Flavin-induced photodecomposition of sulfur-containing amino acids is decisive in the formation of beer lightstruck flavor†

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Photooxidation of sulfur-containing amino acids and derivatives readily occurs upon visible-light irradiation in the presence of flavins. The sulfur moiety seems pivotal for interaction, as was determined from kinetic analyses using laser flash photolysis spectroscopy. After photooxidation, the resulting radical intermediates were characterized by addition to a spin trap, followed by electron paramagnetic resonance spectroscopy and evaluation of the coupling constants. The presence of the proposed radical intermediates was strongly supported by the identification of the reaction products using mass spectrometry. Accordingly, feasible degradation pathways for various sulfur-containing amino acids and derivatives were proposed. It was finally proven that flavin-induced photoproduction of sulfhydryl radicals and recombination with a 3-methylbut-2-enyl radical, derived from the photodegradation of hop-derived isohumulones, are decisive in the formation of beer lightstruck flavor.

Introduction

Flavor quality of beer is compromised by exposure to light, a phenomenon which is generally referred to as 'lightstruck flavor' (LSF). Although the occurrence of off-flavor formation in light-exposed beer was already recognized in 1875,[1] the mechanism has not been fully elucidated to date. It is well-known that LSF is predominantly characterized by the formation of 3-methylbut-2-ene-1-thiol (MBT),[2] which produces a foul smell already at concentrations of few ng L^{-1}.[3] Formation of MBT, also called *skunky thiol* due to its resemblance to compounds occurring in the secretions of skunks anal glands, is unique to beer, as it is not present in other light-sensitive beverages such as milk, fruit juice, wine, and champagne.[4] Indeed, the use of hops (*Humulus lupulus* L.) for beer brewing is pivotal in the development of LSF,[5] as MBT formation results from the photodegradation of isohumulones,[6] a set of hop-derived five-membered-ring compounds which mainly account for the typical bitter taste, but also for bacteriostatic activity[7] and for stable foam build-up.[8] Despite the fact that isohumulones were found to decompose following absorption of 300 nm light,[9,10] blue light (350–500 nm) was observed to be most efficient in generating LSF.[6] Since isohumulones do not absorb in this wavelength range, the intervention of a photosensitizer such as riboflavin (vitamin B_2) seemed essential. Indeed, riboflavin and derivatives (flavin mononucleotide and flavin adenine dinucleotide), which show absorption maxima at 375 nm and 445 nm, are present in beer in concentrations of few hundreds of $\mu g\ L^{-1}$.[11] The high molar absorptivities ($>10^4\ M^{-1}\ cm^{-1}$) are characteristic for π–π^* transitions that initially produce the short-lived singlet-excited state.[12] Subsequent intersystem crossing to the triplet-excited state is associated with potent electron-acceptor properties that lead to photooxidation of various organic substrates.[13–19] Previous studies indicated that a similar interaction with isohumulones leads to the formation of a 3-methylbut-2-enyl radical,[20–22] which eventually recombines with a sulfhydryl radical to form MBT. However, unlike for the photodegradation pathway of isohumulones that has been studied in detail,[23,24] little insight exists as to the nature and the role of the sulfur source. Cysteine, as well as sulfur-containing peptides and proteins, have been suggested to act as potential sulfur donors,[25] but a mechanism involving the intervention of a sulfhydryl radical has not been elucidated to date.

The purpose of this study was to investigate the involvement of sulfur radicals as a final step in the photoinduced formation of MBT. Therefore, relevant sulfur-containing amino acids and derivatives were employed in model systems involving flavin-mediated photooxidation. Highly reactive, short-lived intermediates were analyzed using laser flash photolysis spectroscopy and the technique of spin trapping with subsequent electron paramagnetic resonance spectroscopy. Furthermore, photoreactions were the subject of a comprehensive product analysis by mass spectrometry in order to provide support for the existence of the proposed reaction intermediates.

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Experimental

Chemicals

N-Acetyl-L-cysteine, DL-alanine, *N*-*tert*-butoxycarbonyl-L-alanine (*N*-BOC-L-alanine), *N*-*tert*-butoxycarbonyl-L-methionine (*N*-BOC-L-methionine), cysteamine hydrochloride (2-mercaptoethylamine hydrochloride), L-cysteine, L-cysteine ethyl ester hydrochloride, dimethylaminoethanethiol hydrochloride,

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glutathione, 3-mercaptopropionic acid, L-methionine, and L-penicillamine (3,3-dimethyl-L-cysteine) were supplied by Acros (Beerse, Belgium). *S*-Methyl-L-cysteine and DL-homocysteine were obtained from MP Biomedicals Europe (Brussels, Belgium), while 2-(methylthio)ethylamine, L-methionine-*carboxy*-^{13}C, flavin mononucleotide sodium salt, 5,5-dimethylpyrroline *N*-oxide, 2-methyl-2-nitrosopropane, dihydrogen sulfide, and dimethyl disulfide were purchased from Aldrich (Bornem, Belgium). Methanethiol was available from Fluka (Bornem, Belgium). Isohumulones, formulated as an aqueous solution (*ca.* 30% w/v) of their potassium salts, were a kind gift by Botanix (Eardiston, Near Tenbury Wells, Worcestershire, UK). Pure 3-methylbut-2-ene-1-thiol was obtained from Oxford Chemicals (Hartlepool, UK).

