Design of an Antioxidant Nitrone Scaffold Based on Plant Pigments

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ABSTRACT

Nitrones derived from natural antioxidants are emerging as highly specific therapeutics for the treatment of various human diseases, including stroke, neurodegenerative pathologies, and cancer. However, the development of useful pseudo-natural nitrones requires the judicious choice of a secondary metabolite as precursor. Betalains are nitrogen-containing natural pigments that exhibit marked antioxidant and pharmacological properties and, hence, are ideal candidates for the design of multifunctional nitrones. In this work, we describe the semisynthesis and properties of a biocompatible and antioxidant betalain-nitrone named OxiBeet. This bio-based compound is a better radical scavenger than ascorbic acid, gallic acid and most non-phenolic antioxidants and undergoes concerted proton-coupled electron transfer. The autoxidation of OxiBeet gives rise to a persistent nitroxide radical, which was studied by electron paramagnetic resonance spectroscopy. Femtosecond transient absorption spectroscopy reveals that excited state formation is not required for the oxidation of OxiBeet. The results are compared with those obtained using betanin, a natural betalain, and pBeet, the imine analogue of OxiBeet. The findings in this study will enable the development of antioxidant nitrones based on the novel *N*-oxide 1,7-diazaheptamethinium scaffold and betalain dyes with enhanced hydrolytic stability in aqueous alkaline media

Keywords – nitrones, nitroxide, antioxidant, betalain, pseudo-natural compounds.

Introduction

The formation of stable and persistent nitroxide adducts of free radical reactive species endows nitrones with marked antioxidant properties and support their use as therapeutic agents to treat disorders related to oxidative stress.[1-3] The same process promotes the use of nitrones as building blocks for the construction of nitrogenous compounds[4] and as electron paramagnetic resonance (EPR) spin traps.[5] Although natural products containing the nitrone functional group are scarce,[6] antioxidant and pharmacologically active compounds of natural origin can be used as starting materials for the semisynthesis of novel nitrones with high potential for application.

Antioxidants based on natural product scaffolds help to reduce the demand for non-renewable hydrocarbon resources and are valuable in medicinal chemistry and drug discovery.[7, 8] The nitrone derivatives of ligustrazine, a pyrazine found in the Chinese herb *Ligusticum wallichii* Franch., have antioxidant and thrombolytic properties and are effective therapeutics for treating ischemic stroke.[3] Another example is the nitrone derived from the water-soluble analogue of vitamin E, Trolox, which shows enhanced radical scavenging and neuroprotective properties.[9]

Betalains are non-toxic water-soluble pigments found in plants, fungi and bacteria[10] that have been consumed in foods for millennia.[10-12] Indeed, betanin (5-*O*-β-glucosyl betanidin, EFSA/E162 and FDA/73.40) and indicaxanthin (L-proline-betaxanthin), the main pigments in red beetroots (*Beta vulgaris* L.) and prickly pears [*Opuntia ficus-indica* (L.) Mill.], respectively, have positive effects on the overall redox state in vivo.[13-17] Both phenolic and non-phenolic betalains are highly efficient dietary antioxidants,[14] outperforming vitamin C, vitamin E and many flavonoids in terms of antioxidant capacity (*thermodynamics*, number of radicals scavenged) and activity (*kinetics*, reactivity towards radicals).[14, 18-20]

Here, we describe the semisynthesis and properties of OxiBeet, the first betalain-nitrone. This bioinspired compound is stable towards hydrolysis under neutral and slightly alkaline conditions and is not cytotoxic to the human hepatic cell line HepaRG at concentration up to 1 mM. OxiBeet has high radical scavenging capacity and low reduction potential. Plus, it can be converted into a persistent *N*-oxide 1,7-diazaheptamethinium radical cation via autoxidation in aqueous solution. Comparison of the characteristics of OxiBeet with those of natural betalains and pBeet provide new insights into the use of polymethine dyes to develop resonance-stabilized radicals.

Materials and methods

General information

All chemicals were purchased from Sigma-Aldrich and used without further purification unless otherwise stated. Betalamic acid was extracted from base-hydrolyzed red beetroot juice and processed as described previously.[21, 22] Aqueous solutions were prepared using deionized water (18.2 MΩ cm at 25 ºC, TOC ≤ 4 ppb, Milli-Q, Millipore). Additional experimental details are available in the ESI.

