Reactivity of Beer Bitter Acids Toward the 1-Hydroxyethyl Radical as Probed by Spin-Trapping Electron Paramagnetic Resonance (EPR) and Electrospray Ionization–Tandem Mass Spectrometry (ESI–MS/MS)

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S *Supporting Information*

ABSTRACT: The iso-α-acids or isohumulones are the major contributors to the bitter taste of beer, and it is well-recognized that they are degraded during beer aging. In particular, the *trans*-isohumulones seem to be less stable than the *cis*-isohumulones. The major radical identified in beer is the 1-hydroxyethyl radical; however, the reactivity between this radical and the isohumulones has not been reported until now. Therefore, we studied the reactivity of isohumulones toward the 1-hydroxyethyl radical through a competitive kinetic approach. It was observed that both *cis*- and *trans*-isohumulones and dihydroisohumulones are decomposed in the presence of 1-hydroxyethyl radicals, while the reactivities are comparable. On the other hand, the tetrahydroisohumulones did not react with 1-hydroxyethyl radicals. The apparent second-order rate constants for the reactions between the 1-hydroxyethyl radical and these compounds were determined by electron paramagnetic resonance (EPR) spectroscopy and electrospray ionization–tandem mass spectrometry [ESI$^+$–MS/MS]. It follows that degradation of beer bitter acids is highly influenced by the presence of 1-hydroxyethyl radicals. The reaction products were detected by liquid chromatography–electrospray ionization–ion trap–tandem mass spectrometry (LC–ESI–IT–MS/MS), and the formation of oxidized derivatives of the isohumulones was confirmed. These data help to understand the mechanism of beer degradation upon aging.

KEYWORDS: Isohumulones, reduced isohumulones, oxidized isohumulones, beer, 1-hydroxyethyl radical, kinetics

INTRODUCTION

Beer is an alcoholic beverage made from water, barley malt, hops (*Humulus lupulus*), and yeast.[1,2] Indeed, hop-derived compounds are essential for the typical bitter taste and attractive flavor of beer.[3] During wort boiling, the α-acids or humulones (**1a**–**1c**; Figure 1) are extracted from hops and, subsequently, isomerized to a mixture of *trans*- and *cis*-isocohumulones (**2a** and **3a**; Figure 1), *trans*- and *cis*-isohumulones (**2b** and **3b**; Figure 1), and *trans*- and *cis*-isoadhumulones (**2c** and **3c**; Figure 1). In general, the mixture of these six isomers and analogues is called "isohumulones". It has been demonstrated that *trans*-isohumulones are readily degraded during beer aging by oxidative and photochemical processes, while *cis*-isohumulones seem to better resist oxidative degradation.[1,4,5] Recently, autoxidation products have been characterized for both *cis*- and *trans*-isohumulones in beer stored in oxygen-permeable polyethylene terephthalate (PET) bottles.[2,3]

Reduced iso-α-acids or isohumulones, mainly dihydroisohumulones (**4**) and tetrahydroisohumulones (**5**) (Figure 1), are increasingly being used in beer manufacturing, because they prevent or retard oxidative and photochemical decompositions.[1,6] These compounds have additional desirable properties, including foam stability and cling quality.[1,7]

Spin-trapping electron paramagnetic resonance (EPR) experiments have demonstrated that the 1-hydroxyethyl radical is the predominant radical in beer.[8,9] The reaction between ethanol

and hydroxyl radicals is proposed to give rise to the formation of this radical in the presence of hydrogen peroxide and transition-metal ions, such as iron(II).[10] In addition, Elias at al. have shown that the 1-hydroxyethyl radicals are the main radicaloid species responsible for wine oxidation.[11]

Therefore, the objective of the present study was to investigate the reactivity of isohumulones (**2**–**5**) toward the 1-hydroxyethyl radical as probed by spin-trapping EPR and electrospray ionization–tandem mass spectrometry (ESI–MS/MS) using a competitive kinetic approach.

MATERIALS AND METHODS

Chemicals and Materials. Chemicals were obtained commercially: ethanol and hydrogen chloride (from J. T. Baker, Xalostoc, Mexico); ferric chloride (FeCl$_2$·4H$_2$O) and 30% hydrogen peroxide (from Merck, Darmstadt, Germany); acetonitrile, methanol, and ethyl acetate (from Tedia, Fairfield, OH); formic acid, catalase from liver bovine, α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN), sodium borohydride, 10% palladium on carbon, quercetin, *p*-coumaric acid, tetrabutylammonium perchlorate, and ferrocene (from Sigma-Aldrich, Steinheim, Germany); isomerized hop extract [30% (w/w) iso-α-acids/
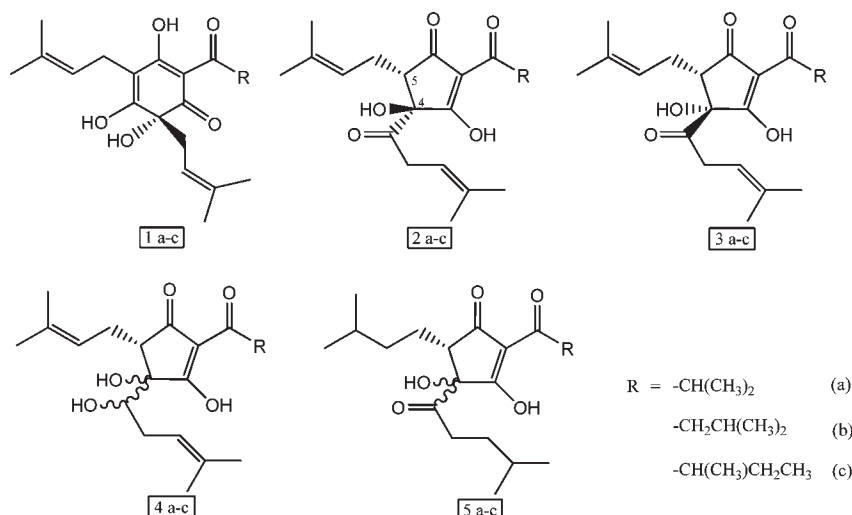
Figure 1. Chemical structures of the humulones (**1a**−**1c**), *trans*-isohumulones (**2a**−**2c**), *cis*-isohumulones (**3a**−**3c**), *cis*- and *trans*-dihydroisohumulones (**4a**−**4c**), and *cis*- and *trans*-tetrahydroisohumulones (**5a**−**5c**).

isohumulones] (from Hopsteiner, Mainburg, Germany); water purified (18 MΩ cm^{-1}) by means of a Milli-Q purification system (from Millipore, Bedford, MA); argon (purity grade of 5.0) and nitrogen (from White Martins, Sertãozinho, São Paulo, Brazil); and sodium sulfate (from Chemis, São Paulo, São Paulo, Brazil). All solvents used were of analytical-grade high-performance liquid chromatography (HPLC).

