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Kinetics of DPPH[•] scavenging by bacterioruberin from *Haloferax alexandrinus* GUSF-1 (KF796625)

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Abstract

This is the first account of the kinetics of free radical scavenging by bacterioruberin obtained from cells of *Haloferax alexandrinus* GUSF-1 (KF796625), grown at optimum conditions of 25% NaCl, pH 7, 42 °C, 150 rpm in NaCl Tryptone yeast extract medium and light. Bacterioruberin separated from methanolic extract displayed characteristics absorption peaks at 368, 386, 463, 492 and 525 nm and gave an m/z value of 740.4 ($C_{50}H_{76}O_4$) in Liquid Chromatography-Mass Spectroscopy validating its purity. Bacterioruberin (13 μ M) decolorized and decayed 0.2 mM 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH[•]) monitored at 517 nm and reached a steady state within 30 min. An EC_{50} of $6.50 \mu\text{M} \pm 0.27$ ($4.81 \mu\text{g/mL} \pm 0.2$) was deduced for the 0.2 mM DPPH[•]-bacterioruberin reaction using the GraphPad Prism 9 statistical software and employing the right-angled triangle technique. The study also revealed a comprehensive information of the total kinetic activity of bacterioruberin with DPPH[•]: the antioxidant activity index was 16.38 ± 0.67 ; time needed to reach the steady state with the added EC_{50} —30 min; the antiradical power 30.77 ± 1.27 and the antiradical efficiency of $54.7 \times 10^{-3} \pm 2.24$, thus reflecting the strong antioxidant nature of bacterioruberin. Scavenging of DPPH[•] by bacterioruberin was a pseudo-first-order reaction with a rate constant k_2 of $2.76 \times 10^{-5} \pm 0.001 \mu\text{M}^{-1} \text{s}^{-1}$ calculated at $t=0$ or initial time and $t=30$ min. The knowledge of the kinetics of bacterioruberin to scavenge DPPH[•] adds to its effective application as an antioxidant in medicinal use, pharmaceutical products and others. Additionally, the use of simple conventional method of DPPH[•] free radical scavenging, monitored using an easily available laboratory spectrophotometer, will certainly help in the effective use of any antioxidant compound.

Keywords: Bacterioruberin, DPPH[•], Free radical scavenging, Kinetics, *Haloferax alexandrinus* GUSF-1

Introduction

Oxidative stress in a biological system is the inability of the body to eliminate the free radical reactive species through the use of endogenous antioxidants (Yusuff et al. 2019). Natural antioxidants are gaining importance as nutraceuticals, dietary supplements (Guerin et al. 2003) and as therapeutic medicines (Firuzi et al. 2011). They are preferred over synthetic antioxidants because of their

inability to accumulate in the human body and cause harm (Deng et al. 2011).

Antioxidants relieve oxidative stress via: hydrogen atom transfer (HAT), proton coupled electron transfer (PCET), single electron transfer followed by proton transfer (SET-PT), sequential proton loss electron transfer (SPLET), radical adduct formation (RAF) and sequential proton loss through hydrogen atom transfer (SPLHAT) (Marković 2016).

Free radical scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH[•]) at fixed endpoint has been widely used to evaluate the antioxidant properties of natural compounds capable of scavenging free radicals (Brand-Williams et al.

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1995) and is preferred since it simulates reactive oxygen and nitrogen-free radical species, which affects the biological systems (Arnao 2000). The use of DPPH[•] provides an easy and rapid method to evaluate the potential of an antioxidant molecule scavenging free radicals which can be measured as a change in optical density/absorbance and referred to as the strength of the antioxidant. (Brand-Williams et al. 1995).

Knowledge of kinetic parameters such as antiradical efficiency and time required to reach steady-state provides additional information about antioxidant behavior. Hence, they are more informative than mere total antioxidant capacity determinations at fixed endpoint even if carried out by different methods (Sánchez-Moreno et al. 1998).

Bacterioruberin of C₅₀ carotenoid is reported in members from different genera of haloarchaea such as *Halo-terrigena*, *Haloarcula*, *Halobacterium*, *Halococcus*, *Halogeometricum*, *Haladaptatus*, *Haloplanus*, *Halope-lagius* and *Halogramum* (Squillaci et al. 2017; Yatsunami et al. 2014; Abbes et al. 2013; Mandelli et al. 2012; Hou and Cui 2018); in *Haloferax volcanii* (Ronnekleiv and Liaaen-Jensen 1995; Hou and Cui 2018), in genetically modified *Haloferax volcanii* strain (HVLON3) by Zalazar et al. (2019), *Haloferax alexandrinus* strain TM^T (Asker et al. 2002) and in *Haloferax alexandrinus* GUSF-1 (KF796625) (Alvares and Furtado 2021), respectively.

Bacterioruberin, of *Halobacterium salinarium*, is reported to protect cells in vitro against oxidative damage of UV light and hydrogen peroxide (Shahmohammadi et al. 1998); of *Haloferax alexandrinus* GUSF-1 (KF796625) to scavenge free radicals (Alvares and Furtado 2018) and of genetically modified *Haloferax volcanii* strain (HVLON3) in scavenging free radicals and enhancing ram sperm velocity (Zalazar et al. 2019).

Recently, through separation and chemical profiling of the hexanolic cell extracts of *Haloferax alexandrinus* GUSF-1 (KF796625), the antioxidant property of bacterioruberin and other C₃₀, C₄₀, and C₅₀ isoprenoids has been confirmed (Alvares and Furtado 2021). Although the authors Zalazar et al. 2019 investigated the antioxidant activity of bacterioruberin obtained from genetically modified *Haloferax volcanii* strain (HVLON3) and arrived at EC₅₀ value using the decolorization of DPPH[•] monitored through electron paramagnetic resonance analysis, they did not investigate other kinetic parameters or delve into the kinetics of the DPPH[•]-bacterioruberin interaction.

Therefore, the present study used bacterioruberin from cells of *Haloferax alexandrinus* GUSF-1 (KF796625), studied the kinetic parameters and behavior of this bacterioruberin to scavenge the 1,1-diphenyl-2-picrylhydrazyl (DPPH[•]) free radicals using the conventional

spectrophotometric method which is simple, reproducible, reliable, can be carried out routinely and does not require sophisticated instrumentation.