Semisynthesis of OxiBeet

N-Phenylhydroxylamine (13.5 mg, 125 μmol, 5.0 equiv) was added to an aqueous solution of betalamic acid (5.3 mg, 25 μmol, 1.0 equiv) acidified with HCl (25 mL, pH 3). The mixture was kept at 4 ± 1 °C for 1 h and the resulting red-orange solution was subjected to flash gel permeation column chromatography (Sephadex LH-20, water, 1.5 × 20 cm, 20 psi). The orange-colored fraction was lyophilized (–80 °C, 0.08 mbar) to obtain OxiBeet (phenylhydroxylamine-betaxanthin, 3.8 mg, 50%) as an orange solid.

^{1}H-NMR (800 MHz, D_2O): δ 8.32 (d, *J* = 10.4 Hz, 1H), 7.60 (d, *J* = 7.9 Hz, 2H), 7.52 (t, *J* = 7.9 Hz, 2H), 7.46-7.41 (m, 1H), 7.09 (d, *J* = 10.4 Hz, 1H), 6.50 (bs, 1H), 4.27 (bs, 1H), 3.22 (bs, 1H), 3.10 (dd, *J* = 17.1, 7.5 Hz, 1H).

^{13}C-NMR (200 MHz, D_2O): δ 179.84, 170.39, 160.41, 153.81, 146.46, 132.24, 131.53, 122.67, 118.30, 109.77, 57.02, 30.51.

HRMS (ESI(+)-TOF): Calc'd for $C_{15}H_{15}N_2O_5^+$, $[M]^+$, 303.0975; found 303.0976; diff. 0.3 ppm.

UV-Vis: λ_{abs}^{max} = 460 nm, $\varepsilon^{460\ nm}$ = 35,000 ± 2400 L mol^{-1} cm^{-1} (pH 6) (Figs. S1 and S2).

Fluorescence (Fl): λ_{Fl}^{max} = 562 nm (pH 6, λ^{EX} = 460 nm).

MTT assay

HepaRG cells were cultured until confluence in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). Cells were seeded in a 96-well plate (5×10^3 cells/well) and treated with OxiBeet in 1% FBS DMEM (mmol L^{-1} to μmol L^{-1}) for 6 h at 37 °C. Negative and positive control experiments were carried out using 1% FBS DMEM and Triton X-100 1% (v/v) in phosphate buffer saline (PBS), respectively. A solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in 1% FBS DMEM (5 mg mL^{-1}, 10% (v/v)) was added to each well. After incubating for 30 min at 37 °C, the medium was removed and replaced with DMSO (100 μL). The absorption of the resulting purple formazan solution was measured at 550 nm, and the cell viability was calculated as the percentage of absorbance relative to the negative control.

Radical scavenging capacity

Radical scavenging capacity was determined using the TEAC/$ABTS^{+\bullet}$ assay.[23] A solution of $ABTS^{+\bullet}$/ABTS in phosphate buffer (50 mmol L^{-1}, pH 7.4) with an absorbance of 0.7 at 734 nm (46.7 μmol L^{-1} $ABTS^{+\bullet}$) was prepared. The bleaching of $ABTS^{+\bullet}$ by the antioxidant (μmol L^{-1} concentration range) was monitored at 734 nm for 6 min at 25 °C. The slope (α) of the linear correlation between the change in absorption (ΔAbs) and the antioxidant concentration was measured. The Trolox equivalent antioxidant capacity (TEAC) was calculated by dividing the value of α for the sample with that of the Trolox standard ($\alpha_{Sample}/\alpha_{Trolox}$ ratio).

Cyclic voltammetry

Measurements were carried out at 25 °C on a Metrohm Autolab PGSTAT101 potentiostat/galvanostat equipped with a 10-mL electrochemical cell and controlled using NOVA software. A glassy carbon working electrode (diameter of electrode disk = 2 mm), a platinum wire auxiliary electrode, and an Ag/AgCl (KCl sat.) reference electrode were used; potential range: –0.2 to 0.8 V, scan rate: 50 mV s^{-1}; [analyte] = 88 mmol L^{-1} in aqueous KCl (0.1 mol L^{-1}) at pH 7.0. Before each experiment, the working electrode was polished with 0.05-μm-sized alumina particles (60 cycles) and washed with water under ultrasound irradiation for 1 min.

