Enhanced Molecular Dimension of a Humic Acid Induced by Photooxidation Catalyzed by Biomimetic Metalloporphyrins

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Synthetic water-soluble meso-tetra(2,6-dichloro-3-sulfonatophenyl)porphyrinates of Mn(III) chloride [Mn−(TDCPPS)Cl] and iron(III) chloride [Fe−(TDCPPS)Cl] were employed as biomimetic catalysts in the photooxidative coupling of a terrestrial humic acid (HA). The changes in molecular dimension of HA induced by irradiation with UV light for different periods were followed by high-performance size-exclusion chromatography (HPSEC), with both spectrophotometric and refractive index (RI) detectors. Enhancement of apparent weight-average molecular weight (Mwa) of sodium humate solutions at both pH 7 and 3.5, occurred after irradiation with UV light after 5.5 and 13 h by catalysis with either manganese or iron porphyrin. A significant increase in Mwa values was also found when samples were kept in the dark for 8 and 11 days after the end of irradiation, thereby suggesting a progression of free-radicals coupling with time course after photoinduction. The enhancement of absorptivity of humic matter with catalyzed photooxidation was confirmed by the significant increase in apparent molecular mass as shown by the RI detector. The latter also indicated a slightly larger effect by iron-porphyrin than by manganese-porphyrin in the photooxidative coupling of humic molecules. These findings suggest that photooxidation may represent a valid alternative to oxidizing agents, such as H2O2, in the polymerization of natural organic matter catalyzed by biomimetic metalloporphyrins.

Introduction

Humic substances (HS) are ubiquitous natural compounds, arising from the chemical and biological degradation of plant and animal residues and occurring in soils, sediments, coals, waters, and other natural materials.1 Approximately 40–60% of dissolved organic carbon (DOC) in freshwaters and 60–70% of the total soil C occurs in humic materials.2 Therefore, HS are important contributors to the C cycle as a major source of CO2 and as a C reservoir in the biosphere.3,4

It has been suggested that humic compounds rather than being macromolecular polymers should be better described as supramolecular associations of relatively small heterogeneous molecules held together mainly by weak dispersive forces, such as van der Waals, π-π, and CH-π interactions, in only apparent large molecular dimensions.5-7 The molecular mass of single humic molecules was found to be around 600 Da on average8 and hardly larger than 1000 Da.9,10

Within this novel understanding, it could be reasoned to stabilize these compounds by increasing the number of intermolecular covalent bonds and thus enhance their resistance to further biotic or abiotic degradation.11 Previous works have shown that the humic superstructure can be stabilized by oxidation in the presence of oxidoreductive enzymes (containing natural metalloporphyrins), such as horseradish peroxidase.11,12 The enzymes are thought to catalyze the oxidative coupling of the phenolic components in humic matter by means of a radical mechanism.13-15

Since the isolation and purification of any enzyme is rather problematic, synthetic biomimetic catalysts are a favorable alternative to be applied to mimic the activity of natural oxidative enzymes in an efficient and economic way.16 To overcome the disadvantages of natural metalloporphyrins, resulting from their instability in the presence of excess oxidants (due either to self-destruction or to the formation of inactive μ-oxo complexes PFeIII−O−FeIII),17 bulky substituents, such as 2,6-dichloro sulfonatophenyl groups, may be attached to the porphyrin ring to increase both their redox potential (i.e., resistance to oxidants) and their water solubility.18-20

Synthetic metalloporphyrins have been successfully applied to mimic catalytic oxidation of various hydrocarbons, such as polychlorinated aromatics and other pollutants,21,22 drugs,23 various lignin models,24 and humic substances.25 In all cases, singlet oxygen donors, such as hydrogen peroxide, were required to produce the highly reactive o xoiron(IV)-porphyrin radical cations. These o xo species, for the first time characterized as the key intermediates in the catalytic cycle of natural oxygenases by Groves et al.,26 show strong oxidizing ability.27 Therefore, once the o xoiron(IV)-porphyrin radical cations are formed, the organic substrate can undergo an oxidation giving unstable free radicals that are stabilized by spontaneous mutual coupling without additional involvement of a catalyst.28