Experimental

Chemicals and reagents

Analytical grade chemicals and reagents from Himedia laboratories-India, 1,1-diphenyl-2-picrylhydrazyl (DPPH[•]) from Sigma, USA and silica gel GF254 plates from Merck, Darmstadt, Germany, were used for thin-layer chromatography (TLC). A 0.2 mM solution of (DPPH[•]) was prepared in methanol (97% purity) and used in the study, further dilutions were done with methanol, as and when required.

Standard curve of DPPH[•] solution

The linearity range of the DPPH[•] solution was determined. Varying concentrations of DPPH[•] (0- 0.2 mM) were prepared from 0.2 mM stock solution in methanol and incubated at room temperature (28 °C) in the dark for 30 min. A linear regression was obtained from the curve by plotting the concentrations v/s absorbance obtained at 517 nm (Additional file 1: Fig. S1).

Culturing of *Haloferax alexandrinus* GUSF-1 (KF796625) for bacterioruberin

Haloferax alexandrinus GUSF-1 with accession number KF796625, an isolate from a salt pan (Sequeira 1992), was cultured in Tryptone Yeast Extract containing 25% (w/v) NaCl (NTYE) (Raghavan and Furtado 2004) and as indicated in results, at different concentrations of NaCl, temperatures, pH and in the presence of light. Culture broth was withdrawn daily and cells separated out using the centrifuge (Biofuge-Heraeus Stratos; 8000 rpm, 4 °C, 10 min) were washed with 15% (w/v) NaCl. Growth was monitored as dry weight of washed cells. The cells were then extracted in methanol and clear methanolic extracts were obtained on centrifugation. The culture was also grown under conditions yielding optimum growth, and 100 mg of wet washed cells was used to prepare the methanolic extract which was stored at -20 °C till further use. All experiments were carried out in triplicates.

Estimation of antioxidant potential of methanolic extracts

The methanolic extract was checked for DPPH[•] free radical scavenging, according to Alvares and Furtado (2021). Here, methanolic extract was mixed with 1 ml of 0.2 mM methanolic DPPH[•] (1:2 v/v) and incubated in the dark (28 °C) for 30 min; thereafter, the absorbance was read at 517 nm. The antioxidant capacity, expressed as the % DPPH[•] radical scavenging activity (%DPPH[•] RSA), was calculated using the following equation:

$$\% \text{DPPH}^+ \text{RSA} = \frac{A_B - A_S}{A_B} \times 100 \quad (1)$$

where A_B is the absorbance of 1 mL of 0.2 mM DPPH⁺ + 0.5 mL of methanol and A_S is the absorbance of 1 mL of 0.2 mM DPPH⁺ + 0.5 mL of methanolic extract after incubation for 30 min.

Preparation of bacterioruberin

The wet pellet obtained by growing culture under physicochemical parameters yielding maximum antioxidant was treated with methanol, and the cell debris were then separated by centrifuging to obtain a clear cell-free methanolic extract of cells. Components from this were then fractionated with distilled hexane and distilled water according to the method of Asker et al. (2002). The pigment was recovered in the hexane layer, washed several times with distilled water and dried over anhydrous sodium sulfate and other impurities were removed using acetone precipitation. The resulting pigmented residue was re-dissolved in hexane. The orange-colored hexanolic extract, containing bacterioruberin, was purified by preparative TLC using a developing system of methanol-chloroform (7: 93, v/v) in minimum light (Asker et al. 2002). Bacterioruberin was checked for purity by scanning between 300 and 800 nm using the UV-Vis dual-beam spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) in quartz cuvettes of 1 mL volume and 1 cm path length and methanol as the reference solution. The identity and purity were further re-confirmed and validated using Applied Biosystems API 2000 Liquid Chromatography–Mass Spectroscopy (LC–MS) system with Ion spray Voltage (IS) – 45,000 V; Ion source gas (GS1) – 20.0 psi, 1 ppm lowest sensitivity and analyst software Q1/MS (A1).

Free radical scavenging activity of bacterioruberin by steady-state measurement

Bacterioruberin in methanol corresponding to 13 μM and 1 mL of 0.2 mM DPPH⁺ reagent was mixed in the spectrophotometric quartz cuvette and incubated in the dark at 28 °C. The decrease in absorbance of DPPH⁺ radical was spectrophotometrically monitored at 517 nm, at initial 1 min and then every 5 min (1–35 min) until the reaction reached a plateau/steady state. The reaction mixture was also scanned at appropriate intervals using the UV-Probe 2.42 software. The percentage of DPPH⁺ remaining at different intervals was determined according to Mishra et al. 2012 by using Eq. (2)

$$\% \text{DPPH}^+ \text{ Remaining} = \frac{A_f}{A_0} \times 100 \quad (2)$$

where A_0 is the absorbance of mixture at 517 nm containing 1 mL of 0.2 mM DPPH⁺ + 0.5 mL of methanol and A_f is the absorbance of the reaction mixture at 517 nm containing 1 mL of DPPH⁺ + 0.5 mL of bacterioruberin.

Potency and kinetics of DPPH⁺-bacterioruberin interaction

(i) The effective concentration (EC₅₀)

GraphPad Prism 9 (San Diego, CA) statistical program with the built-in equation for nonlinear regression, i.e. Asymmetric (five-parameter, 5P) was the statistical model used to derive the plot of scavenging of 0.2 mM DPPH⁺ by bacterioruberin by plotting log concentration of bacterioruberin on the X-axis and % DPPH⁺RSA on the Y-axis (Chen et al. 2013). The EC₅₀ was then calculated from this log concentration–response curve by using a mathematical method based upon the principle of a right-angled triangle (Alexander et al. 1999). The theoretical value was calculated by using the concentration–effect curve shown in Fig. 4a, wherein the maximum response chosen is a true representation of the E_{\max} and by selecting the two concentrations corresponding to two recorded points on either side of the 50% maximal response. From the raw data/curve, the values of A, B, C, and D are known, and the 50% maximal response is calculated from minimum (baseline) and maximum responses selected from the raw data.