Isolation of Isohumulones and Preparation of Reduced Isohumulones. Following a literature procedure described by Khatib and Verpoorte,[12] *cis*- and *trans*-isohumulones were isolated from a commercially available extract of isohumulones (Hopsteiner). The purities of the *trans*- and *cis*-isohumulones were 95 and 90%, respectively, as determined by high-performance liquid chromatography−mass spectrometry (HPLC−MS) and ^{1}H nuclear magnetic resonance (NMR).

Tetrahydroisohumulones (**5**) were obtained according to a procedure described by Verzele and De Keukeleire,[13] resulting in a purity of 90%, as determined by HPLC−MS and ^{1}H NMR.

Dihydroisohumulones (**4**) were obtained from isohumulones (1.1 × 10^{-3} mol) in methanol (27 mL) by adding sodium borohydride (92 mg) under stirring for 23 h at room temperature. Then, the reaction mixture was acidified to pH 1 by the addition of hydrogen chloride (12 mol L^{-1}), and the solvent was removed under reduced pressure. The residue was dissolved in water (20 mL) and extracted with ethyl acetate (3 × 20 mL). The organic phase was then dried over Na$_2$SO$_4$, and the solvent was removed under reduced pressure, yielding a mixture of dihydroisohumulones in a purity higher than 98%, as determined by HPLC−MS and ^{1}H NMR.

Formation of 1-Hydroxyethyl Radicals and Spin Trapping by 4-POBN. The formation of 1-hydroxyethyl radicals and spin trapping by 4-POBN were assayed by mixing 80 μL of a H$_2$O$_2$ solution (58.0 × 10^{-3} mol L^{-1}) with the reaction mixture containing 1 mL of 4-POBN [3.2 × 10^{-3} mol L^{-1} in aqueous solution containing 12% (v/v) of ethanol] and 60 μL of FeCl$_2$·4H$_2$O (2.0 × 10^{-3} mol L^{-1}) after exhaustive purging with high-purity argon. The incubation time was 5 min at ambient temperature (25 °C), and the reaction was finally quenched by the addition of 100 μL of catalase (47.4 mg mL^{-1}).

Competitive Kinetic Studies. To overcome the fact that the 1-hydroxyethyl radical has a short lifetime, the apparent second-order rate constants of the reactions between isohumulones and reduced isohumulones toward the 1-hydroxyethyl radical were obtained through a competitive kinetic approach employing the spin-trap 4-POBN as a

probe. Thus, the reaction was conducted by the addition of 80 μL of H$_2$O$_2$ (58.0 × 10^{-3} mol L^{-1}) in an argon-saturated solution containing 1 mL of 4-POBN [3.2 × 10^{-3} mol L^{-1} in aqueous solution containing 12% (v/v) of ethanol], 60 μL of FeCl$_2$·4H$_2$O (2.0 × 10^{-3} mol L^{-1}), and varying concentrations of the bitter acids. The reaction was then incubated for 1 min at 25 °C and, finally, quenched by mixing 100 μL of catalase (47.4 mg mL^{-1}).[14] The initial analytical concentrations of isohumulones and reduced isohumulones were determined by electronic absorption spectroscopy (isohumulones, $\varepsilon_{279\ nm}$ = 11 150 L mol^{-1} cm^{-1}; dihydroisohumulones, $\varepsilon_{279\ nm}$ = 11 150 L mol^{-1} cm^{-1}; tetrahydroisohumulones, $\varepsilon_{254\ nm}$ = 17 690 L mol^{-1} cm^{-1}).[13]

The competitive kinetics were conducted by monitoring the content of 1-hydroxyethyl/4-POBN by EPR or ESI$^+$−MS.

Identification of Degradation Products of Isohumulones in the Presence of 1-Hydroxyethyl Radicals. The reaction was started by adding 100 μL of H$_2$O$_2$ (58.0 × 10^{-3} mol L^{-1}) in a solution containing isohumulones (1.0 × 10^{-5} mol L^{-1}) in ethanol and 80 μL of FeCl$_2$·4H$_2$O (2.0 × 10^{-3} mol L^{-1}), incubated for 1 min. The composition of the resulting solution was analyzed by liquid chromatography−electrospray ionization−ion trap−tandem mass spectrometry (LC−ESI−IT−MS/MS.

LC−MS/MS. The LC−ESI−IT−MS/MS analysis was carried out using Shimadzu Prominence HPLC equipped with two LC-20AD solvent delivery units, a online Shimadzu degasser model DGU20A3, a manual Rheodyne model 8125 sample injector valve with a sample loop of 20 μL, and a CBM-20A Shimadzu Prominence communications bus module. Samples were separated using an Agilent C-18 reverse-phase column (2.1 mm × 150 mm × 5 μm). The mobile phase with a flow rate of 0.3 mL min^{-1} consisted of a mixture of solvents: A [99.9:0.1 (v/v) water/formic acid] and B [99.9:0.1 (v/v) acetonitrile/formic acid] using the following linear eluting gradient: 0−15 min, 0−25% B in A; 15−20 min, 100% B in A; 20−23 min, 100% B in A; and 23−25 min, 0% B in A . The electrospray mass spectra were collected in the positive-ion mode for the identification and quantification of the target compounds using a Bruker Daltonics ion-trap mass spectrometer model Esquire 4000 (Bremen, Germany).

