



Application Note

Quantitative analysis of singlet oxygen production via a 4+2 endoperoxide cycloaddition by ¹H benchtop nuclear magnetic resonance spectroscopy







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Introduction

Singlet oxygen production has a variety of biosynthetic, ecological remediative, and defense-related utilities as a means of introducing reactive oxygen species into chemical systems. Singlet oxygen has been harnessed by nature to perform highly selective oxidations in key biosynthetic routes, and this has been emulated in biomimetic synthetic approaches to natural products.¹ Moreover, reactive oxygen species have the ability to induce cell death via oxidative damage of key biomolecules, including deoxyribonucleic acid (DNA).² However, singlet oxygen is notoriously difficult to quantify, as singlet oxygen is a colorless, odorless substance with an incredibly short lifetime. It has been previously reported that cyclic 1,3-dienes engage singlet oxygen in 4+2 cycloadditions, and singlet oxygen has also been known to participate in ene reactions with electron rich alkenes.³⁻⁶ Production of singlet oxygen is mediated by photosensitizing agents, which undergo initial photoexcitation, followed by intersystem crossing and phosphorescence to excite diatomic oxygen from the ground triplet state to an excited singlet state.⁷ Here, we report the use of benchtop nuclear magnetic resonance (NMR) spectroscopy to monitor the production of singlet oxygen by various putative singlet oxygen photosensitizers, berberine and its reduced analogs, dihydroberberine and tetrahydroberberine.

In order to quantify singlet oxygen production, we utilized α -terpinene, a commercially available monoterpene with a cyclic 1,3-diene functionality, to trap singlet oxygen species in a Diels-Alder-like hetero 4+2 cycloaddition, whereby the resulting endoperoxide product could be determined with quantitative NMR (qNMR) spectrscopy.⁸



Figure 1. Reaction scheme depicting Diels-Alder-like 4+2 like reaction between singlet oxygen and α -terpinene. The diene of the α -terpinene reacts with the singlet oxygen to trap it into an endoperoxide, which can be monitored using NMR and quantified by integrating endoperoxide and cymene peaks.

We performed photochemical reactions in acetone- d_{δ} , since the vibrational frequency of the carbon-deuterium bond of the solvent does not vibrationally quench singlet oxygen, unlike its protonated counterpart.⁹ Dimethyl terephthalate was used as an internal calibrant to provide a basis for quantitative NMR analysis of singlet oxygen generation, and compounds were irradiated under ultraviolet (UV) light due to increased overlap between their absorbance & UV light sources.





Figure 2. (a) Synthesis, experimental design, and photoirradiation of NMR tubes. NMR tubes were flushed with oxygen and then irradiated for 60-minute intervals while monitored with ¹H NMR in between each interval of irradiation. Such an experimental setup allowed for the tracking of endoperoxide formation in α -terpinene over time, allowing us to view our tested singlet oxygen photosensitizers' efficiency. (b) Irradiation of NMR tubes under UV and LED light.



Figure 3. Synthesis of reduced berberine analogs through borohydride reduction of the C-ring.

We harnessed this 4+2 cycloaddition between singlet oxygen and *a*-terpinene to compare the production of singlet oxygen over time when photoirradiated in the presence of methylene blue, rose bengal, berberine, or its reduced analogs.

Results

¹H (60 MHz) NMR spectroscopy was used to quantify singlet oxygen production as a function of time, taken in 60-minute intervals. Reduced berberine analogs were irradiated with UV light, while berberine, rose bengal and methylene blue were irradiated with a light-emitting diode (LED) light. The emission spectra of the UV and LED light sources as well as the absorption spectra of the photosensitizers are shown below.



Figure 4. (a) Ultraviolet-visible spectra of rose bengal, methylene blue, berberine, dihydroberberine and tetrahydroberberine. (b) Emission spectra of LED irradiation source. (c) Emission spectra of UV irradiation source.



Figure 5. All of the protons and corresponding peaks of interest are highlighted. Singlet oxygen production was quantified through the integration of a doublet with a chemical shift of 6.44 ppm and doublet of doublets with a chemical shift of 7.13 ppm, which are highlighted in green and purple, respectively. The aromatic protons of cymene highlighted in purple, the minor oxidative product of α -terpinene, generated a doublet of doublets at 7.13 ppm. In comparison, the vinyl protons of ascaridole highlighted in green generated a doublet at 6.44 ppm. Vinyl protons of α -terpinene highlighted in yellow generated a doublet at 5.5 ppm, and dimethyl terephthalate's aromatic protons highlighted in blue generated a doublet of doublets at about 8.1 ppm while its methoxy protons (highlighted in red) generated a singlet at about 4.0 ppm.

Endoperoxide and p-cymene concentrations were measured over time and the sum of their integrations was used to determine total effective singlet oxygen production over time. Endoperoxide and p-cymene were revealed through the appearance of peaks with a chemical shift of 6.44 ppm and 7.13 ppm, respectively, and were used to calculate the concentrations of products through gNMR. The derived concentrations of endoperoxide and p-cymene were used to graph the singlet oxygen production over time.



Figure 6. The formation of ascaridole was monitored and quantified through ¹H (60 MHz) NMR spectroscopy. Formation of ascaridole was compared between berberine and commercially available photosensitizers under LED conditions (a) and UV conditions (b). Moreover, ascaridole formation of berberine against reduced berberine analogs was monitored under UV conditions (c). Formation of cymene was compared between berberine and commercially available photosensitizers under LED conditions (e). Moreover, cymene formation of berberine against reduced berberine analogs was monitored under UV conditions of berberine against reduced berberine analogs was monitored under UV conditions (f).

Discussion

The results from this study suggest that berberine is a comparable singlet oxygen photosensitizer to methylene blue, but is a worse photosensitizer than rose bengal. Moreover, reduction in conjugation of the C-ring of the berberine scaffold changes the photochemical properties and UV-visible absorbance spectrum of the compound, ultimately making berberine a worse photosensitizer. The results from this work prompt future studies on the photodynamic biological activity of the two berberine analogs studied here, as well as other analogs of berberine with modified chromophores. Such studies are currently underway in our laboratory. The content in this application note was derived from a paper published by Su, *et al.* (2021) in the *Journal of Emerging Investigators*.org.

Materials and Methods

Physical Methods

All NMR data was obtained using a Nanalysis 60PRO benchtop NMR spectrometer in acetone- d_{\circ} . The ¹H experiments were performed using the following acquisition parameters: spectral width, 20 ppm; spectral center, 5 ppm; number of points, 2048; number of scans, 16; dummy scans, 0; interscan delay, 2.4 seconds; receiver gain, 40.

The emission spectra of LED lights and UV lights used in this study were collected on an Ocean Optics spectrometer. The UV-visible spectra were collected in acetone with a BioRad SmartSpec 3000 UV-visible spectrophotometer or a Thermo Electron Genesys 5 UV-visible spectrophotometer in quartz cuvettes. Tubes were photo irradiated in 60-minute intervals with LED and UV light, respectively. Endoperoxide formation was quantified by ¹H (60 MHz) NMR at 60-minute intervals for 5 hours by integrating the endoperoxide peak at 6.44 ppm (d, J = 2.28 Hz). Cymene formation was quantified by ¹H NMR at 60-minute intervals for 5 hours by integration of the cymene peak at 7.13 ppm (dd, J = 3.6, 2.4 Hz). Dimethyl terephthalate was used as an internal standard and was chosen because its signature resonances (8.1 ppm, Ar-H; 3.95 ppm, -OCH₃) did not overlap with signals from α -terpinene or ascaridole. NMR spectra were processed and visualized on the MestreNova software package.

References

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