All solutions were prepared using Milli-Q (Millipore, Bedford, Massachusetts, USA) double-distilled water ($R = 18$ mΩ cm^{-2}). Buffers at 0.1 M were freshly prepared from potassium dihydrogen phosphate and sodium hydroxide (pH 7.0) or from citric acid and trisodium citrate (pH 4.2), respectively.[26]

Laser flash photolysis spectroscopy

The third harmonic (355 nm) of a pulsed Q-switch Nd:YAG laser was applied to pump a dye laser system (Spectron Laser Systems, Rugby, UK) containing a Coumarin 440 (Exciton, Dayton, Ohio, USA) solution in methanol. The resulting 440 nm laser flash (pulse width ~8 ns) served to excite solutions, consisting of flavin mononucleotide (125 μM) and selected compounds that were added in concentrations varying from 0.5 mM up to 50 mM. The analytical light beam, generated by a pulsed xenon lamp (Applied Photophysics, Leatherhead, UK), was sent through a filter (cut-off wavelength: *ca.* 610 nm) before entering the sample in order to avoid absorption interference. Subsequently, the analyzing light passed through a monochromator (resolution: 4 nm) and the transient absorption at 720 nm was measured by a R928 photomultiplier tube (Hamamatsu Photonics, Hamamatsu City, Japan). Absorbances were measured at 293 K, while the laser energy amounted to approximately 2.6 mJ pulse^{-1}.

Electron paramagnetic resonance spectroscopy

Solutions were prepared by dissolving amino acids or derivatives (10 mM) and flavin mononucleotide (250 μM) in a buffer at pH 4.2 or 7.0. Either 5,5-dimethylpyrroline *N*-oxide (DMPO, 7.5 mM) or 2-methyl-2-nitrosopropane (MNP, 1.3 mM up to 4 mM) were added as spin traps, followed by degassing by purging with nitrogen. Subsequently, samples were transferred into a flat quartz cell by a peristaltic pump in order to avoid oxygen intake. Irradiation experiments were carried out inside the EPR resonator cavity using a focused light beam generated by a wavelength-adjustable snake light (wavelength: 440 nm, power: 2 mW).

A Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany) was used for EPR spectroscopy. The following settings were applied: center field: 3475 G; sweep width: 80 G; microwave power: 10 mW; modulation frequency: 100 kHz; modulation amplitude: 1.0 G; conversion time: 20.48 ms; sweep time: 21 s. Spectra of spin adducts were the result of a 4 scan accumulation, while direct observation of radicals resulted from addition of 10 scans. Simulation and fitting of EPR spectra to calculate hyperfine coupling constants were performed by the PEST WinSIM program.[27]

Irradiations

Photoreaction mixtures, containing amino acids or derivatives (3 mM) and flavin mononucleotide (1 mM) dissolved in water, buffer pH 4.2 or buffer pH 7.0, were prepared in 5 mL transparent Gerstel headspace vials. For the study of MBT formation, analogous model systems were prepared with 5 mM isohumulones added. Samples were degassed by purging with nitrogen before capping and were protected from light prior to irradiation. Identical but non-irradiated samples served as controls, while samples without flavin mononucleotide were analyzed in order to exclude potential product formation from thermal decomposition in the heating turret of the headspace analysis setup. Irradiations (2 h) were carried out in a photoreactor equipped with Philips Cool White lamps (8 × 9 W; 2720 lx), followed by headspace GC-MS analysis.

For analyses by electrospray ionization coupled to a mass spectrometer (ESI-MS) solutions were prepared in acetonitrile–water (v/v, 1 : 1) to improve atomization. Final concentrations of flavin mononucleotide and amino acids (and derivatives) were 1 mM and 5 mM, respectively. Time-dependent formation of reaction products was monitored by direct irradiation of the infusion syringe with a commercially available 50 W incandescent lamp.

Gas chromatography-mass spectrometry (GC-MS)

Volatiles resulting from photooxidation reactions were analyzed by GC-MS using a headspace sampler and a CIS-4 liquid-nitrogen cooled injection system (Gerstel, Mülheim-an-der-Ruhr, Germany). The GC-MS system consisted of an Agilent 6890 series GC system, connected to a HP5973 mass-selective detector (Agilent, Palo Alto, California, USA). Prior to injection, vials were preheated for 10 min at 60 °C. Injection of 1 mL headspace volume was followed by cryofocusing (−50 °C) in the injector liner on Tenax TA (20/35 mesh, Alltech, Deerfield, Illinois, USA). Subsequent flash heating (to 250 °C) of the cold trap transferred the analytes to the capillary column. The injector split ratio during desorption was 0.7 : 1, increasing to 40 : 1 at completion of the injection. Chromatographic separation was carried out on a Chrompack fused-silica CP-Select 624 (6% cyanopropylphenyl-dimethylsilicone) capillary column (41 m × 0.25 mm id, 2.1 μm film thickness) using the following temperature program: 10 min at 38 °C, raised to 170 °C at 10 °C min^{-1}, and 10 min at 170 °C. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. The mass spectrometer was operated in the scan mode (mass range: m/z 34–300 u) or in the single-ion mode for specific analytes (*vide infra*). Major reaction products were identified by comparing retention times and mass spectra with reference compounds. Furthermore, experimental spectra obtained from minor compounds were compared to library mass spectra using a NIST Mass Spectral Database v. 2.0 (National Institute of Standards and Technology, US Secretary of Commerce, Gaithersburg, Maryland, USA).

Electrospray ionization-mass spectrometry (ESI-MS)

Reaction mixtures were irradiated simultaneously with a continuous flow injection in the electrospray ionization source (Z-spray) of a quadrupole time-of-flight (Q-TOF) hybrid mass spectrometer (Waters, Manchester, UK).[21] A Harvard syringe pump, equipped

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with a 0.25 mL Hamilton glass syringe, was used at an infusion rate of 10 μL min^{-1}. Source and desolvation temperatures were 80 °C and 120 °C, respectively; desolvation gas: 450 L h^{-1}; nebulizer gas 20 L h^{-1}; capillary voltage: 2900 V; cone voltage: 40 V. Mass spectra were acquired over a mass range from 35 u to 750 u, both in the positive and the negative ionization modes.