The experimental conditions used for the identification of the oxidized reaction products were the same as described above, using the following linear eluting gradient: 0−5 min, 0−30% B in A; 5−20 min, 50% B in A; 20−35 min, 65% B in A; 35−55 min, 100% B in A; and 55−60 min, 0% B in A. The electrospray mass spectra were collected in the negative-ion mode.
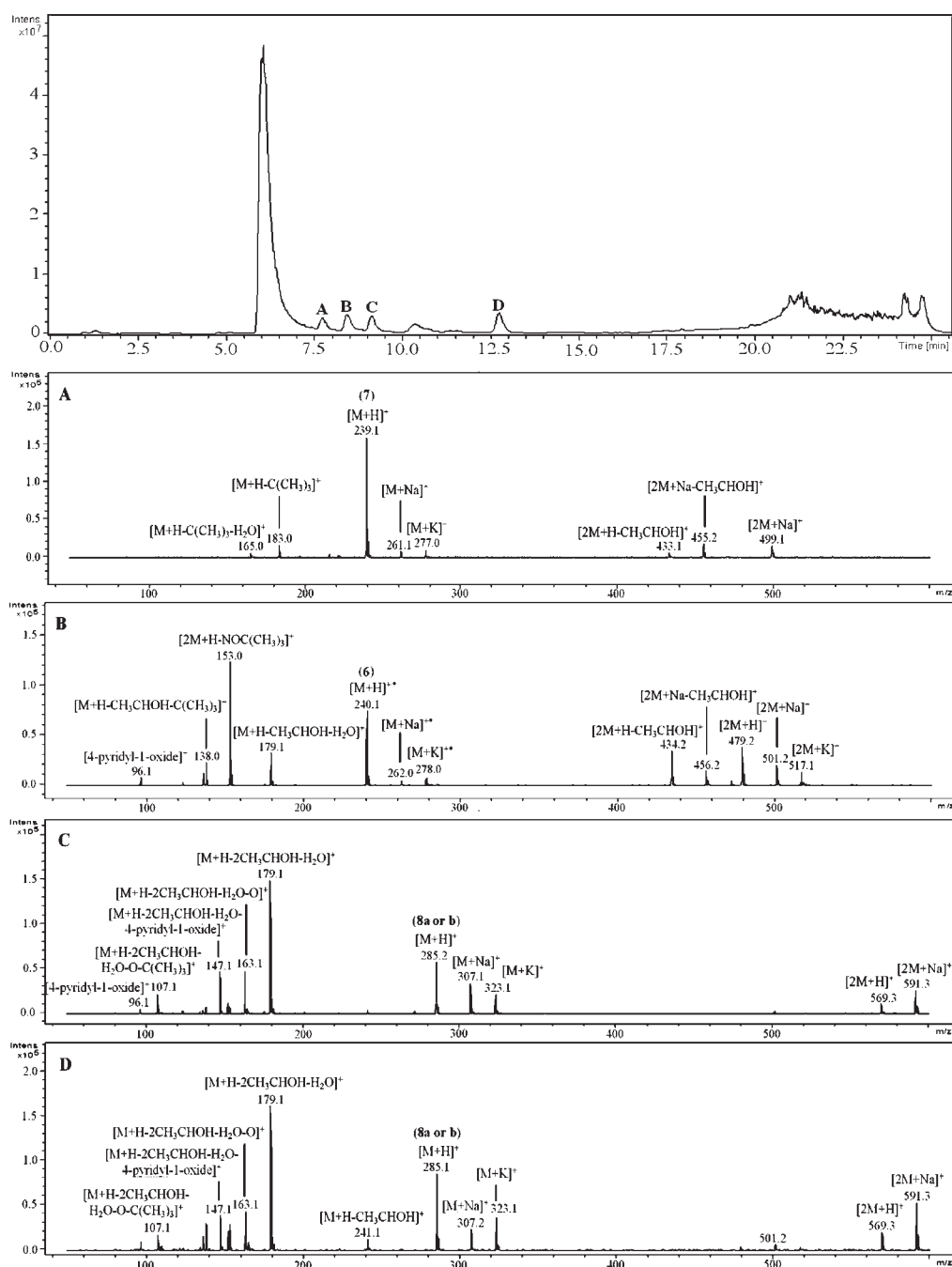
Figure 2. LC—ESI—IT—MS/MS chromatograms of the products of the reaction between 4-POBN and the 1-hydroxyethyl radical after 5 min at 25 °C under argon, operating in the positive-ion detection mode using an electrospray ion source. (Peak A) Mass spectrum of the oxidized spin adduct; MW = 238. (Peak B) Mass spectrum of the spin adduct radical; MW = 239. (Peaks C and D) Mass spectra of the addition product of two molecules of the 1-hydroxyethyl radical to 4-POBN; MW = 284. The peak notations refer to the chemical structures given in Figure 4.

EPR Spectroscopy. Analysis of the spin adduct radical was performed with a Bruker EMX spectrometer (Rheinstetten, Germany), operating in the band X, with center field of 3378 G, frequency of 9.53 GHz, power of 1 mW, frequency modulation of 100 kHz, and amplitude modulation of 1 G, using a quartz capillary (inner diameter of 0.75 mm) sample cell (Wilmad Glass, Buena, NJ).

Electrochemical Studies of *trans*-Isohumulones. Cyclic voltammetry of the *trans*-isohumulones in undissociated forms (**2**) was carried out with a AUTOLAB GPES Eco Chemie BV potentiostat/galvanostat (Metrohm, Utrecht, The Netherlands) using a diamond electrode doped with boron (8000 ppm) as a working electrode and platinum wire as an auxiliary electrode. The couple ferrocene/ferrocenium (Fe^+/Fe) was used as an internal reference. Compounds **2a**−**2c** (5.0×10^{-3} mol L^{-1}) were dissolved in the supporting electrolyte (0.1 mol L^{-1} of tetrabutylammonium perchlorate in acetonitrile), and the solution was degassed with high-purity nitrogen before measurements. The electrochemical cell was thermostatted at 25 °C, and the sweep rate was varied from 10 to 200 mV s^{-1}. The measured potential is reported against the normal hydrogen electrode (NHE) using E^o = +630 mV versus NHE for the Fe^+/Fe couple in acetonitrile.[15]
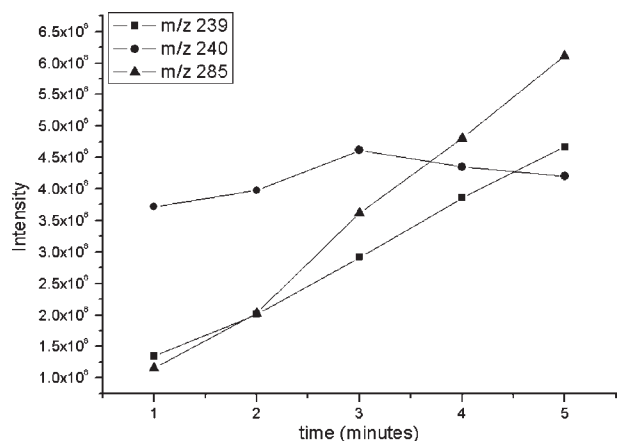
Figure 3. Quantitative profiles of the oxidized spin adduct (m/z 239), the spin adduct radical (m/z 240), and the species containing two molecules of the 1-hydroxyethyl radical linked to 4-POBN (m/z 285) as a function of the reaction time, at 25 °C, under argon.