EPR spectroscopy and simulation

X-band EPR spectra were obtained at room temperature (20 ± 2 °C) using a Bruker EMX spectrometer equipped with a standard cavity and operated at approximately 9.7 GHz with a 100 kHz modulation frequency. The spectrometer settings were 20 mW microwave power, 0.5 G modulation amplitude, 20.48 ms time constant, 1024 points, and 100 G scan range. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (*g*-value = 2.0036) was used to calibrate the magnetic field. pBeet and OxiBeet solutions (1 mmol L^{-1}) were prepared immediately before use

in aerated phosphate buffer (50 mmol L^{-1}, pH 7.4) and kept at room temperature. All measurements were carried out using 200 µL of the respective solution in a suprasil flat cell (Wilmad). EPR simulations were carried out using the Easyspin toolbox in MATLAB.[24] The simulation was fitted to the experimental spectra using the Nelder-Mead simplex method. The following parameters were used: *g*-value = 2.0059; a_{α}^{N} = 33.6 MHz, a_{β}^{H} = 17.6 MHz, a_{γ}^{H} = 9.2 MHz and $a_{\gamma}^{2\times H}$ = 8.2 MHz; line width for isotropic broadening and full width at half maximum (FWHM) = 0.01% Gaussian and 0.29% Lorentzian.

Transient absorption spectroscopy

Femtosecond pump-probe transient absorption measurements were performed using a regenerative amplified Ti:sapphire laser system (Libra Ultrafast Amplifier System designed by Coherent; 795 nm, FWHM ≈ 40 fs, 1 mJ/pulse and 3 kHz repetition rate) as the laser source and a Newport MS260i imaging spectrograph. Briefly, the 795 nm output pulse from the regenerative amplifier was split into two parts. The transmitted part was directed into an optical parametric amplifier (TOPAS-prime, Light Conversion) to generate a pump beam of 485 nm (1 mW ± 2%), which was focused on the sample with a beam size of ca. 300-µm in diameter. The reflected beam was passed through a delay stage (8.5 ns; 1-2 fs step size) and focused into a sapphire crystal to generate a white light continuum, which was used as the probe beam. The pump pulses were chopped by a synchronized chopper at 1500 Hz and the absorbance change was calculated with two adjacent probe pulses (pump-blocked and pump-unblocked). The samples were placed in 1-mm quartz cuvettes and measured under ambient conditions.

Results and discussion

OxiBeet was semisynthesized as shown in Fig. 1a. Betalamic acid (**1**) was produced by the hydrolysis of the betalains in red beetroot juice, but it can also be obtained by enzymatic oxidation of L-DOPA[25]. The coupling between **1** and *N*-phenylhydroxylamine (**2**) in acidified water is quantitative and gives rise to OxiBeet (**3**). The product was isolated in 50% yield after purification in the presence of oxygen, which is twice as high as the values reported for other betalains.[20, 26] The high-resolution mass spectrum was consistent with the postulated structure of OxiBeet (Fig. 1b). Notably, both the semisynthesis and purification of **3** are carried out using water as solvent and **2** can be produced using environmentally benign methods.[27]

The high persistence and solubility of OxiBeet in water enables its characterization using nuclear magnetic resonance (NMR) spectroscopy (Fig. 1c), which is rarely used to study betalains because of their lability and low solubility in organic media.[28, 29] The coupling constant between the H7 and H8 atoms of OxiBeet ($^3J_{H7,H8}$ = 10.4 Hz) is slightly smaller than the typical values typically reported for natural betalains ($^3J_{H7,H8}$ ~ 12 Hz).[30] The H7 and C5 atoms of OxiBeet are deshielded (by ca. 1 ppm and 10 ppm, respectively) compared to those of betanin and indicaxanthin[28, 30]. Hence, the mesomeric donation of electron density by the oxygen atom of the nitrone group into the π-system decreases the electrophilicity of the C8 and increases the hydrolytic stability of OxiBeet compared to natural analogues.