Results

In order to establish the feasibility of generating sulfenyl or sulfhydryl radicals by photooxidation, the kinetics of the interaction of sulfur-containing amino acids and derivatives with excited flavin molecules were initially investigated by laser flash photolysis spectroscopy. Since these reactions were studied in aqueous model systems, the sodium salt of flavin mononucleotide (FMN) was selected as light-absorbing species. Indeed, the solubility of FMN is superior to that of riboflavin, while the photochemical properties remain unaltered in the presence of the phosphate moiety.[28] Furthermore, FMN shows an absorption band with a maximum at 445 nm, whereas the selected sulfur-containing compounds are transparent in the visible. Triplet-excited flavin mononucleotide (^{3}FMN*), generated by a 440 nm laser pulse, shows a characteristic absorption at 720 nm,[15] which allows the monitoring of the lifetime of ^{3}FMN* in the presence of various compounds. It was concluded that increasing concentrations of the substrates proportionally affected the ^{3}FMN* decay rate (Fig. 1). As a result, bimolecular rate constants for the interaction could be determined from the slope of the linear plot of the pseudo-first-order rate constants (observed by transient absorption spectroscopy at 720 nm) as a function of the concentration of sulfur compounds. The substrates included amino acids, as well as judiciously chosen derivatives thereof, since the influence of structural disparities on reaction kinetics allows to retrieve the role of functionalities in the interaction mechanism. Bimolecular rate constants were investigated at pH 4.2, which is a typical pH value for lager beers, and at pH 7.0 (Table 1).

Next to kinetic investigations, continuous wave electron paramagnetic resonance spectroscopy (CW-EPR) was applied to model systems that were irradiated at 440 nm inside the cavity of the spectrometer. All systems containing FMN and a particular amino acid (or derivative) showed a broad signal with distinct hyperfine couplings (peak-to-peak linewidth ~18.5 G) after irradiation for 10 min (Fig. 2). This radical appeared relatively stable, since it persisted after irradiation was interrupted. However, as a similar signal was observed for each compound investigated, mechanistic information could not be extracted. Subsequently, spin traps including 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and 2-methyl-2-nitrosopropane (MNP) were added to convert highly reactive radicals that escape detection by CW-EPR analysis into relatively stable, EPR-active spin adducts.

Table 1 Bimolecular rate constants k (10^6 L mol^{-1} s^{-1}) for the interaction of amino acids (and derivatives) with ^{3}FMN*

Substrate	pH 4.2	pH 7.0
Alanine	N.q.[a]	0.03
N-BOC-Alanine	N.q.[a]	N.q.[a]
Cysteine	1.4	40.5
Penicillamine	1.6	55.4
3-Mercaptopropionic acid	36.7	45.6
Cysteamine	1.1	103.5
Dimethylaminoethanethiol	1.5	217.1
N-Acetylcysteine	12.1	10.7
Cysteine ethyl ester	2.5	132.5
Homocysteine	15.3	13.1
Glutathione	7.3	14.3
S-Methylcysteine	328.6	120.8
2-(Methylthio)ethylamine	48.1	33.5
Methionine	48.6	21.5
N-BOC-Methionine	713.6	108.6

[a] No quenching observed.

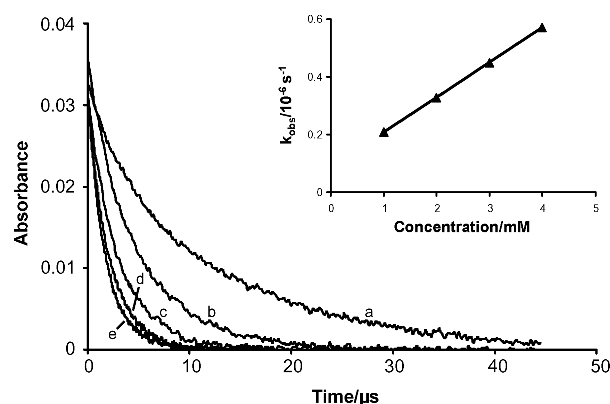

Fig. 1 Transient absorption trace of ^{3}FMN*, observed at 720 nm, in the presence of varying concentrations of *S*-methylcysteine (a: 0 mM, b: 1 mM, c: 2 mM, d: 3 mM, e: 4 mM) at pH 7.0. Inset: linear plot of the pseudo-first-order rate constants (k_{obs}) for the decay of ^{3}FMN* as a function of the concentration of *S*-methylcysteine.

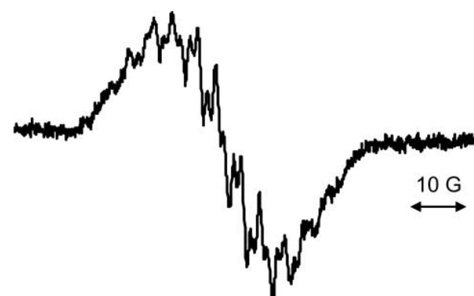

Fig. 2 EPR spectrum observed during 440 nm irradiation of FMN in the presence of methionine.

Spin trapping with DMPO

First, potential interferences during irradiation of model systems, either from photodecomposition of the spin trap or from radical formation from buffer constituents, were evaluated by recording spectra under identical reaction conditions in the absence of substrate. Since systems buffered at pH 4.2 suffered from an excessive EPR background signal (most likely originating from secondary reactions involving citrate), analysis in the inorganic, EPR-silent phosphate buffer (pH 7.0) was preferred. Fig. 3 shows a selection of spin patterns with corresponding simulated spectra, resulting from photooxidation of various substrates. Spectra were recorded shortly after irradiation was started, as prolonged reaction times (>5 min) strongly reduced the intensity of the observed signals for several compounds. Furthermore, no