■ RESULTS AND DISCUSSION

Spin Trapping by 4-POBN. After reaction (5 min) between the spin-trap 4-POBN and 1-hydroxyethyl radicals, the reaction products were analyzed by LC−MS/MS. The chromatographic separation is shown in Figure 2. LC−ESI−IT−MS/MS displays five peaks eluting at t_r = 6.0, 7.9 (indicated by "A"), 8.6 (indicated by "B"), 9.2 (indicated by "C"), and 12.8 min (indicated by "D"). The first peak was identified as 4-POBN ($[M + H]^+$ = m/z 195 and $[M + Na]^+$ = m/z 217). Fragmentation of the quasi-molecular ion at m/z 195 gave ions at m/z 139 $[M + H−C(CH_3)_3]^+$, 122 $[M + H−C(CH_3)_3−H_2O]^+$, and 95 $[M + H−C(CH_3)_3−H_2CNO]^+$. Peaks A−D refer to reaction products. Peaks A and B were identified as the oxidized spin adduct (**7**) ($[M + H]^+$ = m/z 239) and the spin adduct radical (**6**) ($[M + H]^+$ = m/z 240), respectively. For the oxidized spin adduct (peak A), the collisionally induced dissociation (CID) spectra of the quasi-molecular ion at m/z 239 showed fragment ions at m/z 183 $[M + H−C(CH_3)_3]^+$ and 165 $[M + H−C(CH_3)_3−H_2O]^+$, while for the spin adduct radical (peak B), the quasi-molecular ion at m/z 240 showed fragment ions at m/z 179 $[M + H−CH_3CHOH−H_2O]^+$, 153 $[M + H−NOC(CH_3)_3]^+$, 138 $[M + H−CH_3CHOH−C(CH_3)_3]^+$, and 96 $[4\text{-pyridyl-1-oxide}]^+$. The remaining peaks C and D were tentatively assigned to the combination of two 1-hydroxyethyl radicals and 4-POBN ($[M + H]^+$ = m/z 285). For these compounds, the CID spectra of the quasi-molecular ion at m/z 285 produced fragment ions at m/z 241 $[M + H−CH_3CHOH]^+$, 179 $[M + H−2CH_3CHO−H_2O]^+$, 163 $[M + H−2CH_3CHOH−H_2O−O]^+$, 147 $[M + H−2CH_3CHOH−H_2O−4\text{-pyridyl-1-oxide}]^+$, and 107 $[M + H−2CH_3CHOH−H_2O−O−C(CH_3)_3]^+$.

Figure 3 illustrates the reaction time profile for the relative content of the spin adduct radical (m/z 240), the oxidized form of the spin adduct (**7**) (m/z 239), and the species formed by binding 4-POBN with two molecules of the 1-hydroxyethyl radicals (**8a** and **8b**) (m/z 285). The increases of the latter species, oxidized and spin adduct biding two 1-hydroxyethyl radicals, may be associated to subsequent reactions involving the spin adduct radical, thereby leading to species with quasi-molecular ions of m/z 239 and 285.

The possible reaction mechanism is presented in Figure 4. Initially, the spin adduct (m/z 240; **6**) is formed with a second-order
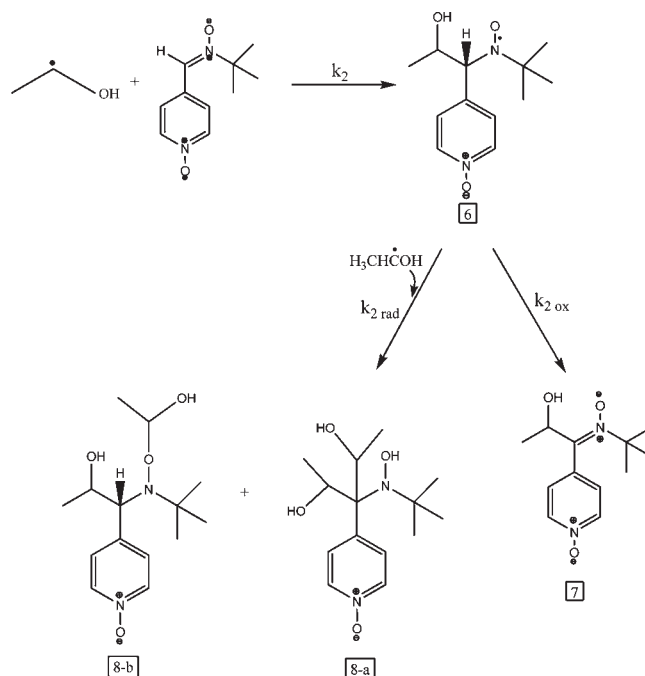

Figure 4. Proposed reaction pathway for the formation of the spin adduct radical (**6**; B in Figure 2), the oxidized spin adduct (**7**; A in Figure 2), and the species containing two molecules of the 1-hydroxyethyl radical linked to 4-POBN (**8a** and **8b**; C and D in Figure 2).
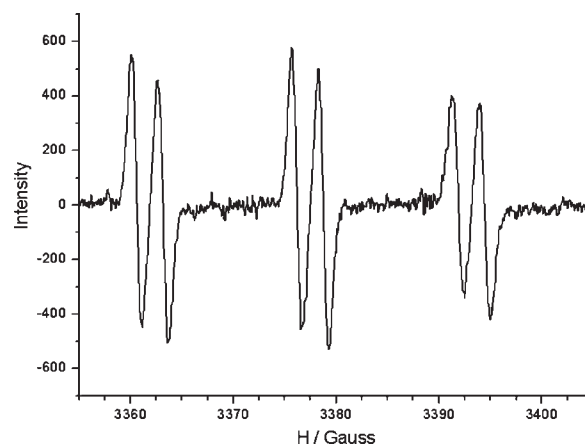

