer from cysteine to triplet-excited FMN. On the other hand, differences in signal amplitude and intensity *versus* delay time (maximum amplitude at *ca.* 800 ns rather than at 400 ns) were noted. Since cysteine is a less efficient one-electron donor compared to EDTA, these variations seem reasonable. Irradiation of samples containing 5 mM FMN, 25 mM cysteine, and 50 mM of the respective hop-derived acids resulted in TREPR spectra that were very similar to those found for samples without addition of cysteine. This confirms the notion that the sulfur source does not interfere significantly with the primary photochemical events.

Product analyses

Our previous efforts to understand the photooxidation of isohumulones and derivatives by excited flavins have mainly been

focused on the identification of products originating from five-membered ring hop derivatives,[16] while the fate of the interacting flavin molecules has hitherto not been examined. Therefore, the aim of this product study was to determine the formation of possible flavin-derived reaction products, in order to obtain further evidence for previously proposed reaction mechanisms.

Photochemistry of flavins in the presence of isohumulones

The presence of 0.05–0.1 mole equivalents of RF induced complete degradation of isohumulones on prolonged irradiation (>8 h). Short exposure (*ca.* 1 h) to visible light of a methanol/water mixture (1 : 1, v : v) containing *trans*-isohumulone (*trans*-**2a**; 1.4 mM) (the implementation of the descriptor *trans* is described in the experimental section) and RF (**1**; 0.066 mM) furnished a distinct number of fast-eluting photoreaction products (Fig. 6) that were identified as riboflavin derivatives (Table 1). Late-eluting peaks (9–18) could be assigned to *trans*-isohumulone and its photodegradation products. Although these compounds have been studied before, a new prominent degradation product could be identified, most likely due to altered reaction conditions (increased irradiation times).

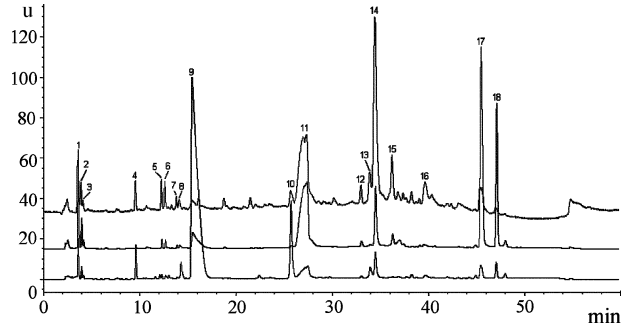


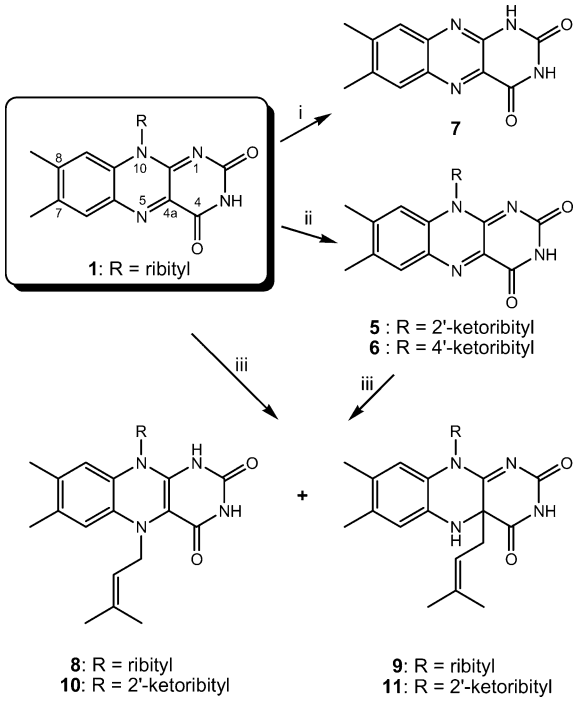
Fig. 6 Photoreaction products after visible-light irradiation of *trans*-isohumulone (*trans*-**2a**) in the presence of riboflavin (lower trace: detection at 320 nm, middle trace: detection at 280 nm, upper trace: total ion chromatogram (positive ionization mode)).