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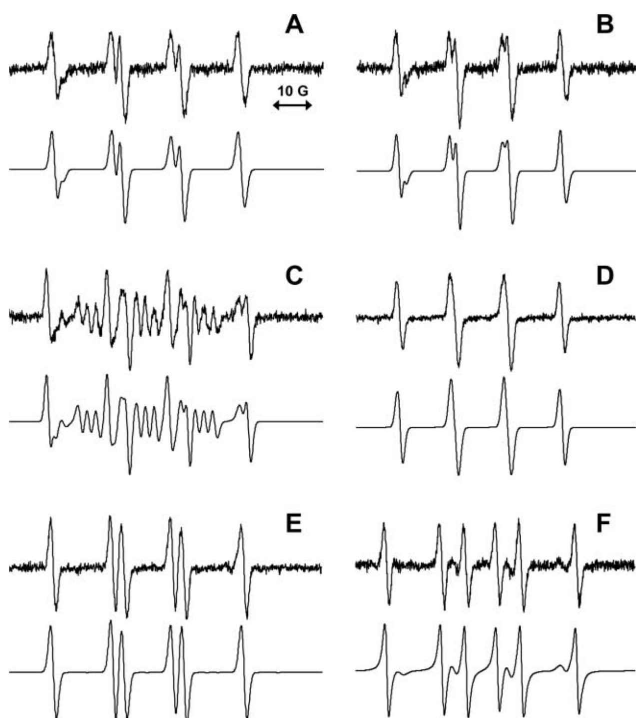

Fig. 3 Experimental (upper) and simulated (lower) EPR signals of spin adducts resulting from photooxidation of sulfur-containing substrates by $^{3}FMN^{*}$ at pH 7.0 and subsequent trapping by DMPO. A: cysteine, B: cysteamine, C: penicillamine, D: glutathione, E: *S*-methylcysteine, and F: 2-(methylthio)ethylamine.

significant spin adduct formation with DMPO could be observed when alanine was irradiated in the presence of FMN. Relevant data including values of coupling constants of spin adducts, obtained by computer simulations, are listed in Table 2.

Table 2 Relevant data from simulations of the EPR spectra of DMPO adducts with radicals derived from photooxidation of various sulfur-containing substrates

Compound	Time/s	RA[a] (%)	a_N[b]/G	a_H[c]/G
Cysteine	30	80.5	15.2	17.2
		19.5	15.0	15.7
Penicillamine	60	23.7	15.2	17.8
		36.1	15.4	20.2
		30.1	14.9; 2.2	—
		10.1	14.9	15.6
3-Mercaptopropionic acid	30	100	15.1	16.7
Cysteamine	30	75.1	15.2	16.9
		24.9	14.9	15.3
Dimethylaminoethanethiol	10	100	15.2	16.9
N-Acetylcysteine	60	94.2	15.2	16.8
		5.8	15.4	22.5
Cysteine ethyl ester	10	100	15.1	16.5
Homocysteine	10	86.9	15.2	17.0
		13.1	15.0	15.7
Glutathione	10	100	15.2	16.2
S-Methylcysteine	60	100	15.3	18.0
2-(Methylthio)ethylamine	60	89.1	15.9	22.7
		10.9	15.4	16.4
Methionine	10	77.3	15.5	23.9
		22.7	15.2	18.0
N-BOC-Methionine	60	100	15.6	21.7

[a] Relative abundance of the observed spin adduct. [b] Coupling constant to nitrogen. [c] Coupling constant to hydrogen.

Table 3 Relevant data from simulation of the EPR spectra of MNP adducts with radicals derived from photooxidation of various sulfur-containing substrates

Compound	Time/s	RA[a] (%)	a_N[b]/G	a_H[c]/G
Alanine	300	100	17.2	—
Penicillamine	30	100	16.0	—
3-Mercaptopropionic acid	30	100	17.8	—
N-Acetylcysteine	60	86.1	18.2	—
		13.9	14.5	14.1
Glutathione	30	50.4	14.5	14.2
		49.6	18.3	—
S-Methylcysteine	300	53	17.2	—
		25.5	18.5	1.5
		21.5	14.6	14.3
2-(Methylthio)ethylamine	300	100	17.1	—
Methionine	300	73.2	14.5	14.1
		26.8	17.2	—

[a] Relative abundance of the observed spin adduct. [b] Coupling constant to nitrogen. [c] Coupling constant to hydrogen.

Spin trapping with MNP

Although MNP is known to be photoactive,[29] no signal was observed when a MNP solution was exposed to 440 nm light. On the other hand, irradiation of a system containing MNP and FMN, buffered at pH 7.0, produced an intense triplet ($a_N \sim 17.2$ G), which was only observed after prolonged exposure (>5 min). Addition of the various substrates led to different observations when compared to trapping with DMPO, as only few compounds (presented in Table 3) gave rise to detectable spin adducts (Fig. 4). Signals for alanine and 2-(methylthio)ethylamine were identical to the background spectrum, while extra signals for *S*-methylcysteine and methionine appeared only after prolonged irradiation times.

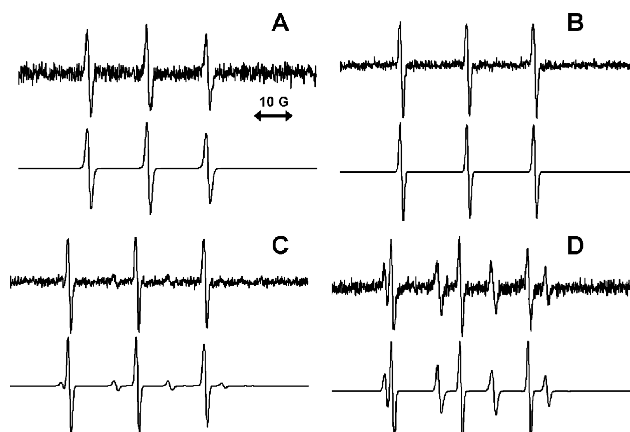

Fig. 4 Experimental (upper) and simulated (lower) EPR signals of spin adducts, resulting from photooxidation of sulfur-containing substrates by $^{3}FMN^{*}$ at pH 7.0 and subsequent trapping by MNP. A: penicillamine, B: 3-mercaptopropionic acid, C: *N*-acetylcysteine, and D: glutathione.