Figure 5. Experimental EPR spectrum of the radical **6** derived from the 1-hydroxyethyl radical trapped by 4-POBN. Reactions are in argon, saturated aqueous solution containing 10% ethanol (v/v) at 25 °C.

rate constant of 3.1×10^7 L mol^{-1} s^{-1} (k_2).[16] Oxidation of the spin adduct radical (**6**) leads to the formation of the oxidized spin adduct (m/z 239; **7**), following a second-order rate constant (k_{2ox}). The addition of another 1-hydroxyethyl radical furnishes compounds **8a** and **8b** (m/z 285) with a second-order rate constant (k_{2rad}). The adduct, compounds **8a** and **8b**, linking two molecules of the 1-hydroxyethyl radical to 4-POBN was identified for the first time, and its formation in companion to the oxidized spin adduct may explain the steady-state condition observed for the radical spin adduct (**6**).

EPR spectroscopy is the technique used for direct detection of species with unpaired electrons and can assist in the determination of the radical identity.[17] Consequently, only the spin adduct

Table 1. Apparent Second-Order Rate Constants of Isohumulones, Dihydroisohumulones, and Tetrahydroisohumulones toward the 1-Hydroxyethyl Radical[a]

	EPR $(L\ mol^{-1}\ s^{-1})$	ESI$^+$−MS/MS $(L\ mol^{-1}\ s^{-1})$
trans-isohumulones	9.7×10^9	8.6×10^9
cis-isohumulones	1.8×10^8	1.7×10^8
isohumulones	1.3×10^9	1.2×10^9
dihydroisohumulones	1.5×10^9	1.4×10^9
tetrahydroisohumulones	nr[b]	nr[b]

[a] Reactions are in aqueous solution (90%), at 25 °C, under argon. [b] nr = not reactive.

radical (m/z 240) is detected, and its spectrum is shown in Figure 5. The presence of a six-line profile in the spectrum and the values of the hyperfine coupling constants obtained (a_N = 15.6 G; a_H = 2.6 G) agree with the values expected for the spin adduct radical formed from the reaction of the 1-hydroxyethyl radical with 4-POBN.[8]

Competitive Kinetic Studies. Using a competitive kinetic approach, the apparent second-order rate constants for the reactions of *trans*-isohumulones, *cis*-isohumulones, the mixture of *trans*- and *cis*-isohumulones, or their reduced derivatives with the 1-hydroxyethyl radical were obtained. Thus, plotting $(F/1 - F)k_2[4\text{-POBN}]$ against the respective concentrations (see the Supporting Information) allows for determination of the second-order rate constant (k_2'), as established by eq 1.[18,19]

$$\left(\frac{F}{1 - F}\right)k_2[4\text{-POBN}] = k_2'[\text{IAA}] \tag{1}$$

where F represent the percentage of inhibition for the formation of the spin adduct and the concentrations of iso-α-acids and 4-POBN were represented by [IAA] and [4-POBN], respectively.

As can be extracted from Figure 3, the concentrations of the oxidized form of the spin adduct (**7**) and the species formed by binding 4-POBN with two 1-hydroxyethyl radicals (**8a** and **8b**) were lower than the concentration of the spin adduct radical (**6**) during the first few minutes of the reaction course. The spin adduct radical shows a fairly constant evolution, which indicates that subsequent reactions are negligible. The apparent rate constants were determined by ESI$^+$−MS/MS and EPR, monitoring the signal of the spin adduct radical formed after 1 min of reaction time. The apparent second-order rate constants are displayed in Table 1. Because the apparent second-order rate constants are similar for all compounds investigated ($p = 0.05$), both approaches are valid and can be applied for our purpose.

The *trans*-isohumulones (average apparent second-order rate constant of 9.2×10^9 L mol^{-1} s^{-1}) showed higher reactivity than the *cis*-isohumulones (average apparent second-order rate constant of 1.8×10^8 L mol^{-1} s^{-1}). The obvious differences in reactivity between these diastereoisomers is apparently connected to the stereochemical arrangement of the side chains at C_4 and C_5. The lower stability of the *trans*-isohumulones is consistent with the spatial proximity of these two chains in the *trans* form, suggesting a higher density of electrons and a dependence of the particular stereochemistry in reaction rates. In this context, the total energies (kJ mol^{-1}) of spatial configurations of the *cis*- and *trans*-isohumulones were evaluated using the the M06/6-31+G(d) methodology. It was found that the *cis* configuration is more stable than the *trans* configuration,

Table 2. Electronic Properties Calculated Using M06/6-31+G(d) for *cis*- and *trans*-Isohumulones in Undissociated Forms

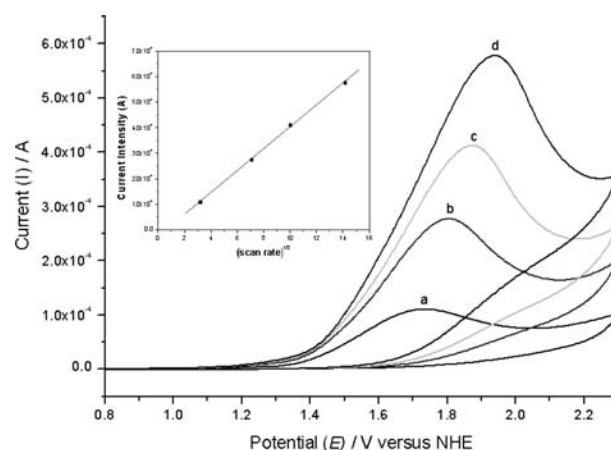|	*cis*-isohumulones	*trans*-isohumulones
dipole moment (debye)	2.2	4.2
E_{HOMO} (au)	−0.2453	−0.2394
BDE of C−H$_{prenyl}$ (kJ mol^{-1})	314	316
BDE of C−H$_{isohexenoyl}$ (kJ mol^{-1})	325	316