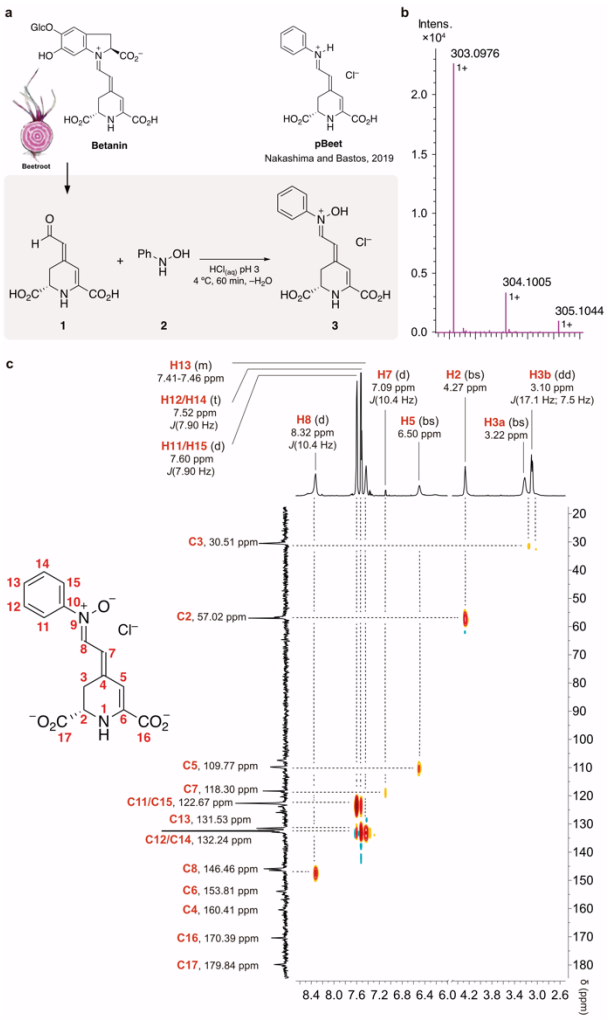

Figure 1. Semisynthesis and characterization of OxiBeet. (a) Acid-catalyzed coupling of betalamic acid (**1**) and **2** in water. The structures of betanin (main starting material of **1**) and pBeet are shown for comparison. (b) High resolution mass spectrum of OxiBeet; *m/z* 303.0976 $[M]^+$; Calc'd.: 303.0975. (c) ^1H, ^{13}C, and $[^1H,^{13}C]$ heteronuclear single quantum coherence (HSQC) spectra of OxiBeet (70 µmol L^{-1}, 800 MHz/200 MHz, D_2O at 288 K). Atom numbering is shown in red; the assignment of the signals in the NMR spectra was supported by quantum mechanical GIAO calculations (Fig. S3).

To examine the behavior of OxiBeet in aqueous solution, we compared the pH dependence of its observed first-order hydrolysis rate constant (k_{obs}) at 60 °C with those of pBeet and betanin (Figs. 2a and S4-S7). Under neutral and slightly alkaline conditions, OxiBeet was found to be far more stable than betanin. However, all compounds are subject to acid-catalyzed decomposition (Fig. 2a). Deprotonation of the nitrone group (pK_a 4.4, Fig. S8) increases the negative charge density at the N1 of the 1,7-diazaheptamethinium system (Fig. 2b) and shifts the absorption maximum of OxiBeet to shorter wavelengths (blue shift, ca. 2000 cm^{-1}, Fig. 2c). Such shift is not observed for pBeet, which ready undergoes hydrolysis in alkaline conditions. Natural betalains hydrolyze under alkaline conditions and the synthesis of a more stable analogue was reported to require extensive modification of the betalain framework.[31]

The biocompatibility of OxiBeet was tested against the human hepatic cell line HepaRG, which is a well-established model for studying drug metabolism and toxicity.[32] Cells incubated with the betalain nitrone (0.1 mmol L^{-1} to 1 μmol L^{-1}) for 6 h remained 100% viable, and 1 mmol L^{-1} OxiBeet was required to reduce cell viability by 8-10% (Fig. 2d).