The EC₅₀ was derived according to Alexander et al. 1999, using Eq. (3) and compared with EC₅₀ of beta-carotene;

$$\text{EC}_{50} = D - \frac{(A - 50\% \text{ maximal response}) \cdot x}{y} \quad (3)$$

Further, the time needed to reach the steady state (TEC₅₀) at which no further scavenging of DPPH⁺ takes place at the added EC₅₀ concentration of bacterioruberin was also calculated graphically from the plot of % DPPH⁺ remaining v/s time.

(ii) **Antioxidant activity index (AAI)** (Scherer and Godoy 2009)

Furthermore, the antioxidant activity index (AAI) was calculated as:

$$\text{AAI} = \frac{\text{Final concentration of DPPH}^+ (\mu\text{g/mL})}{\text{EC}_{50} (\mu\text{g/mL})} \quad (4)$$

(iii) **Antiradical efficiency (ARE)** (Sánchez-Moreno et al. 1998)

Moreover, the antiradical efficiency (ARE) of bacterioruberin was deduced from EC_{50} value converted to $\mu\text{g/mL}$ and expressed as $\text{g BR/kg DPPH}^{\bullet}$ and TEC_{50} at 30 min, as under:

$$\text{ARE} = \frac{1}{EC_{50}} \times \frac{1}{TEC_{50}} \quad (5)$$

(iv) Antiradical power (ARP) (Mishra et al. 2012)

Additionally, antiradical power (ARP), the antioxidant action was calculated as reciprocal of ratio of EC_{50} ($\mu\text{moles}/\mu\text{mole}$ of DPPH^{\bullet})

$$\text{ARP} = \frac{1}{EC_{50}} \quad (6)$$

(v) Reaction stoichiometry (Brand-Williams et al. 1995)

This was expressed as the amount of EC_{50} ratio ($\mu\text{moles}/\mu\text{mole}$ of DPPH^{\bullet}) of bacterioruberin required to reduce 100% DPPH^{\bullet} radicals and was deduced and calculated from EC_{50} value $\times 2$. From this, the number of DPPH^{\bullet} molecules reduced by one mole of bacterioruberin was further calculated thereof.

(vi) Rate constant (Mishra et al. 2012).

The formation of DPPH-H on addition of different μM concentrations of bacterioruberin to fixed 0.2 mM DPPH^{\bullet} was checked, and the rate constant was calculated from the slope of this plot. All reactions were carried out as five independent experiments and expressed as a mean \pm standard deviation ($n=5$) derived using Microsoft Excel 2019.

Results and discussion

Large scale preparation and separation of bacterioruberin

Earlier, Alvares and Furtado (2018) demonstrated the decolorization of the deep purple color of DPPH^{\bullet} by colonies of *Haloferax alexandrinus* GUSF-1 and also of their methanolic extracts as a consequence of free radicals scavenging activity. In a recent report, Alvares and Furtado (2021) attributed this antioxidant activity to the several compounds present in the cells of *Haloferax alexandrinus* GUSF-1 among which, the C_{50} isoprenoid bacterioruberin was most dominant.

As seen in Fig. 1a–d, maximum antioxidant produced by *Haloferax alexandrinus* GUSF-1 grown, separately at 150 rpm for over a period of 6 days in TYE medium with

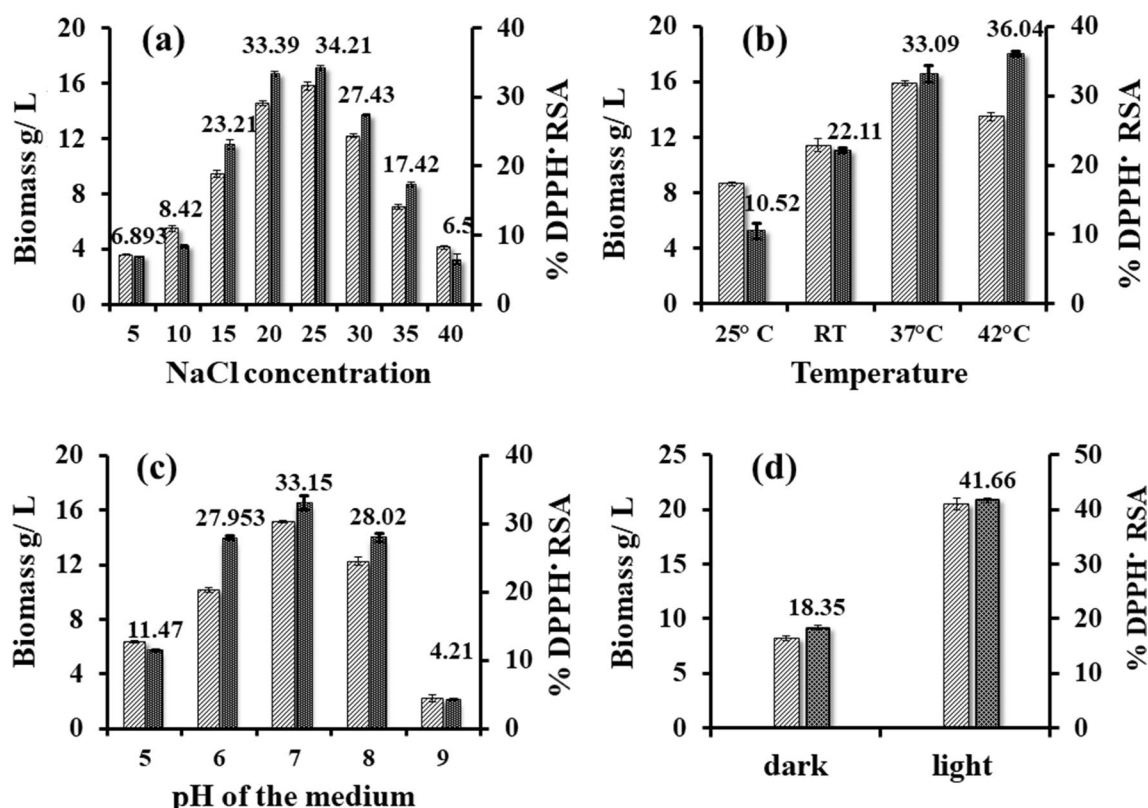


Fig. 1 Biomass and free radical scavenging activity % DPPH^{\bullet} RSA in cells extracts of *Haloferax alexandrinus* GUSF-1 (KF796625) grown in different **a** NaCl concentration, **b** temperature of incubation, **c** pH of growth medium, **d** growth under light and dark conditions. Each value is mean of three replicates \pm SD, ($n=3$)