Figure 6. Cyclic voltammograms of *trans*-isohumulones (5.0×10^{-3} mol L^{-1}) in undissociated forms, supporting electrolyte tetrabutylammonium perchlorate (0.1 mol L^{-1}) in acetonitrile, at 25 °C and different scan rates: (a) 10 mV s^{-1}, (b) 50 mV s^{-1}, (c) 100 mV s^{-1}, and (d) 200 mV s^{-1}.

with an energy difference of 15.9 kJ mol^{-1}. A comparison of the dipole moments (Debye) of *trans*- and *cis*-isohumulones shows that *trans*-isohumulones exhibit a higher dipole moment, suggesting a greater influence of the solvent in reactions involving the *trans* configuration (Table 2). On the other hand, the energies of the highest occupied molecular orbitals (HOMOs) (see the Supporting Information) for both *cis*- and *trans*-isohumulones are quite comparable; hence, these features do not explain the differences in reactivity toward the 1-hydroxyethyl radical.

In a recent study, it was reported that oxidation reactions of methyl esters of unsaturated fatty acids with oxidation potentials around 2.0 V versus NHE occur preferentially by hydrogen atom transfer.[20] This finding can be extended to reactions between the isohumulones and 1-hydroxyethyl radicals, suggesting that hydrogen transfer in our cases occurs rather than electron transfer. It is also important to notice that the difference in reactivity of the dissociated and undissociated isohumulones (**2a**−**2c** and **3a**−**3c**) toward the 1-hydroxyethyl radical was not observed (data not shown), suggesting hydrogen atom transfer from olefinic groups rather than electron transfer from the β-tricarbonyl system to the radical as the operating mechanism.

Tetrahydroisohumulones did not show any reactivity toward the 1-hydroxyethyl radical, because of the lack of accessible hydrogens in allylic positions with respect to the side-chain olefinic bonds and, therefore, the lack of a reactive site. The apparent second-order rate constant for dihydroisohumulones (average of 1.5×10^9 L mol^{-1} s^{-1}) is similar to that of the mixture of the isohumulones (average of 1.3×10^9 L mol^{-1} s^{-1}).
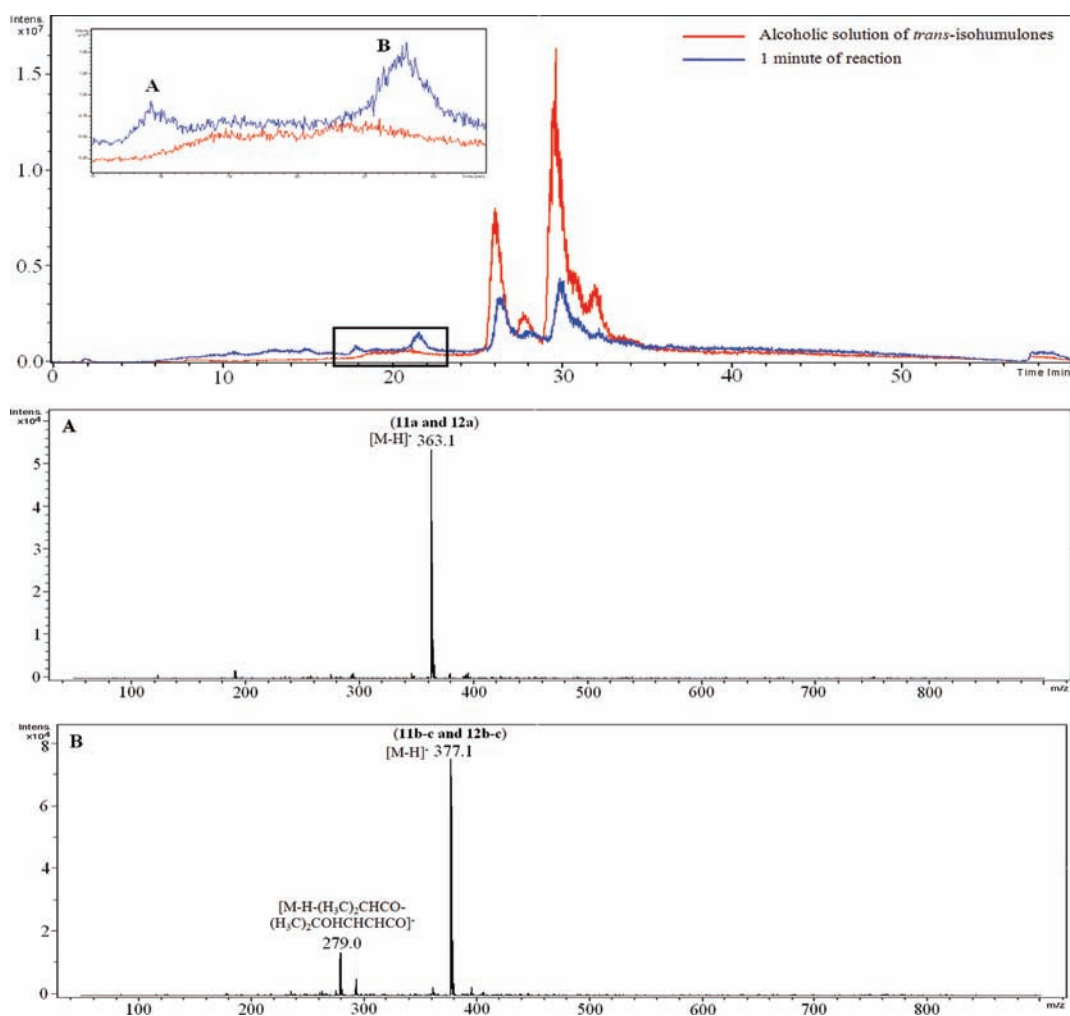
Figure 7. LC−ESI−IT−MS/MS chromatograms of the products of the reactions between *trans*-isohumulones and the 1-hydroxyethyl radical, after 1 min, at 25 °C, operating in the negative-ion detection mode using an electrospray ion source. (Peak A) Mass spectrum of hydroxyl-*trans*-alloisocohumulone; MW = 364. (Peak B) Mass spectrum of hydroxyl-*trans*-alloisohumulone and hydroxyl-*trans*-alloisoadhumulone; MW = 378. The peak notations refer to the chemical structures given in Figure 8.