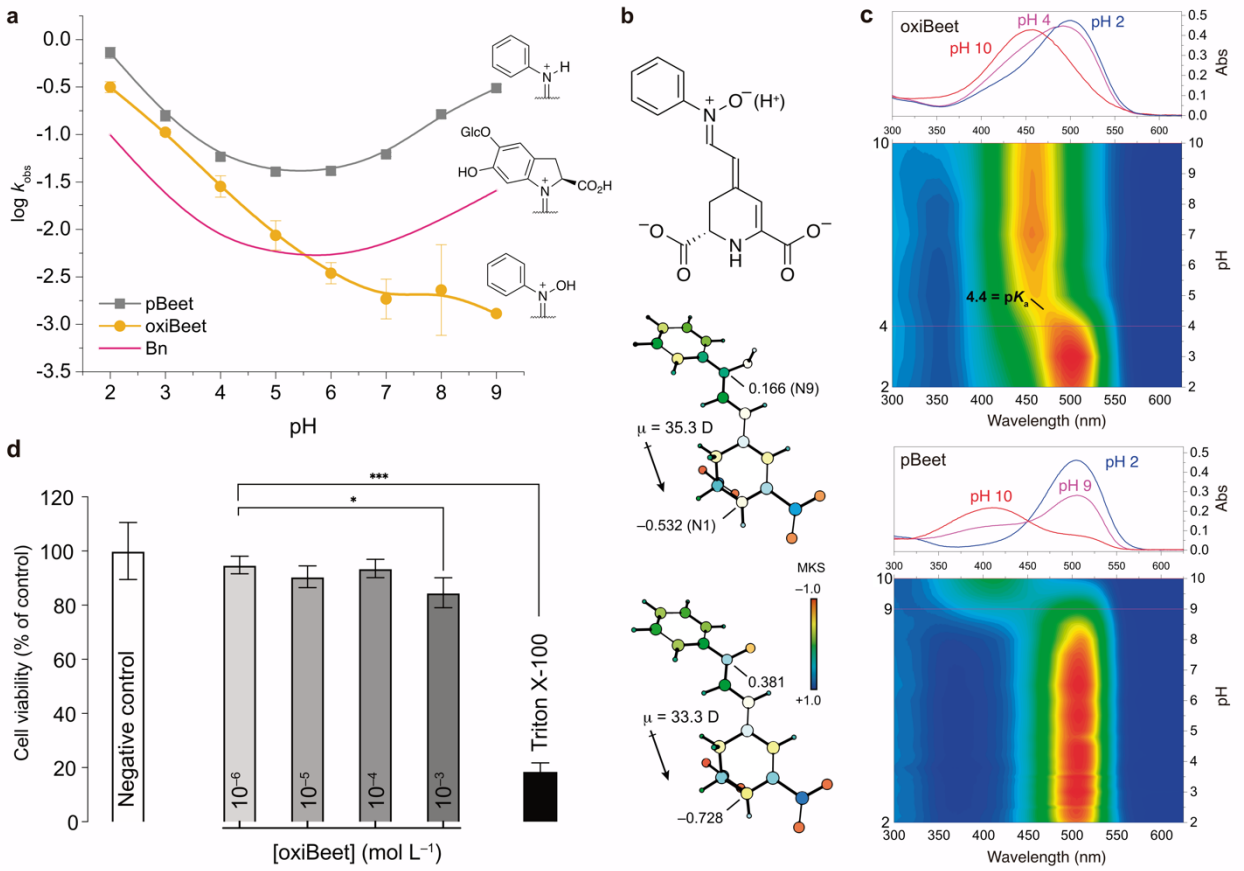

Figure 2. Properties of OxiBeet in aqueous solution. (a) Effect of the pH on the logarithm of observed rate constants (k_{obs}, min^{-1}) for the decomposition of OxiBeet and pBeet at 60 °C. Betanin is shown for reference; the data is reproduced from ref. [33]. (b) Partial charges of the nitrogen atoms of OxiBeet and its protonated (N–O*H*) form according to the Merz-Kollman-Singh (MKS) scheme. (c) Contour plots of the absorbance spectra of OxiBeet and pBeet as a function of the pH; red color indicates Abs ~ 0.5. UV-Vis spectra at selected pH values are shown for clarity. (d) Viability of HepaRG cells treated with OxiBeet (1 mmol L^{-1} to 1 μmol L^{-1}, 6 h) measured by using the MTT assay. Negative control experiments were carried out using 1% FBS DMEM; cells incubated with Triton X-100 (1% v/v in PBS) were used as positive controls. Asterisks indicate the value levels of statistical significance; (*) $P = 0.02$ and (***) $P = 0.0002$,

one-way ANOVA with Geisser-Greenhouse correction and Dunnett's multiple comparison test, $N = 5$.

The ability of nitrones to react with oxidants promotes their broad application.[1, 2] To assess the antioxidant potential of OxiBeet, we measured its radical scavenging capacity and redox potential. At pH 7.4, the radical scavenging capacities of OxiBeet and pBeet determined using the TEAC/$ABTS^{+\bullet}$ method[23] are identical at the 95% confidence level (TEAC = 3.8 ± 0.1, Fig. 3a). However, the oxidation of the 1,7-diazaheptamethinium system of OxiBeet occurred at a much lower potential than those of other betalains,[14] including pBeet,[34] as evidenced by the intense irreversible anodic response at 357 mV vs. Ag|AgCl. Moreover, OxiBeet nitrone was reversibly oxidized to the corresponding nitroxide (E_{pa} = 166 mV and E_{pc} = 78 mV; $E_{1/2}$ = 124 mV, Fig. 3b). Despite being non-phenolic, OxiBeet is a potent reducing agent and its TEAC is higher than those of well-known antioxidants such as gallic acid, vitamin C, vitamin E and many flavonoids.[35, 36]

The occurrence of concerted PCET,[37, 38] that is, hydrogen atom transfer (HAT) or concerted proton-electron transfer (CPET), is the preferred thermodynamic pathway for the oxidation of OxiBeet (Fig. 3c). The homolytic bond dissociation energy (BDE) of the N*O*–*H* bond was at least 70 kJ mol^{-1} lower than the energy required for the electron transfer in sequential proton loss electron transfer (SPLET) or electron transfer followed by proton transfer (ET-PT)[39] (Fig. 3c). The change in enthalpy of the isodesmic reaction between the phenoxyl radical and OxiBeet was –65 kJ mol^{-1}, which was much lower than that of pBeet (–11 kJ mol^{-}

[1]).[34] These results agree with the electrochemical and theoretical data and highlight the ease with which OxiBeet can be oxidized.