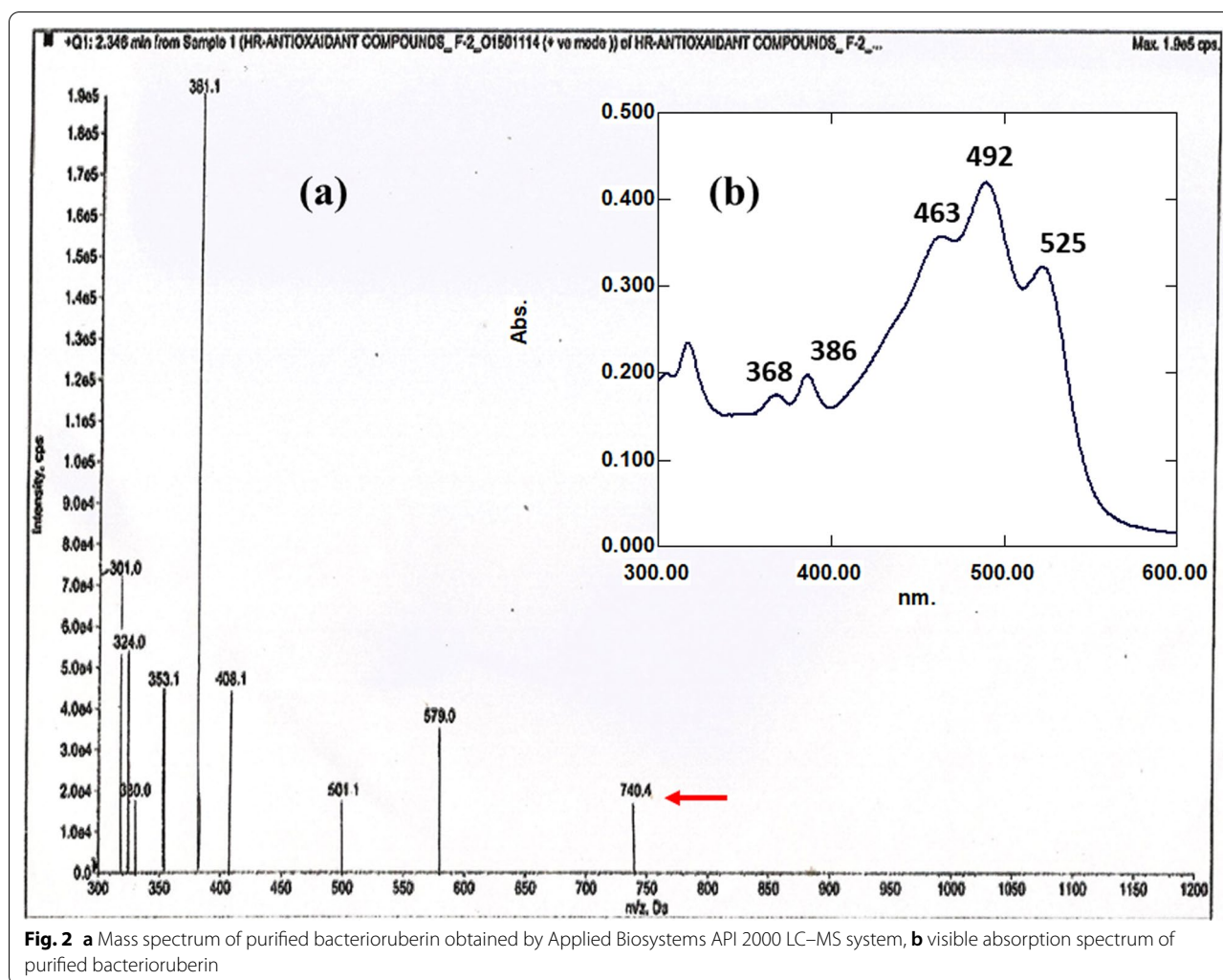


Fig. 2 **a** Mass spectrum of purified bacterioruberin obtained by Applied Biosystems API 2000 LC–MS system, **b** visible absorption spectrum of purified bacterioruberin

different concentrations of NaCl, temperature, pH, light and dark gave a maximum of 34.21% by cells grown in 25% NaCl, 36.04% at 42°C, 33.15% in pH 7 and 41.66% by cells grown in the presence of light and amount of 25 µg of bacterioruberin was easily separated at R_f 0.24 and purified by preparative thin-layer chromatography (PTLC). The recovered bacterioruberin in methanol exhibited characteristic spectral peaks at 368, 386, 463, 492 and 525 nm; and the m/z 740.4 ($C_{50}H_{76}O_4$) of the molecular ion (M^+) detected in LC–MS (Fig. 2a, b) validated its purity. These features were consistent with those reported by Asker et al. (2002) and Alvares and Furtado (2021).

Free radical scavenging activity of bacterioruberin

The two characteristics absorption peaks of 0.2 mM DPPH $^{\bullet}$ in methanol were observed at 517 nm and 325 nm, while purified bacterioruberin displayed peaks at 368, 386, 463, 492 and 525 nm. Spectrophotometric

monitoring was employed to decipher the interaction of bacterioruberin with DPPH $^{\bullet}$ at 517 nm. The addition of bacterioruberin in methanol with one of the absorption peaks at 525 nm when in contact with the chromogen DPPH $^{\bullet}$ in a 1:2 ratio instantly reduced the absorption peak of DPPH $^{\bullet}$ at 517 nm as displayed in Fig. 3a(i–v). Further the peaks at 463, 492 and 525 nm corresponding to bacterioruberin were completely abolished thus indicating that the interaction of bacterioruberin had no interference in the measurement of DPPH $^{\bullet}$ at 517 nm. The incremental decrease of the DPPH $^{\bullet}$ peak at 517 nm on the addition of bacterioruberin reflected receipt of H^+ by DPPH $^{\bullet}$ from the added bacterioruberin and highlighted the free radical scavenging property of bacterioruberin carried out using similar method of DPPH $^{\bullet}$, as reported by Biswas et al. (2016), Squillaci et al. (2017) and Hou and Chi (2018). The extent of change in color corresponded to the H^+ received from the donor bacterioruberin molecule.