This is consistent with the fact that both contain reactive allylic sites next to olefinic groups. Even the data of the apparent second-order rate constants for the mixture of the isohumulones is in agreement with the ratio of *cis*-/*trans*-isohumulones (7:3, v/v) (Table 1).

The apparent second-order rate constants obtained are close to the diffusion limits in aqueous media, thus confirming the high reactivity of isohumulones and dihydroisohumulones toward the 1-hydroxyethyl radical.

To compare our experimental approach with literature data, we determined the apparent second-order rate constant for the reaction between the 1-hydroxyethyl radical and quercetin in 90% ethanol using the same experimental conditions described previously, except for the absence of catalase. The apparent second-order rate constant was 3.1×10^4 L mol^{-1} s^{-1}, which is close to the literature rate constant of 4.0×10^4 L mol^{-1} s^{-1}.[21] Therefore, our newly developed method is suitable for our purpose. In analogy, we studied the reactivity between the 1-hydroxyethyl radical and *p*-coumaric acid, a beer constituent, and found a value of the apparent second-order rate constant of 1.0×10^7 L mol^{-1} s^{-1}. The second-order rate constants of the

reactions of other beer polyphenols with the 1-hydroxyethyl radical are much lower, in fact, around 10^4 L mol^{-1} s^{-1}, as reported in the literature.[21] The contents of (poly)phenolic substances in beer are about 10-fold higher than the concentrations of isohumulones,[22,23] yet the higher oxidative reactivity of bitter acids derived from hops primarily accounts for the decay of beer upon aging.

Electrochemistry of *trans*-Isohumulones. It is known that the β-tricarbonyl group of the isohumulones presents a prime target for oxidation, because it possesses a number of π and p electrons within a conjugated system that incorporates three oxygen and four carbon atoms. In this context, the anions of *trans*-isohumulones have an oxidation potential around 1.4 V versus NHE, which results in an irreversible process and confirms the high reactivity of the one-electron oxidized species produced.[6] However, the electrochemical properties of the corresponding undissociated (acid) forms have not been reported until now.

To determine the oxidation potential of the *trans*-isohumulones in undissociated forms, we used a diamond electrode doped with boron as a working electrode, as described in the experimental procedure. Thus, the oxidation potential obtained
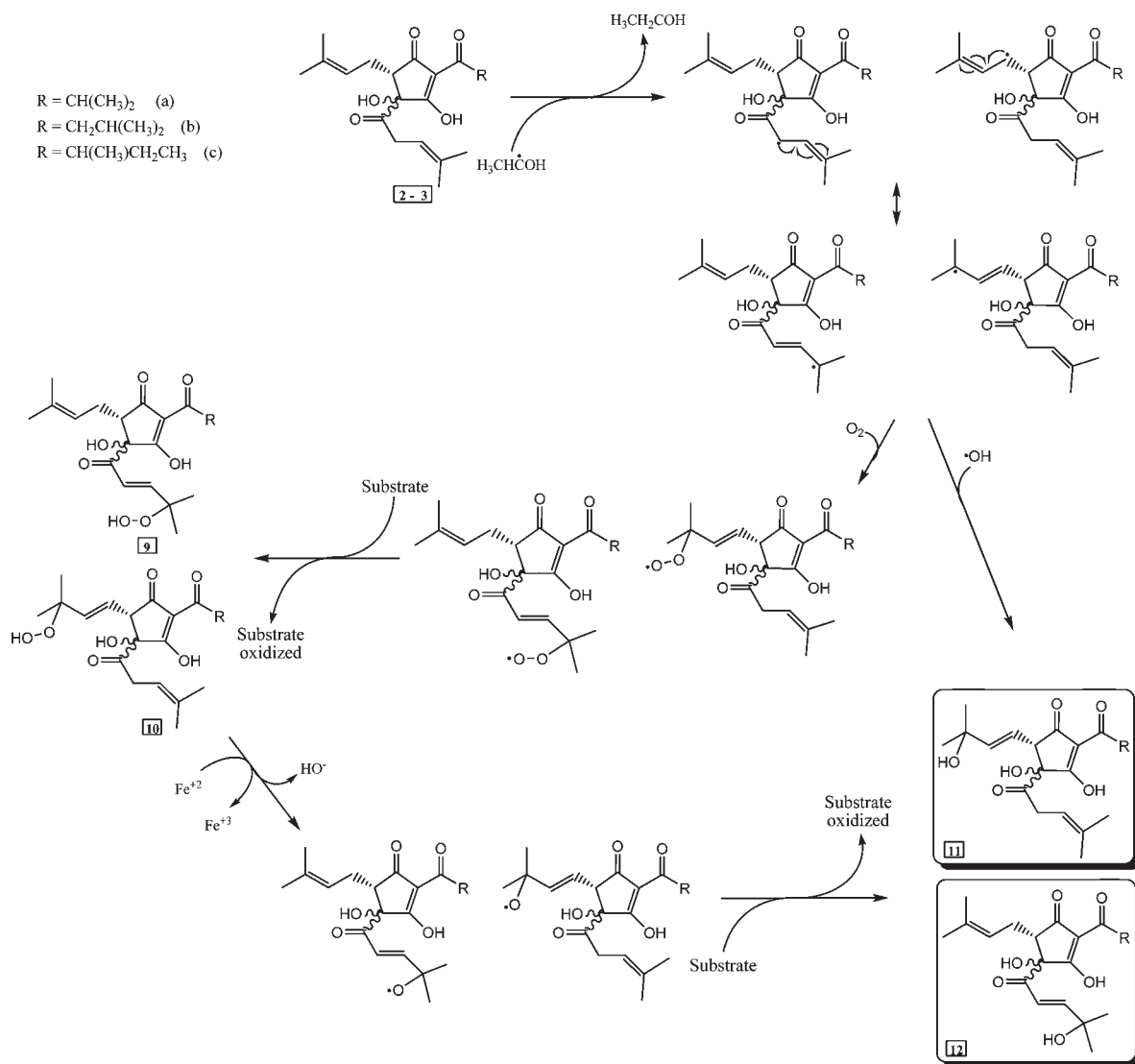
Figure 8. Proposed reaction pathway for the formation of hydroxyl-*cis*- and hydroxyl-*trans*-alloisocohumulones (**11a**), hydroxyl-*cis*- and hydroxyl-*trans*-alloisohumulones (**11b**), and hydroxyl-*cis*- and *trans*-alloisoadhumulones (**11c**). Compounds **12a**–**12c** refer to hydroxylated isohumulones. Peak A contains the co-derivatives, and peak B contains the n- and ad-derivatives. Compounds **2** and **3** refer to *trans*- and *cis*-isohumulones, respectively, whereas compounds **9** and **10** refer to hydroperoxy-alloisohumulones.