Identification of reaction products by GC-MS and ESI-MS

As photooxidation most likely initiated a radical degradation pathway, formation of volatile low-molecular-weight reaction products seemed obvious. Thus, visible-light irradiation of model systems, prepared in water or buffered at pH 4.2 or at pH 7.0, was followed by headspace GC-MS analysis. Identification of

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major analytes was confirmed by comparison with retention times and mass spectra of authentic reference compounds, while minor reaction products were tentatively characterized from fitting the mass fragmentation patterns to library spectra. After irradiation of alanine in the presence of flavin mononucleotide, minor levels of carbon dioxide and acetaldehyde were identified as reaction products. Formation of carbon dioxide was also observed after photooxidation of sulfur-containing substrates possessing a carboxylate group. Moreover, compounds with a thiol group, such as cysteine, cysteamine, penicillamine, *N*-acetylcysteine, homocysteine, and cysteine ethyl ester resulted invariably in formation of dihydrogen sulfide (H_2S), which was more pronounced in non-buffered systems. A thorough analysis of minor reaction products derived from penicillamine resulted in the identification of 2-methylpropanal (Fig. 5), while, on the other hand, cysteine, cysteamine, and *N*-acetylcysteine produced low amounts of acetaldehyde. Apparently, no particular volatiles were detected following photooxidation of glutathione, whereas only traces of ethanethiol (next to carbon dioxide) resulted from 3-mercaptopropionic acid. Major volatile reaction products derived from compounds bearing a methylthio group, such as *S*-methylcysteine, 2-(methylthio)ethylamine, and methionine, were methanethiol and dimethyl disulfide. Furthermore, acetaldehyde was identified as a minor reaction product derived from *S*-methylcysteine as well as from 2-(methylthio)ethylamine, while methionine-*carboxy*-^{13}C gave rise to formation of $^{13}CO_2$ (detected at m/z 45).

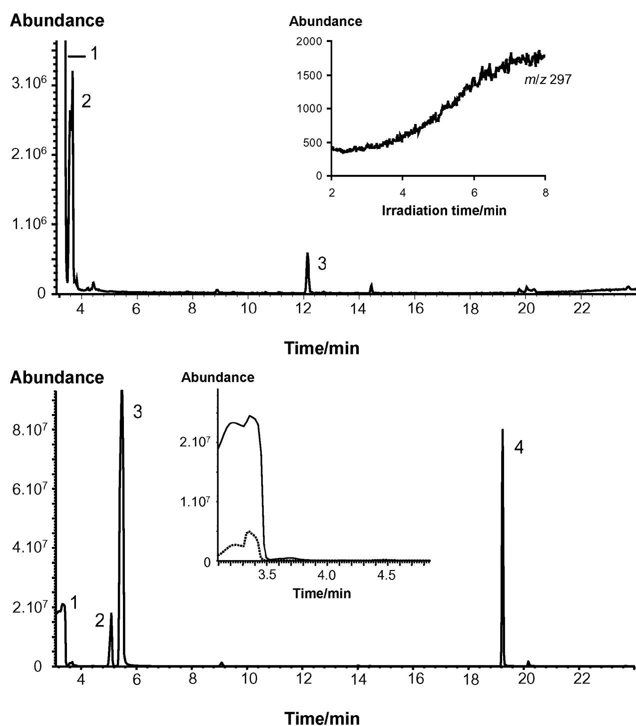

Fig. 5 Upper panel: headspace GC-MS total ion chromatogram of a reaction mixture containing FMN and penicillamine at pH 7.0 after visible-light irradiation for 2 h (peak annotation: 1: carbon dioxide, 2: dihydrogen sulfide, 3: 2-methylpropanal). Inset: extracted ion chromatogram of m/z 279 observed during irradiation concurrent with analysis by ESI-MS. Lower panel: headspace GC-MS total ion chromatogram of a reaction mixture containing FMN and *S*-methylcysteine at pH 4.2 after visible-light irradiation for 2 h (peak annotation: 1: carbon dioxide, 2: acetaldehyde, 3: methanethiol, 4: dimethyl disulfide). Inset: carbon dioxide levels before (· · ·) and after (——) photooxidation of *S*-methylcysteine at pH 4.2.

Formation of non-volatile product formation could not be observed when applying ESI-MS in the negative ionization mode. Changing the ionization polarity produced traces of reaction products resulting from photooxidation of penicillamine and cysteamine (extracted ions at m/z 297 and m/z 153, respectively), while glutathione showed product formation at m/z 231 and m/z 233. Other substrates failed to give reaction products that could be detected by ESI-MS in the positive ionization mode.

In order to confirm that photooxidation of cysteine is part of the mechanism of LSF formation, model systems, containing cysteine, flavin mononucleotide, and isohumulones, were irradiated followed by headspace GC-MS analysis. Thus, MBT could be observed as a minor reaction product, as was confirmed by the comparison of the retention time and the mass spectrum to those of standard MBT. Substituting *S*-methylcysteine for cysteine as a potential sulfur source, followed by photooxidation, produced the methylthio ether of MBT, which was identified from the comparison of its molecular mass (m/z 116) and the corresponding mass fragmentation pattern to that of MBT (m/z 102), as depicted in Fig. 6.