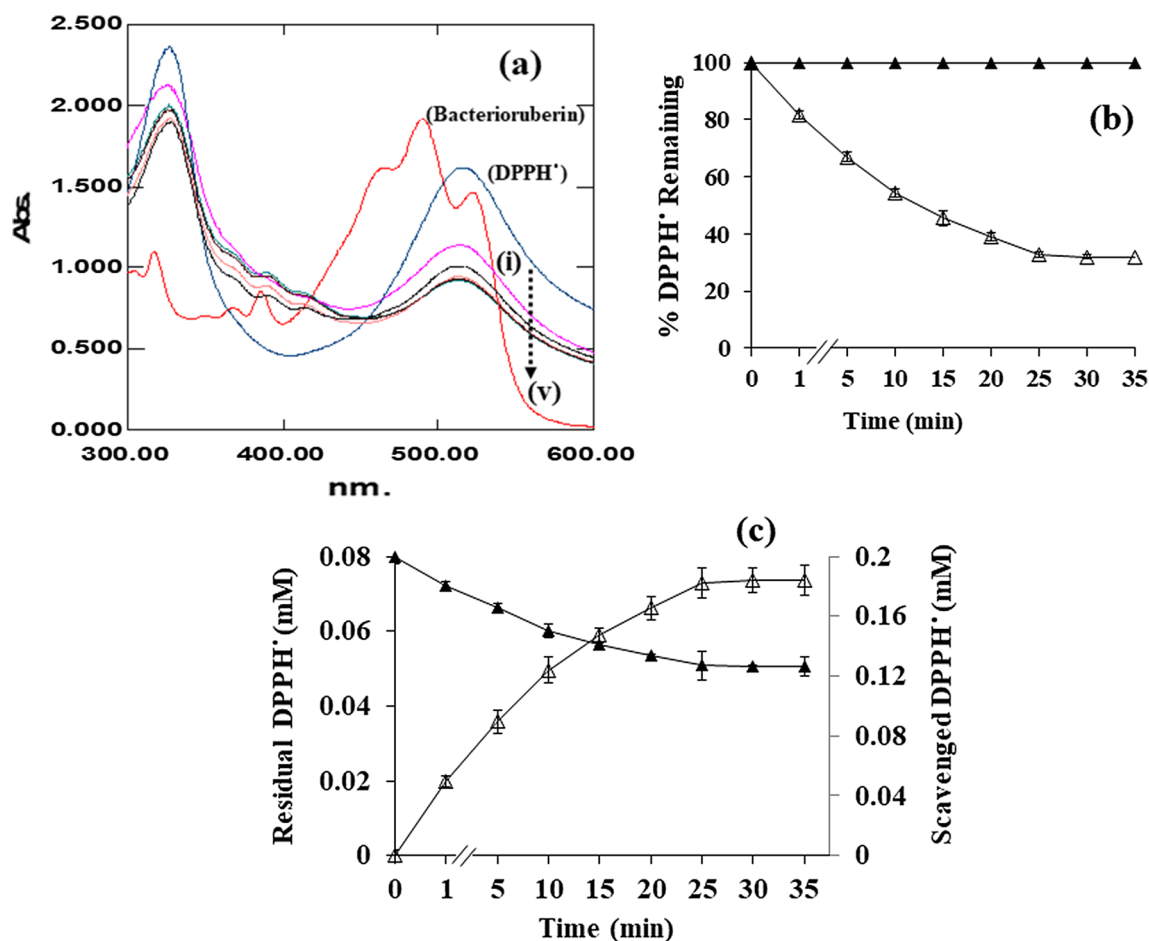


Fig. 3 **a** Visible absorption spectrum of 13 μM bacterioruberin in methanol, 0.2 mM methanolic DPPH \cdot and (i–iv) bacterioruberin–DPPH \cdot interaction; **b** 0.2 mM DPPH \cdot remains steady at 517 nm in the absence of bacterioruberin (filled triangle) and 0.2 mM DPPH \cdot in the presence of 13 μM of bacterioruberin at 517 nm (open triangle); **c** residual concentration of DPPH \cdot (mM) monitored at A_{517} in the presence of bacterioruberin: residual DPPH \cdot (filled triangle) and scavenged DPPH \cdot (open triangle). Data are expressed as mean \pm SD of three independent experiments

Free radical scavenging activity of bacterioruberin was monitored as a function of time. The addition of 13 μM of bacterioruberin to 0.2 mM DPPH \cdot decreased its absorbance at 517 nm, in the dark at 28 $^{\circ}\text{C}$. In contrast, the absorbance of control DPPH \cdot at 517 nm remained unchanged for over 30 min (Fig. 3b) and indicated that it did not undergo auto-oxidation nor was affected by the environment in which the reaction was carried out. The purple color of DPPH \cdot changed to yellow with time, as DPPH \cdot received an electron or a H^{+} from bacterioruberin, the donor molecule. The simultaneous decrease in % DPPH \cdot remaining was noted; 81% on addition of bacterioruberin (13 μM /0.013 mM) within 1 min, 66% at 5 min, 54% at 10 min, 45% at 15 min, 38% at 20 min, 32% at 25 min and remained steady at 31% from 30 min onwards (Fig. 3b).

Further, as seen in Fig. 3c, the DPPH \cdot was converted to DPPH-H molecule inversely proportional to the decay of radical till steady state. At 30 min, an amount of 0.073 mM DPPH \cdot was converted to DPPH-H.

The subsequent slower rate pointed to the role of slow secondary reactions, possibly involving dimerization of phenol-derived radicals. The dependence of absorbance of $A_{\text{B}}(517 \text{ nm})$ and $A_{\text{S}}(517 \text{ nm})$ ($t=x$) is therefore exponential and a power function, obtained by nonlinear regression analysis. The reaction occurs via the HAT radical mechanism wherein H^{+} is donated by bacterioruberin as in $\text{ArOH} + \text{DPPH}\cdot = \text{ArO}^{-} + \text{DPPH-H}$ and is possibly similar to the reactivity of the antioxidant present in *Corchorus olitorius* (*C. olitorius*) and *Vernonia amygdalina* (*V. amygdalina*) towards DPPH \cdot (Yusuff et al. 2019).