Even though hydrogen peroxide is considered to be an environmentally clean oxidant, and hence a suitable singlet...
oxygen donor, iron porphyrin in the presence of H₂O₂ may form unreactive oxoiron(IV)porphyrin complexes, resulting in lower product yields. Therefore, a different singlet oxygen donor should be used or the system should be exposed to light for photooxidation. The photocatalytic effect of synthetic metalporphyrins has been demonstrated using hydrocarbons, such as alkanes and alkenes in the presence of dioxygen. In this case, it is believed that an intramolecular electron transfer, induced by light, leads to the formation of M(II)porphyrin (where M = Fe or Mn).

\[ \text{M}^{\text{III}}(\text{TDCPPS})\text{Cl} \rightarrow \text{M}^{\text{II}}(\text{TDCPPS}) + \text{Cl}^- \]

The reduced metal may thus coordinate a dioxygen molecule, generating M(III)-peroxoalkyl complex, which can undergo subsequent reactions resulting in a formation of oxoferryl porphyrinate \( \pi \) cation radicals, inasmuch as in “dark” reactions.

Our previous work has shown that iron porphyrin successfully catalyzed the oxidative polymerization of dissolved HS when H₂O₂ was used as oxidant. The resulting increase in conformational rigidity of HS was proved by spectroscopic evidence and by high performance size exclusion chromatography (HPSEC), that is a reliable and reproducible method for the appraisal of HS molecular size distribution. The objective of this work was to verify whether photooxidation could act as a substitute for oxidant species in the polymerization of dissolved humic material catalyzed by biomimetic catalysts such as synthetic iron or manganese porphyrins.

**Experimental Section**

**Humic Substances.** A humic acid (HA) was isolated from North Dakota leonardite (Mammoth, Int. chem. Co.) by common procedures. The original material was shaken overnight in a 0.5 M NaOH and 0.1 M Na₃P₂O₇ solution under N₂ atmosphere. The HA was precipitated from alkaline extracts by adding 6 M HCl until pH 1 and extensively purified under N₂ atmosphere by three cycles of dissolution in 0.1 M NaOH and subsequent precipitation in 6 M HCl. The HA was then treated with a 0.5% (v/v) HCl–HF solution for 36 h, dialyzed (Spectrapore 3, 3500 Mw cutoff) against distilled water until chloride-free, and freeze-dried. The humic material redissolved in 0.5 M NaOH was passed through a strong cation-exchange resin (Dowex 50) to further eliminate divalent and trivalent metals and freeze-dried again.

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After homogenization, 30 mg of HA were suspended in 100 mL of distilled water and titrated (automatic titrator, VIT 90 Videotitratior, Radiometer, Copenhagen, Denmark) to pH 7 with a CO₂-free solution of 0.1 M NaOH under N₂ atmosphere and continuous stirring. After having reached the constant pH 7, the resulting sodium humates were left under titration for 2 h, filtered through a Millipore 0.45 µm filter, freeze-dried, and homogenized in agate mortar.

**Size Exclusion Chromatography.** The HPSEC system consisted of a Shimadzu LC-10ADVP solvent pump and two detectors in series, a UV–Vis variable wavelength detector (Perkin-Elmer LC-295) set at 280 nm and a RI detector (Fisons Instruments, Refractomonitor IV). A rheodyne rotary injector, equipped with a 100 µL sample loop, was used to load the calibration standards and humic solutions. Size exclusion separation occurred through a Polysep-GFC–P 3000 (600 mm per 7.8 mm i.d.) column (Phenomenex), preceded by a Polysep-GFC-Guard column (35 mm per 7.8 mm i.d.), and by a 0.2 µL stainless steel inlet filter. Both columns were packed with rigid spherical silica gels chemically bonded with hydrophilic compounds. Phosphate buffer (Na₂H₃PO₄, 0.0625 M, pH 7, ionic strength 0.104 M) was used as eluent at 0.6 mL min⁻¹ flow rate. The void volume \( V_v = 10.96 \text{ mL} \) and the total permeation volume \( V_p = 25.88 \text{ mL} \) of column were determined by Blue dextran (2000 kDa) and water (18 Da), respectively. The HPSEC system was calibrated using sodium polystyrenesulfonate standards (Polymer Standard Service, Germany) with molecular weight ranging from 1100 to 130 000 Da.