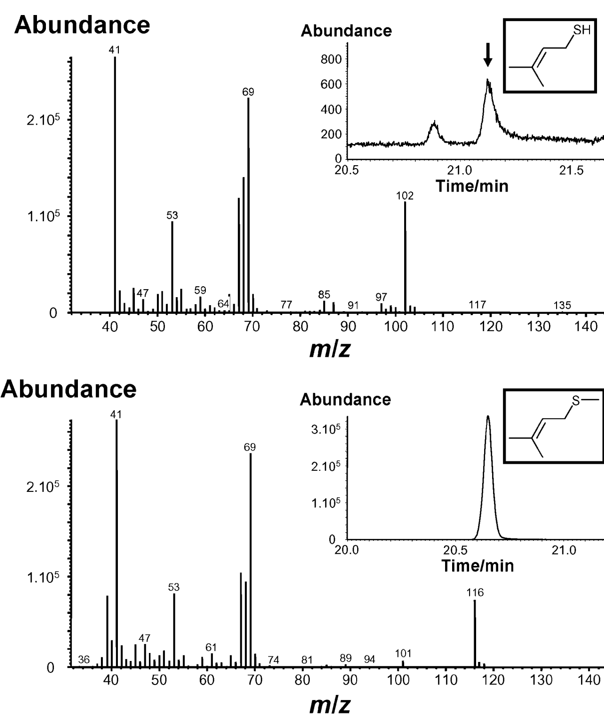

Fig. 6 Upper panel: mass spectrum of MBT, formed by visible-light irradiation (2 h) of a mixture containing FMN, cysteine and isohumulones in water (inset: monitoring of m/z 102 in the headspace. The peak corresponding to MBT is indicated with an arrow). Lower panel: mass spectrum of the methylthio ether of MBT, formed by visible-light irradiation (2 h) of a mixture containing FMN, *S*-methylcysteine and isohumulones at pH 4.2 (inset: monitoring of m/z 116 in the headspace).

Discussion

Research on formation of beer lightstruck flavor (LSF) has mainly focused on the photodegradation of isohumulones, while, remarkably, the origin of the sulfur moiety in MBT has escaped

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attention. Nevertheless, formation of a sulfhydryl radical (•SH) is pivotal in the Kuroiwa-mechanism, which formally describes the development of LSF in beer.[6] It appears that sulfur-containing amino acids (as individual entity, but also as part of peptides or proteins) may act as potential sulfur donors,[25] although details on the reaction mechanism have not been disclosed until now. Amino acids such as cysteine do not directly absorb visible light, but, clearly, triplet-excited flavins may interact with sulfur-containing amino acids (and derivatives) *via* electron abstraction,[19] thus furnishing the corresponding sulfur-centered radical cations.

As no significant quenching of ^{3}FMN* by alanine was observed in the laser flash photolysis study, it was concluded that the pronounced reactivity of cysteine and derivatives must be attributed to the presence of the thiol moiety. Remarkably, other functionalities seem to influence the oxidation rates considerably, as significant differences in bimolecular rate constants were observed. In particular, the reactivity decreased at lower pH for most thiol-containing derivatives, however not for compounds having a modified amino group. Indeed, rate constants for 3-mercaptopropionic acid (α-amino group absent), *N*-acetylcysteine and glutathione (α-amino group converted to an amide), and homocysteine (α-amino group distant from the thiol moiety) were relatively unaffected by pH variations. Therefore, it was suggested that at low pH the proximity of the protonated amino group (ammonium) hampers electron abstraction from sulfur to produce a sulfur-centered radical cation. On the other hand, cysteine and penicillamine prevail in the zwitterionic form (no net charge) at neutral pH[30] and electrostatic interactions between the ammonium and the carboxylate leave the thiol moiety freely accessible for oxidation. Furthermore, the high rate constants observed for cysteamine, *N*,*N*-dimethylaminoethanethiol, and cysteine ethyl ester appear to be merely the result of a decrease in the pK_a values of the thiol moiety.[30,31] Thus, higher levels of the readily oxidizable thiolate become available at pH 7.0.

Remarkably, substituting a methylthio ether for a thiol in cysteine greatly enhances the reaction rate, as was observed for *S*-methylcysteine, but not for 2-(methylthio)ethylamine, which suggests anchimeric assistance by the carboxy group. Such effect has been reported for the oxidation of methylthio ethers in pulse radiolysis studies[32,33] and is ascribed to the tendency of sulfur radical cations to form two-center three-electron bonds with lone pair p-electrons of heteroatoms (including also dimerization with formation of a two-center three-electron sulfur–sulfur bond). Although these interactions can be spectroscopically analyzed, characterization was prevented by severe interferences due to the presence of the strongly absorbing flavins. Participation of the carboxyl seems more pronounced at a low pH value, resulting in a rate that exceeded that of cysteine 300-fold. Indeed, on photooxidation at pH 7.0, electrostatic interactions between the ammonium and the carboxylate groups in the zwitterionic form of *S*-methylcysteine hinder non-bonding p-orbitals of oxygen to affect the sulfur-centered radical cation. On the other hand, at pH 4.2, the partly protonated carboxyl group no longer suffers from such interaction and stabilization of the oxidized sulfur moiety leads to a higher reaction rate at lower pH.[32]

Neighbouring group effects have been reported also for methionine.[34–36] The sulfur-centered radical cation interacts with the free electron pair on nitrogen, which leads to formation of a stable five-membered-ring intermediate. Since the amino group prevails mainly as ammonium at the pH values of the model systems, the non-bonding electron pair of nitrogen is not available and the resulting effect is minimized. Conversely, the lone pair remains accessible when the amino function is converted into a urethane moiety, hence, the reaction is much faster for *N*-BOC-methionine.

Radical precursors for photoreaction products

Further details on the photooxidation reaction mechanism were collected from investigation of intermediates by electron paramagnetic resonance spectroscopy. Attempts to directly observe sulfur-centered radicals failed, most likely due to their high reactivity, but also as a result of their *g*-factor anisotropy that causes extensive line broadening.[37] Still, a broad EPR signal, typical for the formation of the reduced flavin mononucleotide radical,[38] was observed, providing evidence for the electron transfer mechanism. On the other hand, photooxidation of sulfur-containing amino acids in the presence of spin traps furnished details on reactive intermediates. Although only indirect information can be extracted from the resulting spin adducts, their coupling constants, a_N and a_H, have characteristic values and allow recovering structural data information on incipient radicals.