Table 1 Evaluation of EC₅₀ from bacterioruberin-DPPH[•] interaction

Log (M) [Bacterioruberin]	% DPPH [•] RSA					Mean	SD
	Experiment No						
	1	2	3	4	5		
− 6.091	6.5*	7.1*	6.98*	7.2*	6.68*	6.89	0.29
− 5.966	9.8	9.2	9.21	9.5	9.7	9.48	0.27
− 5.869	13.65	14.02	14.61	14.8	13.76	14.16	0.51
− 5.568	25.47	23.44	24.32	24.8	23.2	24.24	0.94
− 5.267	36.48	38.98	37.65	38.8	36.6	37.70	1.17
− 5.091	42.7	43.21	44.52	42.8	44.6	43.56	0.92
− 4.966	57.7	59.8	60	58.6	60.2	59.26	1.07
− 4.869	66.78	68.9	70	68.4	72.2	69.25	2.01
− 4.693	75.29	75	79	78.2	76.4	76.77	1.76
− 4.568	80.77	81.77	81.23	82.4	80.6	81.35	0.74
− 4.471	93.82	94.22*	93.21	93.2	94.6*	93.81	0.61
− 4.392	93.87	92.11	93.22*	92.4	93.6	93.04	0.75
− 4.267	93.87*	93.12	92.23	93.4*	92.8	93.08	0.61
Log EC ₅₀	− 5.15	− 5.18	− 5.19	− 5.20	− 5.20	− 5.18	0.01
EC ₅₀ [M]	6.93 × 10 ^{−6}	6.60 × 10 ^{−6}	6.45 × 10 ^{−6}	6.26 × 10 ^{−6}	6.28 × 10 ^{−6}	6.50 × 10 ^{−6}	0.27
EC ₅₀ [μM]	6.93	6.60	6.45	6.26	6.28	6.50	0.27

*Minimum and maximum responses used for computing the 50% maximal response

The % DPPH[•]RSA values are obtained from five independent experiments and results expressed as mean ± SD

The Log [M] EC₅₀ values for bacterioruberin is computed from Fig. 4a, b

Free radical scavenging kinetics at different concentrations of bacterioruberin

Data at column 1 under experiment no in Table 1 was used to construct a concentration-effect curve (Fig. 4a) using the GraphPad Prism 9 statistical model with the built-in equation; Asymmetric (five-parameter, 5P), as also employed by Chen et al. (2013). From the minimum and maximum response, the amount required to scavenge 50% of the original concentration or 50% maximal response was calculated. The 50% maximal response was interpolated to the x-axis. Also, A and B were selected on the y-axis as two closest points on either side of the 50% maximal response from the raw data. These points were then interpolated as D and C values on the x-axis.

From Fig. 4a, b and Table 1, the following values were obtained:

100% response calculated from the baseline (minimum) and maximum data was 87.37% and hence the 50% maximal response was fixed at 43.68%; A = 58.33%; B = 36.67%; C = – 5.25 log [M]; D = – 4.97 log [M].

Equation (3) was applied to experimental data from column 1 of Table 1, and the following values were calculated:

Response interval (y) between responses A and B = 21.66%; the interval between 50% maximal response and the next highest concentration (y') = 14.65%; x = 5.25 – 4.97 = 0.28

$$EC_{50} = D - \frac{(A - 50\% \text{ max response}) \cdot x}{y}$$

$$EC_{50} = -4.97 - \frac{(58.33 - 43.68) \cdot 0.28}{21.66}$$

$$EC_{50} = -4.97 - 0.189 \log \text{ moles}$$

$$EC_{50} = -5.15 \log \text{ moles}$$

The effectiveness of EC₅₀ of bacterioruberin in scavenging 50% of 0.2 mM DPPH[•] free radicals, calculated using the right-angled triangle method of Alexander et al. 1999, was therefore taken as 6.50 μM ± 0.27 (4.81 μg/mL ± 0.2) (Fig. 4a; Table 1) and that reported for beta-carotene was 18 μM ± 0.2 (10 μg/mL ± 0.2) which was three times lower than bacterioruberin. This is a very simple, precise and rapid method for the calculation of the EC₅₀, (Ralevic et al. 1995) and does not require expensive computational aids, thus making the technique particularly useful for laboratory calculations (Alexander et al. 1999).

A number of reports on EC₅₀ of bacterioruberin from haloarchaea are available in literature, but they do not match the concentration of 0.2 mM DPPH[•] which is employed in this study even though the fixed time of 30 min was used. However, from reported EC₅₀ values in μg/mL, we equated the EC₅₀ required to scavenge 0.2 mM DPPH[•] so as to compare the results obtained in this study. Extract from the cells of *Haloterrigena turkmenica*