Irradiation of a reaction mixture including *trans*-isohumulone (*trans*-**2a**) and RF (**1**) resulted in previously described photoreaction products such as 2′-ketoflavin (**5**) and 4′-ketoflavin (**6**) that were formed through photooxidation by abstraction of the α-hydrogen at C-2′ or C-4′, respectively (Scheme 2).[27] Photodealkylation of RF resulted in the formation of lumichrome (**7**),[30] with concurrent characteristic alteration of the UV features from isoalloxazine ($\lambda_{max} = 450$ nm) to alloxazine ($\lambda_{max} = 350$ nm).

Table 1 Overview of characterized compounds on HPLC-MS analysis of photoreaction products formed on visible-light irradiation of *trans*-isohumulone (*trans*-**2a**) in the presence of riboflavin (**1**)

Peak	t_R/min[a]	M/u[b]	$\Delta M/u$	Compound
1	3.62	376	0[c]	**1**
2	3.95	374	−2[c]	**5**
3	4.16	374	−2[c]	**6**
4	9.57	242	−134[c]	**7**
5	12.30	446	70[c]	**8**
6	12.66	446	70[c]	**9**
7	13.88	444	68[c]	**10**
8	14.15	444	68[c]	**11**
9	15.49	280	−82[d]	T[e]
10	25.71	296	−66[d]	T[e]
11	27.17	362	0[d]	*trans*-**2a**
12	33.05	362	0[d]	*cis*-**2a**
13	33.96	316	−46[d]	N.i.[f]
14	34.44	334	−28[d]	**12**
15	36.25	394	32[d]	T[e]
16	39.71	316	−46[d]	N.i.[f]
17	45.44	362	0[d]	T[e]
18	47.06	362	0[d]	T[e]

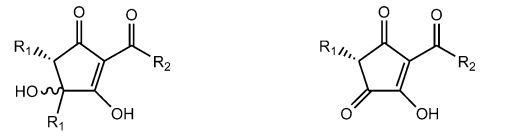
[a] Retention time. [b] Molecular mass. [c] Molecular mass difference with respect to **1**. [d] Molecular mass difference with respect to *trans*-**2a**. [e] Tentative structure assignment is discussed in text. [f] Not identified.



Scheme 2 Photoreaction products derived from riboflavin (**1**): (i) photodealkylation; (ii) photooxidation; (iii) radical recombination.

Four compounds eluting as pairs of peaks at *ca.* 12.5 min with a molecular mass of 446 u and at *ca.* 14 min with a molecular mass of 444 u, respectively, were characterized as flavin derivatives. The increase in molecular mass by 70 u relative to RF (**1**) and 2′-ketoflavin (**5**), respectively, implies addition of a 3-methylbut-2-enyl group and can be envisaged to proceed as follows. In a first step, one-electron transfer (accompanied by instant proton exchange) from the ground state of isohumulones to triplet-excited RF yields a semiquinone radical in combination with the triacylmethyl radical of isohumulones.[14,15] Subsequent degradation of the isohumulone radical affords a 3-methylbut-2-enyl radical (after fast decarbonylation of a 4-methylpent-3-enoyl radical) *via* formal α-cleavage of the side chain at C(4). Finally, recombination of the 3-methylbut-2-enyl radical with a semiquinone radical either from riboflavin or 2′-ketoflavin gives the observed adducts **8/9** and **10/11**, respectively. The pairs of isomeric compounds are, most likely, due to regiodifferentiated addition of a 3-methylbut-2-enyl radical at N(5) or at C(4a). These positions have been reported to carry significant spin density in flavin radicals, hence they are particularly reactive towards radical recombinations.[31]