was 1.8 V versus NHE, and the irreversibility of the process was confirmed (Figure 6). Indeed, the linear dependence of the anodic peak current upon the square root of the scan sweep is consistent with a diffusion-controlled rate-limiting step. As an internal reference, ferrocene was oxidized to the ferrocenium ion.

According to our data, the reaction between the 1-hydroxyethyl radical and the β-tricarbonyl chromophore, which is typical of all compounds studied, is thermodynamically unfavorable, because the reduction potential of the radical is significantly lower ($E = 0.98$ V versus NHE)[24] than the oxidation potential of the compounds involved.

Identification of the Oxidation Products of the *trans*-Isohumulones. To identify the oxidation products resulting from the reaction between the 1-hydroxyethyl radical and *trans*-isohumulones, the reaction mixture containing *trans*-isohumulones, ferric ions, and hydrogen peroxide was analyzed by LC−ESI−IT−MS/MS (Figure 7). The first two peaks are reaction products, while the other peaks at longer retention times refer to the starting *trans*-isohumulones. LC−MS/MS analysis revealed the presence of quasi-molecular ions at m/z 363 and 377 with t_r = 17.8 and 21.4 min, respectively. Recently, compounds that contain a hydroxyl group in the isohexenoyl side chain of the isohumulones were reported in the literature[2,3] as primary autoxidation products from both *cis*- and *trans*-isohumulones present in beer samples. These compounds with quasi-molecular ions of m/z 377 and 363 were referred to as hydroxyl-alloisohumulones.[2] The masses of our reaction products are the same as these oxidation products (one extra oxygen); however, two possible chemical structures arise when a feasible reaction mechanism is considered. Indeed, the bond dissociation enthalpies (BDEs) of relevant allylic hydrogen atoms in the prenyl and isohexenoyl side chains ($C-H_{prenyl}$ and $C-H_{isohexenoyl}$; Table 2) indicate that both groups are susceptible to hydrogen atom abstraction. However, the differences in reactivity between *cis*- and *trans*-isohumulones toward the 1-hydroxyethyl radical cannot be due to these data because they are comparable. Therefore,

the higher reactivity of the *trans* diastereoisomer could be accounted for an entropic compensation because of the spatial proximity of the isohexenoyl and prenyl side-chain group in the *trans* species. The quasi-molecular ion *m/z* 263 refers to hydroxyl-*trans*-alloisocohumulone (**11a**), which contains a hydroxyl function in the isohexenoyl side chain of *trans*-isocohumulone (**2a**). The quasi-molecular ion *m/z* 377 is the result of the addition of a hydroxyl function in the isohexenoyl side chain of *trans*-isohumulone and *trans*-isoadhumulone (**2b** and **2c**), and therefore, the compounds can be identified as hydroxyl-*trans*-alloisohumulone (**11b**) and hydroxyl-*trans*-alloisoadhumulone (**11c**), respectively. However, if one considers the possible reaction mechanism (Figure 8), hydrogen abstraction by the 1-hydroxethyl radical can occur in both the prenyl and isohexenoyl side chains of *trans*-isohumulone, because both possibilities deliver resonance-stabilized radicals. Trapping by hydroxyl radicals derived from the presence of hydrogen peroxide and Fe^{II} ions would result in the formation of the hydroxyl-containing reaction products. Compounds **11** represent the mixture of hydroxyl-*trans*-alloisocohumulone (**11a**), hydroxyl-*trans*-alloisohumulone (**11b**), and hydroxyl-*trans*-alloisoadhumulone (**11c**). Compounds **12** have not been found previously, and they can be considered as novel derivatives of the *trans*-isohumulones. They represent a mixture of positional isomers of hydroxylated *trans*-isohumulones, in which the olefinic double bond is shifted to an allylic position in the prenyl side chain. Another possible path for the reaction involves residual molecular oxygen that reacts with the resonance-stabilized radical derived from isohumulones, yielding the corresponding peroxyl radical. The peroxyl radical may further abstract a hydrogen atom from oxidizable substrates, e.g., ethanol or isohumulones, resulting in the formation of hydroperoxides (**9** and **10**). The presence of a high amount of iron(II) leads to the cleavage of the hydroperoxides, generating alkoxyl radicals, which subsequently abstract a hydrogen atom from oxidizable substrates to give rise to the identified hydroxide compounds (**11** and **12**).

In conclusion, the apparent second-order rate constants of the reactions between the beer bitter compounds, isohumulones and reduced derivatives, and the 1-hydroxyethyl radical were obtained by ESI$^+$−MS/MS and spin-trapping EPR. The apparent rate constants appear to be near diffusion-controlled, thus reassuring the high oxidative reactivities of the isohumulones, in particular of the *trans* compounds. Dihydroisohumulones are equally reactive as the isohumulones, while the tetrahydroisohumulones are fully unreactive toward the radical studied. The reactive sites are the allylic positions in the side chains, and hydrogen abstractions lead to stabilized allylic radicals that are eventually trapped by hydroxyl radicals or molecular oxygen. The results of this study are of great importance for understanding essential processes involved in beer aging and, furthermore, producing beers with improved flavor stability.

■ ASSOCIATED CONTENT

ⓢ Supporting Information. Graph of the competitive kinetic plot ($(F/1 - F)k_2$[4-POBN] versus the concentrations of *trans*-isohumulones) and contour plots of the highest occupied molecular orbital (HOMO) of the isohumulones. This material is available free of charge via the Internet at http://pubs.acs.org.

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