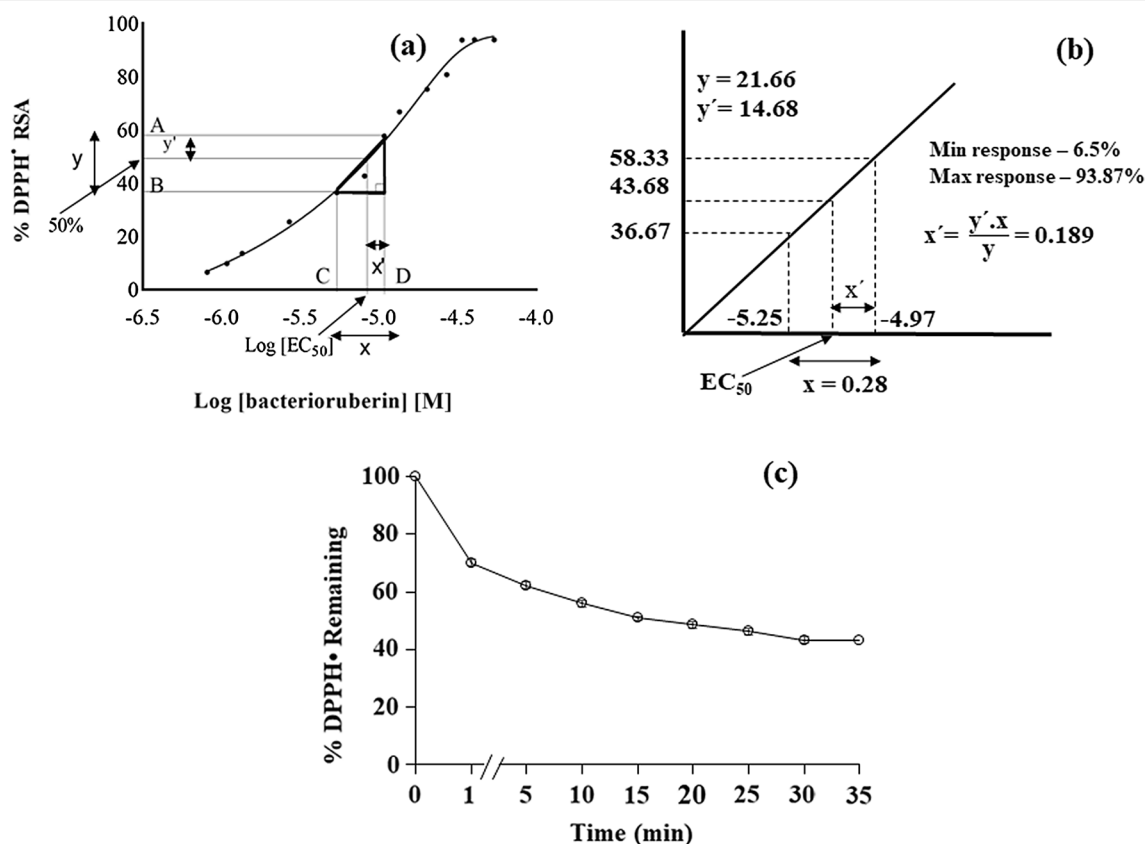


Fig. 4 **a** The concentration–response curve of bacterioruberin obtained GraphPad Prism 9 (San Diego, CA) statistical program with the built-in equation for nonlinear regression, i.e. Asymmetric (five-parameter, 5P) statistical model. The nearest recorded responses (A and B) of each experimental concentration (D and C), nearly equidistant on either side of the EC₅₀ and forming a right-angled triangle as in method of Alexander et al. (1999); **b** graphic magnification of right-angled triangle of data from experiment No. 1 (Table 1) for calculation of the EC₅₀ of bacterioruberin using the right-angled triangle method; **c** time to reach steady state TEC₅₀ with the EC₅₀ concentration of bacterioruberin. Data are expressed as mean \pm SD of five independent experiments

with C₅₀ bacterioruberin (Squillaci et al. 2017) displayed an IC₅₀ value of 8.98 μ g/mL. Hou and Chi (2018), found that 2.22 μ g/mL of bacterioruberin from *Haloferax volcanii* and 160 μ g/mL of bacterioruberin also obtained from *Haloferax* sp. BKW301 (Biswas et al. 2016) was required to scavenge 50% of 0.2 mM DPPH•. Comparing the EC₅₀ value of bacterioruberin obtained in this study, the order of effectiveness of bacterioruberin was from *Haloferax volcanii* > *Haloferax alexandrinus* GUSF-1 > *Haloterrigena turkmenica*. In another study involving the antioxidant activity of bacterioruberin rich extracts, which was assessed using DPPH• assay combined with electron paramagnetic resonance analysis, it was reported that 33.3 μ g/mL scavenged 50% of 0.2 mM of DPPH• (Zalazar et al. 2019), eight times than 4.81 μ g/mL of bacterioruberin from this study.

Figure 4c depicts the DPPH• decolorization the EC₅₀ (6.50 μ M/4.81 μ g/mL) of bacterioruberin, which progressed steadily at 517 nm, till it reached a steady-state

saturation i.e., maximum decrease in DPPH• in 30 min; therefore, the TEC₅₀ for bacterioruberin was fixed at 30 min.

The antiradical efficiency is the reflection of a combination of kinetic and static approaches to characterize the antioxidant efficiency of a molecule (Huang et al. 2005). The antiradical efficiency of bacterioruberin deduced according to Sánchez-Moreno et al. 1998 (EC₅₀ calculated as: g Bacterioruberin/Kg DPPH• = 61.04 ± 2.58) was $54.7 \times 10^{-3} \pm 2.24$, which with TEC₅₀ of 30 min, fitted with an intermediate decay similar to that reported for gallic acid, tannic acid and α -tocopherol and showed a very high efficiency as characterized by Sánchez-Moreno et al. 1998.

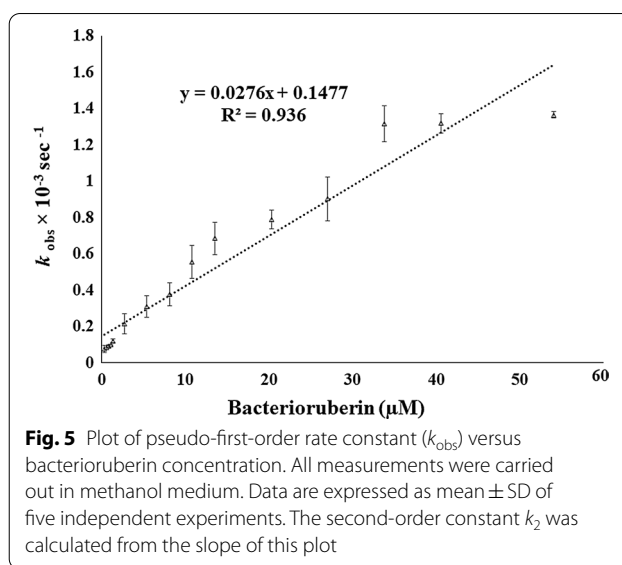
Bacterioruberin had an antiradical power (ARP) of 30.77 ± 1.27 calculated from the EC₅₀ (μ moles of bacterioruberin/ μ mole of DPPH• = 0.03 ± 0.017) using the equation according to Mishra et al. 2012, higher than that reported for all compounds tested in their study. Further

the stoichiometry deduced was found to be 0.06 ± 0.002 and reduced 15.38 ± 0.63 molecules of DPPH \cdot . It is pertinent to note that the stoichiometry and number of reduced DPPH \cdot for bacterioruberin have not been reported earlier.