An important photoreaction product derived from *trans*-isohumulone (*trans*-**2a**) that could not be observed previously was identified as decarbonylated isohumulone (**12**, Scheme 3). Most likely, upon prolonged irradiation, a 3-methylbut-2-enyl was added to dehydrohumulinic acid (**13**; M = 264 u, a primary degradation product resulting from photooxidation of isohumulones[16]), leading to formation of **12**. Minor reaction products have not been studied in detail, since their less pronounced occurrence indicates that they originate from minor side reactions. Feasible pathways include oxygenation of double bonds in dehydrohumulinic acid (**13**) or *trans*-isohumulone (*trans*-**2a**) by singlet oxygen ($^1O_2$) that was generated on interaction of residual oxygen with triplet-excited flavins (compounds corresponding to peak 9 and 15, respectively). However, addition of ground-state oxygen ($^3O_2$) to incipient triacylmethyl radicals of **13** or *trans*-**2a** is also plausible, thereby



12: $R_1 = CH_2CH{=}C(CH_3)_2$, $R_2 = CH_2CH(CH_3)_2$
14a: $R_1 = CH_2CH_2CH(CH_3)_2$, $R_2 = CH_2CH(CH_3)_2$
14b: $R_1 = CH_2CH_2CH(CH_3)_2$, $R_2 = CH(CH_3)_2$

13: $R_1 = CH_2CH{=}C(CH_3)_2$, $R_2 = CH_2CH(CH_3)_2$
15a: $R_1 = CH_2CH_2CH(CH_3)_2$, $R_2 = CH_2CH(CH_3)_2$
15b: $R_1 = CH_2CH_2CH(CH_3)_2$, $R_2 = CH(CH_3)_2$

Scheme 3 Photoreaction products derived from five-membered ring hop derivatives.

forming the respective hydroxide or hydroperoxide derivatives (compounds corresponding to peak 9 (R–OH) and 10 (R–OOH) derived from **13**; peak 15 (R–OOH) derived from *trans*-**2a**). Analogous to the formation of **12**, addition of a 4-methylpent-3-enoyl radical to dehydrohumulinic acid may also occur. Thus, either the starting material *trans*-isohumulone (*trans*-**2a**) or its epimer *cis*-isohumulone (*cis*-**2a**) are formed. Since decarbonylation of a 4-methylpent-3-enoyl is fast, product formation by this pathway is limited. Remarkably, two late-eluting compounds (peak 17 and 18) have an identical molecular mass as *trans*-isohumulone (*trans*-**2a**). The presence of the isomeric, six-membered ring humulone could be excluded based on retention time and UV characteristics (both compounds show an UV spectrum which differs significantly from the humulone spectrum). Other known regioisomers of isohumulones include *anti*-isohumulones,[32] which could in this case be the result of addition of a 4-methylpent-3-enoyl radical to the β-tricarbonyl chromophore in dehydrohumulinic acid. However, this structure assignment is only tentative and preparative-scale experiments should yield higher amounts of these photoproducts for further spectroscopic investigations.

Photochemistry of flavins in the presence of tetrahydroisohumulones

The chromatogram of photoreaction products resulting from irradiation of a mixture of *trans*-tetrahydroisohumulones (*trans*-**3a–c**; 2.7 mM) and riboflavin (**1**; 0.13 mM) shows evident similarities to the chromatogram obtained on analysis of the mixture of photoreaction products formed on sensitized irradiation of isohumulones (Fig. 7). Detailed analysis revealed minor, yet interesting differences. An overview of the characterized constituents and their corresponding molecular masses is given in Table 2. In analogy to sensitized irradiation of isohumulones, photoreaction products can be divided in two series. All riboflavin-derived photoreaction products elute in the most polar part of the chromatogram with retention times between 3 min and 16 min, while photoreaction products originating from *trans*-tetrahydroisohumulones elute with retention times between 15 min and 52 min.