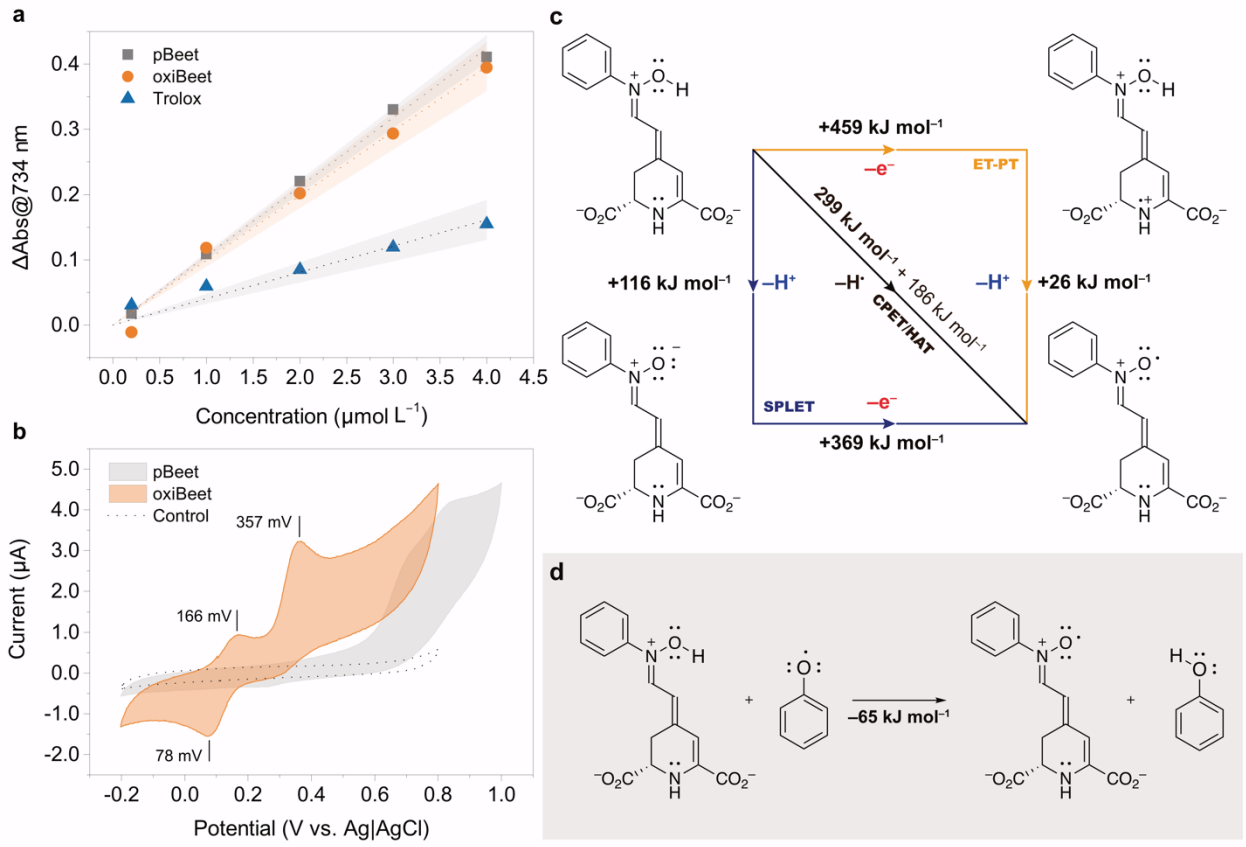

Figure 3. Antioxidant capacity and redox profile of OxiBeet. (a) Change in absorption (ΔAbs) of $ABTS^{+\bullet}$ at 734 nm after 6 min of reaction caused by different concentrations of OxiBeet, pBeet, and Trolox in phosphate buffer (pH 7.4, 50 mmol L^{-1}). (b) Cyclic voltammograms of OxiBeet and pBeet in 0.1 mol L^{-1} KCl and negative control (dotted line, 0.1 mol L^{-1} $KCl_{(aq)}$). Glassy carbon electrode, Ag|AgCl (KCl sat.), scan rate: 50 mV s^{-1}, [betalain] = 88 mmol L^{-1}. (c) More O'Ferrall-Jencks diagrams for the ionization and $1e^-$-oxidation of OxiBeet. Compounds are presented as dicarboxylates since this is the expected

major form in water at pH higher than 4.[33] Energies refer to the enthalpy changes between states; the value of 186 kJ mol^{-1} in the concerted pathway is required for the $1e^-$-oxidation of $H^\bullet$ to produce H^+ and e^-. (d) Isodesmic reaction between the phenoxyl radical and OxiBeet to produce phenol and the $OxiBeet^{+\bullet}$.