Size exclusion chromatograms for both UV and RI detector were evaluated using the Perkin-Elmer Turbochrom v6.2 peak-integration and molecular-weight software applying a SEC noise threshold of 5, an area threshold of 28, and Hamielec calibration. Calculation of the apparent weight-average molecular weight values \( (M_{wa}) \) was carried out by the method of Yau et al. using the following equation:

\[ M_{wa} = \frac{\sum_{i=1}^{N} h_i M_i}{\sum_{i=1}^{N} h_i} \]

where \( h_i \) is the height of the size exclusion chromatogram of each sample at volume \( V \). The relative standard deviation of calculated values among triplicates of each chromatogram varied to a maximum of 5%.

**Biomimetic Catalysts.** The preparation procedure of the meso-tetra-(2,6-dichloro-3-sulfonatophenyl)porphyrinate of Mn(III) [Mn(TDCPPS)Cl] or Fe(III) [Fe(TDCPPS)Cl], (Figure 1), otherwise referred to as Mn–Pha and Fe–Pha, respectively, have been previously described.

**Photooxidative Polymerization of Humic Substances by Biomimetic Catalysis.** A 2.0 mg aliquot of sodium-humate was dissolved in 5 mL of the 0.1 M phosphate buffer solution that was also used as HPSEC eluent. This solution was further treated with 0.24 µmol of Mn(TDCPPS)Cl or Fe(TDCPPS)Cl (2.2 mL of a 1.09 × 10⁻³ M solution in Milli-Q water), and the volumetric flask (covered with aluminum foil) was filled up with the phosphate buffer to reach 10 mL of...
The solutions, transferred into Pyrex tubes, were irradiated with UV light for either 5.5 or 13 h in a photoreactor (Helios Italquartz) equipped with a 500 W high-pressure mercury lamp (through a Pyrex filter, k > 300 nm). The UV lamp was cooled at room temperature by circulating tap water through a double-walled quartz jacket around it. After irradiation, the solutions were filtered through a 0.45 μm Millipore filter and injected in the HPSEC apparatus. The samples already exposed for 13 h were further kept in the dark at 4 °C for other 8 and 11 d, brought to room temperature and injected again. Five control solutions (HA, Mn−Pha, Fe−Pha, HA+Mn−Pha, HA+Fe−Pha) prepared as above were not irradiated with UV light but kept in the dark for the same time as the irradiated solutions. Both control and irradiated samples were then adjusted to pH 3.5 with glacial acetic acid and injected in the HPSEC system. All solutions were prepared in triplicates and triplicate chromatograms were obtained for each solution.

Results and Discussion

The elemental and spectroscopic characteristics of the HA of this study were previously reported and are shown in Table 1. This HA had a C/H ratio of 12.4 and revealed a large content of aromatic and carboxyl carbons. The ratio of hydrophobic/hydrophilic carbons (HB/HI), as determined by CPMAS-13C NMR spectroscopy, was 1.36, thereby indicating a lower hydration capacity than more hydrophilic humic materials.

The UV-detected size exclusion chromatograms of sodium humates solutions both at pH 7 and pH 3.5 before and after oxidative polymerization catalyzed by manganese- and iron-porphyrins are reported in Figures 2 and 3, respectively. At pH 7, the chromatograms of control solution had a similar amount of acetic acid (12.4) indicating a lower hydration capacity than more hydrophilic humic materials. The UV-detected size exclusion chromatograms of sodium humates solutions both at pH 7 and pH 3.5 before and after oxidative polymerization catalyzed by manganese- and iron-porphyrins are reported in Figures 2 and 3, respectively (Table 1).

After exposure to UV radiations, the chromatographic absorption of the catalyst-added solutions was shifted to lower elution volumes, and its intensity significantly enhanced with increasing time of exposure to UV light (Figures 2 and 3). The irradiated humic materials increased the M_w values by 4.1 and 10.7% after 5.5 h and 13.7 and 11.5% after 13 h of exposure to UV light, in the presence of Mn− and Fe−Pha, respectively (Table 1). At pH 7, the intensity of the HPSEC diffused absorption for both manganese- and iron-porphyrin blanks (not shown) did not exceed 10 mV at their elution volume (about 15.8 mL) and thus did not affect the calculations of M_w values for the humic samples.