Additionally, the antioxidant activity index (AAI) for bacterioruberin was calculated from C_f/EC_{50} , (taken as $\mu\text{g/mL}$) as 16.38 ± 0.67 , which is >2 , hence a strong antioxidant as per Scherer and Godoy (2009) antioxidant categories. Furthermore, comparing the AAI of bacterioruberin with AAI values of different standard antioxidants given by Scherer and Godoy 2009, the antioxidant activity index would be between protocatechuic acid $20.17 >$ bacterioruberin (this study) $16.38 >$ quercetin 15.92 .

Many attempts to explain the structure–activity relationship of some polyphenols are reported in the literature. It is known that the monophenols are less efficient than the polyphenols and that the number of hydroxyl groups is an important factor that enhances activity (Cuvelier et al. 1992; Shahidi et al. 1992; Salah et al. 1995). The accessibility of the radical center of DPPH \cdot to each polyphenol also contributes to the antioxidant power obtained (Yoshida et al. 1989). Another important parameter of antioxidant action is the stoichiometry of reactants which is the amount of antioxidant required theoretically to reduce 100% of DPPH \cdot radicals. Bacterioruberin has an extensive system of 13 conjugated double bond, two acyclic ϕ end groups, four OH at positions C-1, C-1', C-3'', C-3'''. The length, conjugated double bonds and functional groups of a molecule are reported to contribute to the antioxidant capacity (Mandelli et al. 2012) and this explains the lower EC_{50} values and corresponding kinetic parameters.

The conversion of DPPH \cdot to DPPH-H was carried out at fixed 0.2 mM concentration of DPPH \cdot and different concentrations of bacterioruberin, and hence represented by the equation $\ln[\text{DPPH}\cdot]_t = \ln[\text{DPPH}\cdot]_0 - k_{\text{obs}}t$, where $[\text{DPPH}\cdot]_{t=0}$ is the concentration of radical at the $t=0$; $[\text{DPPH}\cdot]_{t=30}$ is the concentration of the radical at steady time $t=30$, k_{obs} is therefore the pseudo-first-order rate constant obtained for the fixed reaction time of 30 min. The second-order rate constant k_2 deduced from slope of the plot of k_{obs} v/s concentration of DPPH \cdot (Fig. 5) was $2.76 \times 10^{-5} \pm 0.001 \mu\text{M}^{-1} \text{s}^{-1}$ for the bacterioruberin-DPPH \cdot interaction at a steady state of 30 min. In the absence of studies reporting rate constant for bacterioruberin, the rate constant value of $2.76 \times 10^{-5} \pm 0.001 \mu\text{M}^{-1} \text{s}^{-1}$ obtained in this study was found to be greater than that of curcumin ($2 \times 10^{-5} \pm 0.12 \mu\text{M}^{-1} \text{s}^{-1}$) but lower than the value reported for gallic acid $4 \times 10^{-5} \pm 0.4 \mu\text{M}^{-1} \text{s}^{-1}$ by Mishra et al. 2012,



although both of these molecules being unrelated to bacterioruberin in molecular weight.

Expression of results in terms of the kinetic approach does not take into account only the activity of an antioxidant but also provides information on how quickly the antioxidant acts (Squillaci et al. 2017). Hence, it is pertinent to note that results of antioxidant activity based on kinetic data and on the measurement at a fixed endpoint should be combined so as to provide comprehensive information of the total antioxidant activity of a compound. This is the first study reporting the kinetics of free radical scavenging by bacterioruberin from a genetically unmodified *Haloferax alexandrinus* GUSF-1 investigated by the simple, conventional colorimetric assay for DPPH \cdot chromogen and monitored using an easily available spectrophotometer and highlighting its unique antioxidant potential.

Conclusions

In summary, this study on the kinetic behavior of antioxidant, bacterioruberin, from cells of *Haloferax alexandrinus* GUSF-1(KF796625) on decay of 0.2 mM DPPH \cdot enabled us to fix EC_{50} at $6.50 \mu\text{M} \pm 0.27$ ($4.81 \mu\text{g/mL} \pm 0.2$) and TEC_{50} of 30 min and also the other kinetic parameters such as AAI at 16.38 ± 0.67 , ARP at 30.77 ± 1.27 and the ARE of bacterioruberin was $54.7 \times 10^{-3} \pm 2.24$, showed a stoichiometric value of 0.06 ± 0.002 and reduced 15.38 ± 0.63 molecules of DPPH \cdot . These kinetic parameters are not yet reported for bacterioruberin interacting with DPPH \cdot . Further, the scavenging reaction of DPPH \cdot by bacterioruberin was pseudo-first order with a second-order rate constant

k_2 $2.76 \times 10^{-5} \pm 0.001 \mu\text{M}^{-1} \text{s}^{-1}$ fixed at a fixed time of 30 min.

Additionally, the production of C_{50} carotenoid bacterioruberin by cells of *Haloferax alexandrinus* GUSF-1(KF796625) is economical, easy to extract, purify and is expected to be helpful for use in the food production industry, antioxidant supplements and cosmetics. Besides, the application of the simple conventional method of DPPH[•] free radical scavenging, monitored using easily available instruments and methodology will certainly help in the effective use of any antioxidant compound.

Abbreviations

DPPH[•]: 1,1-Diphenyl-2-picrylhydrazyl; TLC: Thin-layer chromatography; NTYE: Tryptone Yeast Extract containing 25% (w/v) NaCl; % DPPH[•]RSA: % 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity; LC-MS: Liquid Chromatography–Mass Spectroscopy; EC_{50} : Effective concentration; TEC_{50} : Time required to attain the steady state with EC_{50} concentration of bacterioruberin; ARP: Antiradical power; ARE: Antiradical efficiency; AAI: Antioxidant activity index.

Supplementary Information

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Additional file 1: Fig. 1. Standard curve of DPPH[•].

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Authors' contributions

JJA planned and carried out the experiments. IJF and JJA inferred and analyzed the observations. IJF designed the manuscript. Both authors read and approved the final manuscript.

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Availability of data and materials

Haloferax alexandrinus GUSF-1 (GenBank accession number KF796625). Gen bank: <http://www.ncbi.nlm.nih.gov>.

Declarations

Competing interests

The authors declare that they have no competing interests.

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