A solution of OxiBeet in phosphate buffer at pH 7.4 was saturated with molecular oxygen for 1 min at room temperature to promote its autoxidation. The EPR spectrum of the product shows a 14 lines pattern, indicating a delocalized NO radical with the following hyperfine coupling constants (hfcc, in Gauss): $a_\alpha^N = 11.9$, $a_\beta^H = 6.3$, $a_\gamma^H = 3.3$ and $a_\gamma^{2H} = 2.9$ (Fig. 4a). This result is compatible with the delocalized nitroxide 1,7-diazaheptamethinyl radical cation of OxiBeet, as supported by EPR simulation. Control experiments using pBeet did not show an EPR response. The value of k_{obs} for the formation of $OxiBeet^{+\bullet}$ was $(6.5 \pm 1.4) \times 10^{-4}$ s^{-1} (half-life ($\tau_{½}$) = 18 min, excess O_2, pseudo-first order conditions), while its decomposition rate constant was much lower ($k_{obs} = (8.8 \pm 2.7) \times 10^{-5}$ s^{-1}, lifetime (τ) = 2.5 h, $\tau_{½}$ = 106 min) (Figs. 4b and S9). For comparison, the $\tau_{½}$ of the superoxide adducts of the widely used *N*-oxide spin traps DMPO, EMPO and CDMPO are 0.9, 9.9 and 27.5 min, respectively.[40]

The positive spin density of $OxiBeet^{+\bullet}$ is localized on the N1, C5, C7 and N9 atoms (Fig. 4c), providing additional support to the assigned structure. The partial positive charges at the N1 and N9 atoms increase upon $1e^-$-oxidation of OxiBeet, as expected considering charge delocalization arguments. Although studies on the reaction between betanin and radicals were performed using EPR spectroscopy,[41] this report shows for the first time that the 1,7-diazaheptamethinium scaffold increases the stability of nitroxide radicals. Last, these

findings highlight the importance of the 1,7-diazaheptamethinium system of betalains for their striking antioxidant properties.[20, 34]

Ultrafast transient absorption experiments were carried out to determine whether photoexcitation plays a role in the autoxidation of OxiBeet. In aerated water, the electronic excitation of OxiBeet at 485 nm results in ground state bleaching (Fig. 4d). The transient spectra revealed a positive absorption band at 450 nm, which was ascribed to the excited state absorption ($S_1 \rightarrow S_n$; $n > 1$), and a negative band at about 520 nm that was attributed to S_0 depletion. The broad negative absorption band centered at approximately 625 nm was attributed to the $S_1 \rightarrow S_0$ stimulated emission, which was visible after 800 fs and ceased to exist after 10 ps. In fact, full OxiBeet ground state recovery occurs in less than 100 ps after photoexcitation, indicating that $S_1 \rightarrow T_1$ intersystem crossing is precluded and photoproducts were not formed in noticeable amounts, as observed for betanin[42].