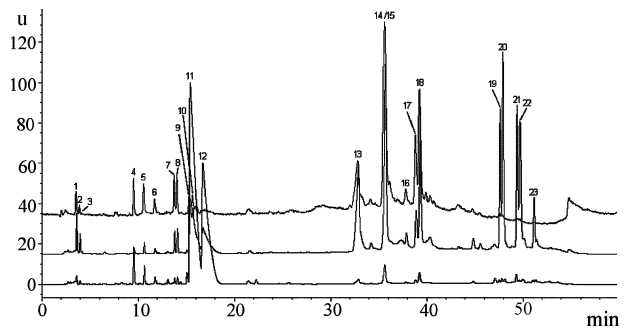


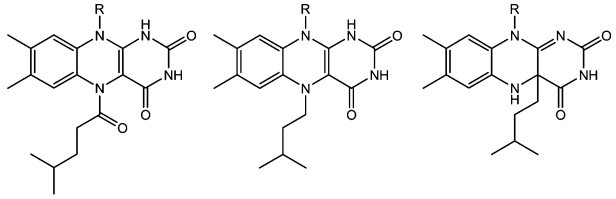
Fig. 7 Photoreaction products after visible-light irradiation of *trans*-tetrahydroisohumulones (*trans*-**3a–c**) in the presence of riboflavin (lower trace: detection at 320 nm, middle trace: detection at 280 nm, upper trace: total ion chromatogram (positive ionization mode)).

Table 2 Overview of characterized compounds on HPLC-MS analysis of photoreaction products formed on visible-light irradiation of *trans*-tetrahydroisohumulones (*trans*-**3a–c**) in the presence of riboflavin (**1**)

Peak	t_R/min[a]	M/u[b]	$\Delta M/u$	Compound
1	3.61	376	0[c]	**1**
2	3.95	374	−2[c]	**5**
3	4.14	374	−2[c]	**6**
4	9.58	242	−134[c]	**7**
5	10.63	476	100[c]	**16**
6	11.75	474	98[c]	**17**
7	13.80	448	72[c]	**18**
8	14.10	448	72[c]	**19**
9	15.37	446	70[c]	**20**
10	15.38	268	−84[d]	T[f]
11	15.69	446	70[c]	**21**
12	16.69	282	−84[e]	T[f]
13	32.87	352	0[d]	*trans*-**3b**
14	35.59	324	−28[d]	**14b**
15	35.59	366	0[e]	*trans*-**3a**
16	37.77	366	0[e]	*trans*-**3c**
17	38.82	366	0[e]	T[f]
18	39.23	338	−28[e]	**14a**
19	47.61	352	0[d]	T[f]
20	47.90	352	0[d]	T[f]
21	49.37	366	0[e]	T[f]
22	49.68	366	0[e]	T[f]
23	51.12	366	0[e]	T[f]

[a] Retention time. [b] Molecular mass. [c] Molecular mass difference with respect to **1**. [d] Molecular mass difference with respect to *trans*-**3b**. [e] Molecular mass difference with respect to *trans*-**3a** or *trans*-**3c**. [f] Tentative structure assignment is discussed in text.

The four first eluting compounds in the mixture of photoreaction products resulting from sensitized irradiation of *trans*-tetrahydroisohumulones (*trans*-**3a–c**) are identical to those derived from sensitized irradiation of *trans*-isohumulone (*trans*-**2a**). In addition to residual RF (**1**), 2′-keto-flavin (**5**), 4′-ketoflavin (**6**), and lumichrome (**7**) were observed as photoreaction products derived from photodegradation of RF. Also, a number of RF derivatives with an increased molecular mass, resulting from recombination processes, could be detected. Indeed, sensitized irradiation of *trans*-tetrahydroisohumulones led to the formation of adducts of the semiquinone radical with a 4-methylpentanoyl radical or a 3-methylbutyl radical, which are also the precursors of volatile photodegradation products of tetrahydroisohumulones.[16] The molecular masses of 476 u and 474 u found for two compounds eluting with $t_R = 10.63$ min and $t_R = 11.75$ min, respectively, are consistent with the incorporation of a 4-methylpentanoyl group in RF (**16**) and 2′-ketoflavin (**17**), respectively (Scheme 4). Most likely, regioselective addition of a 4-methylpentanoyl group to N(5) occurred, however no isomeric pairs were observed. On the other hand, the presence of isomeric photoreaction products with molecular masses of 448 u and 446 u indicates regiodifferentiated addition of a 3-methylbutyl group, most likely, at N(5) and C(4a) in RF (**18/20**) and 2′-ketoflavin (**19/21**), respectively.