As such, all thiol-containing derivatives gave rise to spin adducts with DMPO that are typical for trapping of a sulfenyl radical (RS•) ($a_N \sim 15.2 \pm 0.1$ G and $a_H \sim 17.0 \pm 0.8$ G).[37,39,40] An extra radical was trapped with cysteine, penicillamine, cysteamine, and homocysteine. As such spin adduct has not been recorded in literature, assignment has been attempted. Reaction products arising from photooxidation were identified by headspace GC-MS and ESI-MS in order to characterize their radical precursors. It was found that these substrates produced dihydrogen sulfide (H_2S) as the sole major photoreaction product, most likely as the result of hydrogen abstraction by a sulfhydryl radical (•SH). Since the values of the coupling constants are very similar to the coupling constants reported for a sulfenyl radical, the unknown adduct was considered to have arisen from addition of •SH to DMPO.

Remarkably, penicillamine (**1**) led to two extra EPR signals with respect to cysteine due to the presence of two methyl groups at the carbon attached to the thiol. The most particular signal resulted from trapping of a nitrogen radical, as coupling to a nucleus with a spin of 1 ($I = 1$) gave a triplet of triplets ($a_{N(DMPO)} \sim 14.9$ G and $a_{N(\text{-HN-R})} \sim 2.2$ G), while the adduct with $a_N \sim 15.4$ G and $a_H \sim 20.2$ G has been reported as a penicillamine-derived carbon-centered radical.[40] Observation of these species provides additional information with regard to the elucidation of the decomposition mechanism, as depicted in Scheme 1. After photooxidation, the resulting sulfur-centered radical cation (**i**) is deprotonated to form a sulfenyl radical (**ii**) that readily adds to DMPO or, in the absence of a spin trap, recombines to form a penicillamine dimer ($[M + H]^+ = 297$ u). On the other hand, decarboxylation in concert with elimination furnished a sulfhydryl radical (**iii**), that eventually led to the formation of dihydrogen sulfide (**2**) and dimethylvinylammonium (**3a**), which should lead to imine **4** after deprotonation followed by allylic rearrangement. Subsequent hydrolysis of **4** produced 2-methylpropanal (**5**), as observed by GC-MS analysis, and ammonia (**6**) which escaped detection by the analytical techniques applied. However, photooxidation of penicillamine also resulted in a DMPO adduct with a

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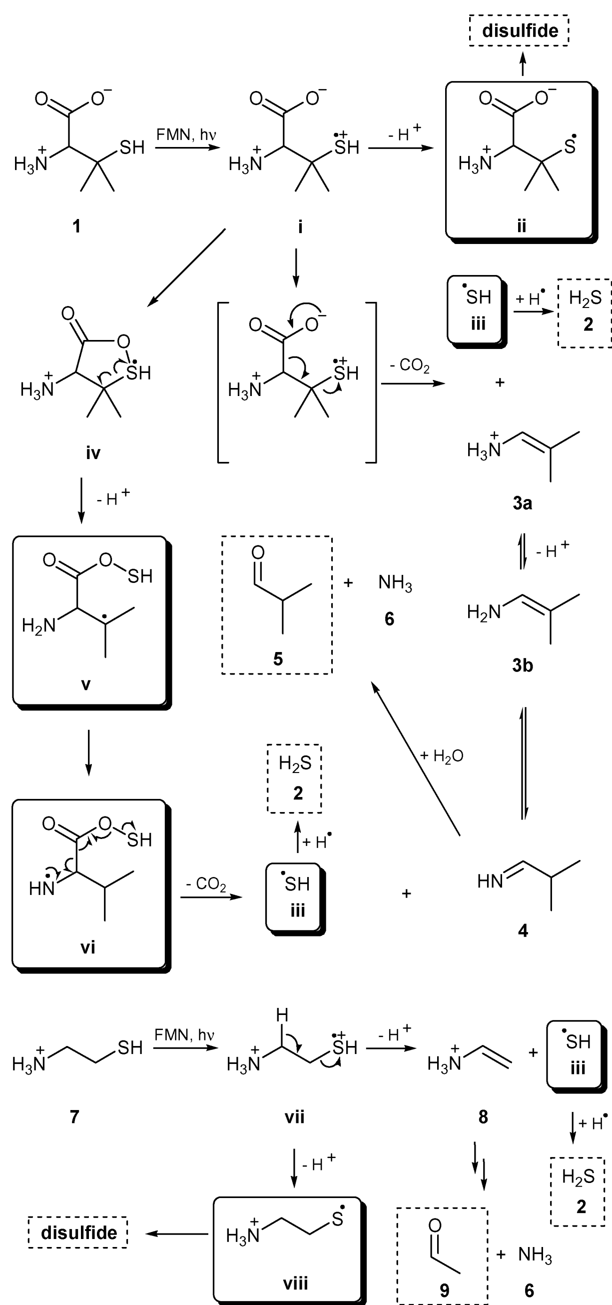

Scheme 1 Photooxidation mechanism of penicillamine (**1**) and cysteamine (**7**). Radicals that produced detectable spin adducts are depicted in a full box, while observed reaction products are boxed with a dotted line.

carbon-centered radical, while spin trapping with MNP indicated that an adduct with a tertiary carbon radical ($a_N \sim 16$ G, a_H not observed) was formed. As no such species was detected with cysteine, the unpaired spin was suggested to be localized on the carbon bearing the two methyl groups. Indeed, homolytic cleavage of the C–S bond in intermediate **iv**, involving a two-center three-electron bond between the neighbouring carboxylate and the sulfur-centered radical cation to form an intermediate five-membered-ring radical cation,[41] is favored by formation of the stabilized tertiary radical **v**. Next, a 1,3-hydrogen shift would result in a nitrogen-centered radical (**vi**), that after decarboxylation produced a sulfhydryl radical (**iii**) and imine **4**, which was eventually hydrolyzed to 2-methylpropanal (**5**) and ammonia (**6**).