To verify whether the catalytic polymerization of humic molecules induced by photooxidation continued in the time course progression after exposure to UV light, further chromatograms of the samples irradiated for 13 h were obtained after 8 and 11 d following irradiation (Figures 2 and 3). For both catalysts, the main diffused absorption of samples showed a further significant increase in intensity and shift to lower elution volumes with progression of time after the exposure to UV light. The relative M_w values reached an increase of 26.9 and 23.2% after 8 d since exposure to UV light, (F) as D but after 11 d since exposure to UV light. Solutions A−D were brought to pH 3.5 with acetic acid and run again by HPSEC.

The chromatograms of the neutral humate solutions, right after exposure to 5.5 and 13 h of UV irradiation, were modified by lowering the solution pH from 7 to 3.5 with acetic acid prior to the injection in the HPSEC system. A similar amount of acetic acid (<10−6 M) did not affect the HPSEC elution profiles of macromolecules such as polysaccharides and polystyrenesulfonates. The treatment with acetic acid decreased the molecular size of humic solutions.


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**Figure 2.** UV-detected HPSEC chromatograms of (A) control HA solution at pH 7, (B) HA solution at pH 7 added with Mn−Pha, (C) as B but exposed to UV light for 5.5 h, (D) as B but exposed to UV light for 13 h, (E) as D but after 8 d since exposure to UV light, (F) as D but after 11 d since exposure to UV light. Solutions A−D were brought to pH 3.5 with acetic acid and run again by HPSEC.

**Table 1.** Distribution of C Intensity in Different Regions (ppm) of 13C−CPMAS NMR Spectrum and Elemental Properties of the Humic Acid Used in This Study

<table>
<thead>
<tr>
<th>Region</th>
<th>Percent C</th>
<th>%H</th>
<th>%N</th>
<th>C/H</th>
<th>C/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–40 aliphatic</td>
<td>44.1</td>
<td>18.6</td>
<td>13.6</td>
<td>8.5</td>
<td>15.2</td>
</tr>
<tr>
<td>40–110 C−O, C−N</td>
<td>8.5</td>
<td>15.2</td>
<td>1.36</td>
<td>22.02</td>
<td></td>
</tr>
<tr>
<td>110–140 aromatic</td>
<td>0.96</td>
<td>12.4</td>
<td>47.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* [(0–40) + (110–140)]/[40–110] + (140–190)]. *b* (110–140)/0–190).
mainly because the intensity of the diffused absorption was reduced in comparison to that at pH 7 and split into 3 peaks at larger elution volume (i.e., larger molecular sizes). This had been previously explained by the increased stability of humic conformations due to formation of new hydrogen bonds with addition of acetic acid. The reduction in absorbance is attributed to a hypochromic effect, by which molecular absorptivity decreases when the mutual orientation of the transition dipole moments of the heterogeneous neighboring chromophores are altered during their repositioning. Moreover, the chromophores, which are not covalently bound in rigid structures, are assumed to be separated during the dynamic chromatographic elution following the acetic acid treatment and elute at larger elution volume.

For the samples added with acetic acid, the chromatograms of the solutions in the presence of catalysts and exposed to UV light (Figures 2 and 3) were similar in shape to that of control solutions (HA and HA+ catalyst). However, the absorptions were more intense and slightly shifted toward lower elution volume (i.e., larger molecular sizes). Calculations of $M_{wa}$ values (Table 1) were limited to the 1st peak because of the restricted interval of elution volumes for the calibration standards employed. These $M_{wa}$ values indicated, for the irradiated materials brought to pH 3.5, an increase in molecular size of 10.3 and 3.1% after 5.5 h and 1.9 and 11% after 13 h of irradiation of humate solutions containing the Mn- and Fe—Pha catalysts, respectively, with respect to the control solution brought to pH 3.5 with acetic acid.