16: R = ribityl
17: R = 2'-ketoribityl
18: R = ribityl
19: R = 2'-ketoribityl
20: R = ribityl
21: R = 2'-ketoribityl

Scheme 4 Riboflavin-derived reaction products resulting from sensitized irradiation of *trans*-tetrahydroisohumulones (*trans*-**3a–c**).

Analogous to *trans*-isohumulone, irradiation of a mixture of *trans*-tetrahydroisohumulones (one homolog and two

isomers) yielded decarbonylated tetrahydroisohumulones (**14a–b**) after addition of a 3-methylbutyl radical to hydrogenated dehydrohumulinic acids (**15a–b**, which have the double bond in the side chain at C(5) reduced with respect to dehydrohumulinic acids (**13**) and were previously identified as primary photoproducts in this reaction).[16] However, since decarbonylation to a 3-methylbutyl radical is slow, its precursor, a 4-methylpentanoyl radical, is more prevalent and may also add to the hydrogenated dehydrohumulinic acids. Thus, either the initial *trans*-tetrahydroisohumulones (*trans*-**3a–c**) or the epimeric *cis*-tetrahydroisohumulones (*cis*-**3a–c**) may result. Since the most abundant compound in the starting material is *trans*-tetrahydroisohumulone (*trans*-**3a**), it is very likely that *cis*-tetrahydroisohumulone (*cis*-**3a**) is the more prevalent epimer to be detected. Minor degradation products (as observed in the total ion chromatogram) are possibly derived from oxidation reactions of hydrogenated dehydrohumulinic acids (compounds corresponding to peaks 10 and 12). Late-eluting compounds (peak 17 and peaks 19–23) were also found to correspond to isomers of the starting material. As was advanced previously for *trans*-isohumulone, it is feasible that these isomeric compounds are the *anti*-derivatives of tetrahydroisohumulones. However, this assignment is only tentative and further spectroscopical investigation should shed light on this issue.

Photoreactivity of dihydroisohumulones under sensitized irradiation conditions

In contrast to the photolyses of *trans*-isohumulone (*trans*-**2a**) and *trans*-tetrahydroisohumulones (*trans*-**3a–c**) in conjunction with formation of a number of major photoreaction products, no important photoreaction products could be detected for the dihydroisohumulones (**4a–c**). Remarkably, results from TREPR and laser-flash photolysis transient absorption spectroscopy[13] clearly showed the occurrence of a one-electron transfer from dihydroisohumulones to triplet-excited RF, while the occurrence of **13** as a major degradation product on sensitized irradiation was evidenced previously.[16] A possible explanation includes the complexity of the mixture of dihydroisohumulones used as starting material in this investigation, as photoreaction products may escape detection due to coelution under the chromatographic conditions used. Still, these contradictory results are a topic of further investigation.

Conclusions

The combination of photostationary and photodynamic experiments has further contributed to the elaboration of reaction mechanisms for the photochemistry between excited flavins and isohumulones and derivatives. The early photochemical event in the riboflavin-sensitized photodegradation of isohumulones (or reduced derivatives thereof) was unequivocally proven to be a one-electron transfer from five-membered ring hop compounds to triplet-excited flavins. The flavin semiquinone radical thus formed could be readily detected, either by steady-state or by time-resolved electron paramagnetic resonance spectroscopy. Superimposed signals in the spectra revealed the presence of radical fragments derived from isohumulones or tetrahydroisohumulones, which produced stable reaction products on recombination with flavin semiquinone radicals. No superimposed signal was observed on irradiation of dihydroisohumulones, since, in this case, radical fragments were, most likely, too reactive to be detected.

Acknowledgements

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