As homolytic cleavage of the C–S bond in the five-membered ring intermediate derived from cysteine would deliver a primary carbon radical, a similar degradation pathway is disfavored and carbon- or nitrogen-centered radicals were not observed. Thus, decomposition mainly resulted from decarboxylation[42] and furnished $^{\bullet}$SH and vinylamine, which was readily converted to acetaldehyde. Cysteamine, however, lacks a carboxylate group, hence, a simplified reaction mechanism must be operative. After photooxidation, the resulting sulfur-centered radical cation (**vii**) either deprotonates to yield a sulfenyl radical (**viii**) or undergoes an inductive cleavage (mediated by proton loss) to produce $^{\bullet}$SH and vinylammonium (**8**). The feasibility of RS$^{\bullet}$ formation is confirmed by the observation of the dimeric derivative of cysteamine by ESI-MS ($[M + H]^+ = 153$ u), whereas dihydrogen sulfide (**2**) and acetaldehyde (**9**) (after allylic rearrangement and subsequent hydrolysis of **8**) were detected by headspace GC-MS analysis. For 3-mercaptopropionic acid, the incipient sulfenyl radical, which formed an adduct with DMPO ($a_N \sim 15.1$ G, $a_H \sim 16.7$ G)[40] as well as with MNP ($a_N \sim 17.8$ G, a_H not observed),[43] induced decarboxylation with formation of minor quantities of ethanethiol. Photooxidation of glutathione furnished an adduct with a glutathionyl radical (GS$^{\bullet}$) (DMPO: $a_N \sim 15.2$ G, $a_H \sim 16.2$ G;[37,40] MNP: $a_N \sim 18.3$ G, a_H not observed[44]), but volatile reaction products could not be detected. However, analysis by ESI-MS revealed the existence of a molecular ion $[M + H]^+ = 233$ u, corresponding to glutathione lacking a glycine residue. Further oxidation could occur, as a significant formation trace for $[M + H]^+ = 231$ u was observed. Moreover, glutathione also produced an extra adduct with MNP ($a_N \sim 14.5$ G, $a_H \sim 14.2$ G) in agreement with observations by Taniguchi.[43] Coupling constants are characteristic for trapping of a hydrogen radical,[45] however, details about its origin remain unclear.

In line with the observations for thiol-containing compounds, *S*-methylcysteine gave a DMPO spin adduct ($a_N \sim 15.3$ G and $a_H \sim 18.0$ G) that was attributed to trapping of a methylthio radical ($^{\bullet}$SMe).[40] Degradation was most likely initiated by decarboxylation, followed by elimination of $^{\bullet}$SMe. As a result, methanethiol and dimethyl disulfide were detected as major reaction products by headspace GC-MS, while observation of acetaldehyde implied initial formation of vinylamine. Similar as for *S*-methylcysteine, a methylthio radical was observed on photodegradation of methionine, although an adduct with a carbon-centered radical ($a_N \sim 15.5$ G and $a_H \sim 23.9$ G) was more prevalent. As this intermediate was not detected after photooxidation of homocysteine, it was attributed to deprotonation of the sulfur-centered radical cation with formation of $H_3N^+–CH(COO^-)CH_2CH^{\bullet}–SCH_3$. Still, methanethiol and dimethyl disulfide were identified as major reaction products and, moreover, decarboxylation was unambiguously proven, as $^{13}CO_2$ (*m/z* 45) was produced after photooxidation of methionine-*carboxy*-^{13}C. According to Yang *et al.*, degradation products include ethylene, methane, and ammonia as well,[46] but, since the lower mass limit was set by saturation of the samples with nitrogen, these compounds escaped detection by headspace GC-MS.

A peculiar distinction with respect to *S*-methylcysteine was noted for 2-(methylthio)ethylamine, as a carbon-centered radical is the major species being trapped.[45] Indeed, as a result of

deprotonation of the sulfur-centered radical cation, the radical must be located on one of the carbons adjacent to the sulfur atom, as was demonstrated previously for the homologous 2-(ethylthio)ethylamine.[32]

Photooxidation of cysteine and MBT formation

Following identification of a sulfhydryl or a methylthio radical as prevalent intermediates, the feasibility of recombination with a 3-methylbut-2-enyl radical (resulting from photooxidation of isohumulones) was examined. Thus, irradiation of a model system containing isohumulones, FMN, and cysteine resulted in the formation of 3-methylbut-2-ene-1-thiol (MBT), the principal contributor to LSF, as a minor reaction product. Substituting *S*-methylcysteine for cysteine provided further mechanistic information, as significant levels of the methylthio ether of MBT were detected. This finding corroborates a recombination mechanism involving sulfur-centered radicals, resulting from photooxidation of sulfur-containing amino acids (or derivatives), and a 3-methylbut-2-enyl radical as the final step in development of lightstruck flavor under visible-light irradiation.

Conclusions

Sulfur-containing amino acids (and derivatives) were found to be prone to photooxidation using visible light in the presence of flavin mononucleotide. Kinetic information, obtained by laser flash photolysis spectroscopy, revealed a pivotal role of the sulfur moiety, while structural features also affected reaction rates. The fate of the resulting sulfur-centered radical cations was elucidated by studying intermediates through spin trapping, followed by electron paramagnetic resonance spectroscopy. Thiol-containing substrates such as cysteine and homocysteine gave rise to formation of sulfhydryl radicals, whereas the corresponding methylthio ethers *S*-methylcysteine and methionine furnished methylthio radicals after photooxidation. The occurrence of these intermediates was proven by GC-MS-based identification of reaction products. Dihydrogen sulfide (from thiol-containing compounds) and methanethiol and dimethyl disulfide (from methylthio ethers) appeared to be the major reaction products. Furthermore, it was demonstrated that formation of 3-methylbut-2-ene-1-thiol is the result of a recombination of a sulfhydryl radical (resulting from photooxidation of cysteine or other thiol-containing derivatives) and a 3-methylbut-2-enyl radical, derived from photodegradation of isohumulones. The characterization of this reaction step was the missing link in the unravelling of the mechanism of visible-light-induced formation of the beer lightstruck flavor. Hence, the overall mechanism has now been fully established as a result of the present work.

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