These results suggested that the photooxidation of sodium humates in the presence of the metalloporphyrin catalysts had increased the molecular sizes of the humic matter. These increased significantly for the irradiation of humate solutions at pH 7 for 5.5 and 13 h in the case of the Fe—Pha catalyst and for 13 h in the case of Mn—Pha. Both catalysts induced a further and similar enhancement of $M_{wa}$ values in the samples which were kept in the dark for 8 and 11 d after irradiation with UV light. Moreover, the increase in molecular dimension due to formation of covalent interactions among humic molecules was confirmed by the significant enhancement of $M_{wa}$ values found for the irradiated samples treated with acetic acid to pH 3.5. The acetic acid treatment is reported to disrupt the humic conformations, which are only stabilized by dispersive forces, whereas it has little effect when an oxidative coupling has occurred among humic molecules.

Unlike the UV detector, that reveals only the molecular absorptivity of chromophore groups absorbing at a chosen wavelength, a RI detector is able to follow the overall mass absorption of the solutions in the presence of catalysts and exposed to UV light (Figures 2 and 3) were similar in shape to that of control solutions (HA and HA+ catalyst). However, the absorptions were more intense and slightly shifted toward lower elution volume (i.e., larger molecular sizes). Calculations of $M_{wa}$ values (Table 1) were limited to the 1st peak because of the restricted interval of elution volumes for the calibration standards employed. These $M_{wa}$ values indicated, for the irradiated materials brought to pH 3.5, an increase in molecular size of 10.3 and 3.1% after 5.5 h and 1.9 and 11% after 13 h of irradiation of humate solutions containing the Mn- and Fe—Pha catalysts, respectively, with respect to the control solution brought to pH 3.5 with acetic acid.

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Unlike the UV detector, that reveals only the molecular absorptivity of chromophore groups absorbing at a chosen wavelength, a RI detector is able to follow the overall mass distribution of humic matter. Thus, RI reveals additional information on the conformational changes of the bulk humic mass that may take place with molecular variations induced by photooxidation.

Table 2. Apparent Weight-Average Molecular Weight ($M_{wa}$) of Lignite Humic Solutions, at pH 7 and pH 3.5, Added with Either Mn—Pha or Fe—Pha Catalyst and Exposed to UV Light for 5.5 or 13 h.

<table>
<thead>
<tr>
<th>HA solutions</th>
<th>UV detector pH=7 $M_{wa}$ ± SD</th>
<th>UV detector 1st peak pH = 3.5 $M_{wa}$ ± SD</th>
<th>RI detector 1st peak pH = 7 $M_{wa}$ ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA, control</td>
<td>5023 ± 25</td>
<td>2977 ± 136</td>
<td>9525 ± 476</td>
</tr>
<tr>
<td>HA+Mn—Pha</td>
<td>4847 ± 196</td>
<td>2997 ± 144</td>
<td>8838 ± 442</td>
</tr>
<tr>
<td>HA+Mn—Pha, UV 5.5 h</td>
<td>5048 ± 38</td>
<td>3306 ± 94</td>
<td>9629 ± 159</td>
</tr>
<tr>
<td>HA+Mn—Pha, UV 13 h 0 d</td>
<td>5510 ± 15</td>
<td>3055 ± 34</td>
<td>9407 ± 251</td>
</tr>
<tr>
<td>HA+Mn—Pha, UV 13 h, 8 d</td>
<td>6150 ± 307</td>
<td>ND</td>
<td>10943 ± 547</td>
</tr>
<tr>
<td>HA+Mn—Pha, UV 13 h, 11 d</td>
<td>6514 ± 325</td>
<td>ND</td>
<td>11630 ± 582</td>
</tr>
<tr>
<td>HA+Fe—Pha,</td>
<td>4954 ± 150</td>
<td>3089 ± 154</td>
<td>8785 ± 439</td>
</tr>
<tr>
<td>HA+Fe—Pha, UV 5.5 h</td>
<td>5486 ± 266</td>
<td>3186 ± 64</td>
<td>9304 ± 278</td>
</tr>
<tr>
<td>HA+Fe—Pha, UV 13 h, 0 d</td>
<td>5526 ± 114</td>
<td>3429 ± 171</td>
<td>9265 ± 22</td>
</tr>
<tr>
<td>HA+Fe—Pha, UV 13 h, 8 d</td>
<td>6104 ± 305</td>
<td>ND</td>
<td>9938 ± 172</td>
</tr>
<tr>
<td>HA+Fe—Pha, UV 13 h, 11 d</td>
<td>6626 ± 330</td>
<td>ND</td>
<td>13216 ± 660</td>
</tr>
</tbody>
</table>