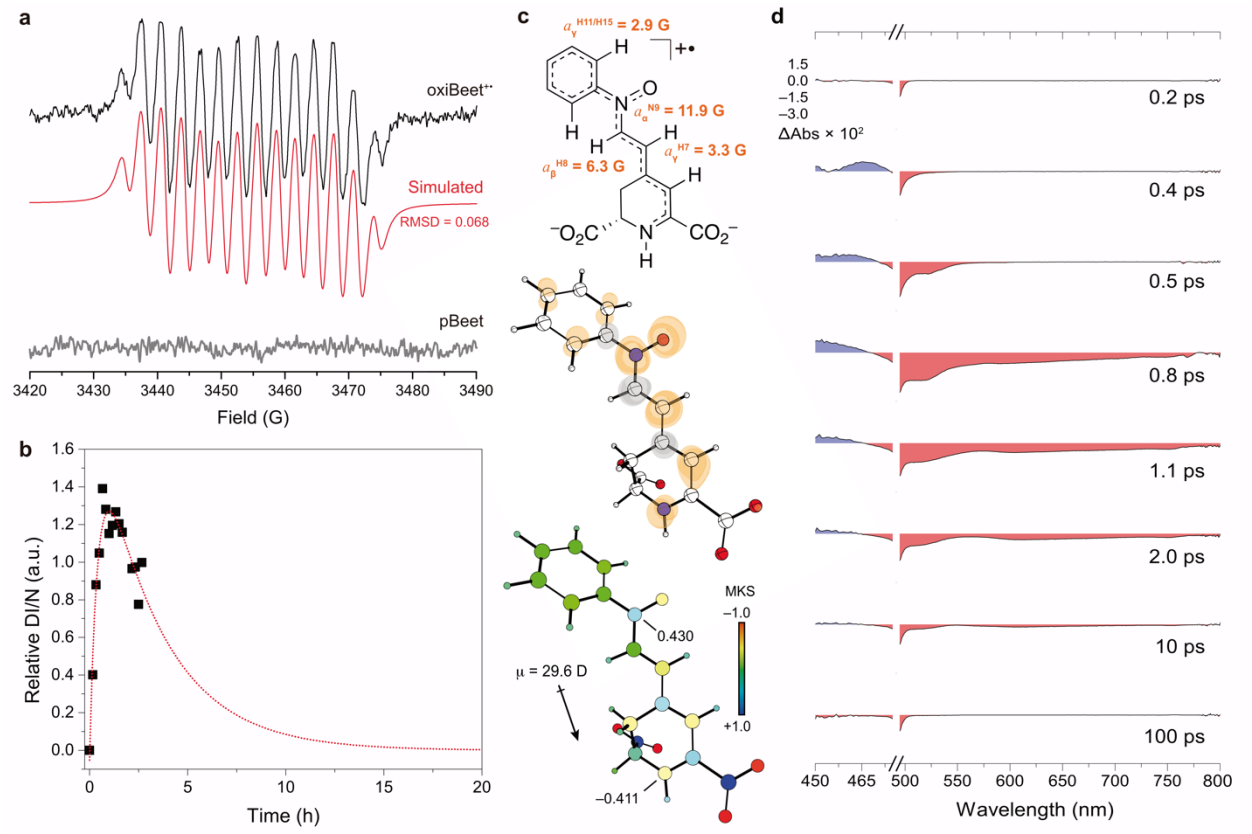

Figure 4. Electronic spin structure of OxiBeet$^{+\bullet}$ and photophysical data. (a) Observed (black) and simulated (red) hyperfine EPR spectra of OxiBeet$^{+\bullet}$ in phosphate buffer, pH 7.4 at 298 K. The lack of response of pBeet is shown for comparison. The color of the solution remained unchanged during the experiment. (b) Kinetics of formation and disappearance of OxiBeet$^{+\bullet}$; the dotted red line represents nonlinear fitting of the data to a biexponential function. (c) Spin density distribution and partial charges of the nitrogen atoms of OxiBeet$^{+\bullet}$; red and blue indicate positive and negative spin densities, respectively. The values of the hfccs of OxiBeet$^{+\bullet}$ calculated from the simulation are presented in orange. (d) Transient absorption spectra of OxiBeet in water; pump wavelength: 485 nm.

Conclusions

The repertoire of multifunctional nitrones has been expanded by the design and synthesis of a prototypical betalain-nitrone based on the *N*-oxide 1,7-diazaheptamethinium scaffold. OxiBeet is based on a chiral starting material obtained from renewable sources and can be conveniently synthesized and purified in water. This pseudo-natural compound is a potent antioxidant that is not cytotoxic within the concentration range typically used in biological assays. Moreover, it is oxidized via concerted PCTE and is stable towards hydrolysis under neutral and alkaline aqueous conditions, which is a clear advantage over its betalain counterparts. The formation of the resonance-stabilized cation radical of OxiBeet upon autoxidation enables the development of antioxidants that can inhibit oxygen-induced changes in industrial products and demonstrates the importance of the 1,7-diazaheptamethinium system for the antioxidant properties of betalains in general. These findings indicate that OxiBeet and its analogue betalain-nitrones can be potentially used as biocompatible antioxidants, redox mediators and spin traps, opening new perspectives for the development of therapeutics for diseases associated with oxidative stress.

Author contribution

E.L.B. conceived the study. E.L.B. and A.C.P wrote the paper. E.L.B., A.C.P., R.B.F., L.C.E., C.O.M., F.A.D., E.P., Y.H., J.S. and A.M.C.F. designed the experiments. A.C.P., R.B.F., L.C.E., C.O.M., F.A.D., and Y.H. performed the experimental work. E.L.B. carried out theoretical calculations. All authors discussed and interpreted the results.

Conflicts of interest

There are no conflicts to declare.

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