* For chromatograms of samples exposed for 13 h and obtained right after exposure (0 days), and after 8 and 11 days after irradiation end. SD = Standard Deviation. Percent $M_{wa}$ difference between irradiated sample and humic solution simply added with catalyst. ND = Not Determined. HPSEC chromatograms were revealed by either UV and RI detector.
The size exclusion chromatograms of HA recorded by RI detector before and after a photooxidative reaction catalyzed by both Mn- and Fe-Pha at pH 7 are shown in Figures 4 and 5. The chromatograms of control humic solution showed only two absorptions of low intensity at about 14.2 and 17.8 mL. The addition of the biomimetic catalysts to HA resulted in different elution volumes (about 15.0 mL and 18.1 mL) and larger intensities for the two absorptions. Since the second absorption was found to reflect mainly the mass of the biomimetic catalyst (chromatograms not shown), the weight-average molecular weight was calculated only for the first absorption of RI chromatograms.

The intensity of the first absorption of the humate chromatograms appeared to slightly increase with time of exposure to UV light and in the presence of both catalysts with respect to the humic solution just added with the catalyst (Figures 4 and 5). The chromatograms of control humic solution showed only two absorptions of low intensity at about 14.2 and 17.8 mL. The addition of the biomimetic catalysts to HA resulted in different elution volumes (about 15.0 mL and 18.1 mL) and larger intensities for the two absorptions. Since the second absorption was found to reflect mainly the mass of the biomimetic catalyst (chromatograms not shown), the weight-average molecular weight was calculated only for the first absorption of RI chromatograms.

The intensity of the first absorption of the humate chromatograms appeared to slightly increase with time of exposure to UV light and in the presence of both catalysts with respect to the humic solution just added with the catalyst (Figures 4 and 5). The irradiated humic materials increased the $M_w$ values by 8.9 and 5.9% after 5.5 h and 6.4 and 5.5% after 13 h of exposure to UV light, in the presence of Mn- and Fe-Pha, respectively (Table 2).

A statistically significant increase in molecular dimension was indeed observed by the RI detector in the samples analyzed after 8 and 11 d following 13 h irradiation with UV light (Table 2). The RI-detected chromatograms of these samples also showed an increase of intensity for the first peak and its shift to lower elution volumes with time course following the exposure to UV light (Figures 4 and 5). This confirmed a persisting reactivity of the catalytic oxidative polymerization of humic molecules even without a continued exposure to UV irradiation. For these RI-detected chromatograms, the $M_w$ values were significantly larger than both the control solution and the humic solution irradiated with UV light for 13 h. In particular, the presence of the Mn–Pha catalyst increased the $M_w$ values of the humic solution by 23.8 and 31.6% after 8 and 13 d from the UV irradiation, respectively, whereas the increase of $M_w$ values obtained with the Fe–Pha catalyst was 13.1 and 50.4% after the same time courses (Table 2). The larger catalytic effect of Fe–Pha in increasing the molecular size of the sodium humates was significant, thereby suggesting that this catalyst may be preferable to Mn–Pha for the photoinduced polymerization of humic acids.

Our results indicate that synthetic water-soluble metalloporphyrins, such as Mn(TDCPPS)Cl and Fe(TDCPPS)Cl are capable of turning weakly associated humic superstructures into more stable covalently bound oligomers or polymers, by means of radical oxidative transformation induced by light. The photooxidative coupling occurred not only direct exposure to UV light but also in time course after the end of irradiation, as suggested by the increase in the apparent weight-average molecular weights. A further evidence for the polymerization of humic molecules was given by the HPSEC chromatograms revealed by a RI detector that showed the actual increase of humic molecular mass with increasing course of the catalyzed reaction. The increased conformational stability of humic matter should be attributed
to the formation of C—O—C and C—C bonds during photooxidation, as previously shown for the catalytic activity of peroxidase\textsuperscript{11,12} and iron-porphyrin,\textsuperscript{15} employing H\textsubscript{2}O\textsubscript{2} as oxidizing agent.

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References and Notes