Useful $^1O_2$ ($^1\Delta_g$) generator, 3-(4′-methyl-1′-naphthyl)-propionic acid, 1′,4′-endoperoxide (NEPO), for dioxygenation of squalene (a skin surface lipid) in an organic solvent and bacterial killing in aqueous medium

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Abstract 3-(4′-Methyl-1′-naphthyl)-propionic acid, 1′,4′-endoperoxide (NEPO) provides singlet state of oxygen ($^1O_2$, $^1\Delta_g$) at 37°C in sodium phosphate buffer (pH 7.2), acetate buffer (pH 4.5), methanol or chloroform, through the retro-Diels-Alder reaction. The total amount of $^1O_2$ generated by NEPO was calculated using the following equation: $[^1O_2]=[NEPO]_0[1-\exp^{-kt}]$, where $[^1O_2]$, $[NEPO]_0$ and k are the total amount of $^1O_2$ produced during the time t, initial concentration of NEPO and the first-order reaction rate constant, respectively. When squalene was exposed to $^1O_2$ which was generated thermolytically from NEPO, it was oxidized to three hydroperoxides, mono-, di- and tri-hydroperoxides, in amounts proportional to the dose of NEPO. The oxidizability of squalene was much more extensive compared with unsaturated phospholipids. Additionally, when wild-type *E. coli* and lycopene-producing mutant *E. coli* were exposed to NEPO-derived $^1O_2$, there was significant loss of viability of wild-type *E. coli* but no significant loss of viability in lycopene-producing strain, suggesting that lycopene by scavenging $^1O_2$ protected *E. coli* against $^1O_2$ toxicity.

Key words: Singlet oxygen; 3-(4′-Methyl-1′-naphthyl)-propionic acid, 1′,4′-endoperoxide (NEPO); Squalene hydroperoxide; Bactericidal activity

1. Introduction

In recent years, considerable interest has been focused on the reactions of $^1O_2$ ($^1\Delta_g$) with organic compounds and its toxicity towards living cells. Known $^1O_2$ generating systems, such as NaOCl-H_2O_2 [1], myeloperoxidase-H_2O_2-halide ions [2] and light-photosensitizer-O_2 [3] systems contain reactive oxygen species other than $^1O_2$ and often generate free radicals, and therefore such systems cannot serve as pure $^1O_2$ generating reactions.

In the light of the aforementioned understanding, Saito et al. [4] have synthesized a novel water-soluble naphthalene endoperoxide, 3-(4′-methyl-1′-naphthyl)-propionic acid 1′,4′-endoperoxide (NEPO), which produces $^1O_2$ thermolytically, not photochemically. However, little is known about the kinetic aspects of NEPO decomposition to produce $^1O_2$ in organic solvents or in aqueous media. The present work was undertaken to understand the stoichiometry of thermolysis of NEPO together with quantification of $^1O_2$ during incubation of NEPO in an organic or aqueous solvent. On the basis of the stoichiometric data obtained, reactivity of $^1O_2$ towards lipids in organic solvents, its toxicity on two *E. coli* strains and on human endothelial cells in aqueous media were examined with NEPO as a pure $^1O_2$ yielding compound.

Additionally, the substance squalene (SQ) with six double bonds is a major component of human skin epidermal lipid [5] and probably the first lipid target in human skin for oxidative stress, for example, during exposure to sunlight [6–8]. Kohno and colleagues [6] were the first to report the presence of squalene hydroperoxide in human skin surface lipids and demonstrated the production of squalene hydroperoxides by dye-sensitized photooxygenation of SQ [7]. Therefore, it is logical to assume that SQ in epidermis is oxygenated by $^1O_2$ produced by photosensitizers (Type II reaction) [9] in the skin. However, other oxidative processes which utilize reactive oxygen species other than $^1O_2$ and sensitizer radicals generated by type I reactions [9] may also participate in the oxidation of SQ. Thus, it is important to know the reactivity of $^1O_2$ towards SQ. NEPO was used as a pure $^1O_2$ generator to examine the products of reaction of $^1O_2$ with other unsaturated lipids.

As a model for studying the action of NEPO in a biological system, Nagano et al. [10] examined the toxicity of $^1O_2$ towards *E. coli*, taking inhibition of cell growth as a measure of toxicity. In this study, we have attempted to examine the protective effect of lycopene (an $^1O_2$ scavenger) on $^1O_2$-mediated bacterial killing, using wild-type *E. coli* and a lycopene-producing mutant *E. coli*.

2. Materials and methods

2.1. Thermolysis of NEPO in aqueous or organic solvents

NEPO, 50–100 μM in a 3-ml cell, which could be shielded to avoid evaporation of the solvent was incubated at 37°C and the absorbance

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Abbreviations: HUVEC, human umbilical vein endothelial cells; LDH, lactate dehydrogenase; NEPO, 3-(4′-methyl-1′-naphthyl)-propionic acid, 1′,4′-endoperoxide; NPA, 3-(4′-methyl-1′-naphthyl)-propionic acid; PAPC, 1-palmitoyl-2-arachidonoyl-phosphatidylcholine; PAPC-OOH, 1-palmitoyl-2-arachidonoyl-phosphatidylcholine hydroperoxide; PBS, phosphate-buffered saline without Ca^{2+} and Mg^{2+}; PLPC, 1-palmitoyl-2-linoleoyl-phosphatidylcholine; PLPC-OOH, 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxide; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; POPC-OOH, 1-palmitoyl-2-oleoyl-phosphatidylcholine hydroperoxide; PC-OOH, phosphatidylcholine hydroperoxide; SQ, squalene; SQ-OOH, SQ hydroperoxide; $^1O_2$, singlet oxygen

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Table 1
Data for thermolysis of NEPO

Solvent	k (10^{-4} s^{-1}) (s^{-1})	ε at 288 nm (M^{-1} cm^{-1})	Half life of NEPO (min)	Time for A_{max} measured (h)
Sodium phosphate (0.1 M, pH 7.2)	4.16	7000	27.8	4
Actate buffer (0.1 M, pH 4.5)	4.65	6890	24.8	4
Methanol	1.64	7100	70.4	8
Chloroform	1.55	7240	74.5	8

of 3-(4′-methyl-1′-naphthyl)-propionic acid (NPA) at 288 nm was continuously recorded during the time quoted. The solvents used were 0.1 M sodium phosphate buffer (pH 7.2), 0.1 M acetate buffer (pH 4.5), chloroform, or methanol.

2.2. Dioxygenation of lipids in organic solvents

1-Palmitoyl-2-oleoyl-phosphatidylcholine (POPC), 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC), 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (PAPC), microperoxidase-11, and isoluminol were obtained from Sigma (St. Louis, MO, USA). SQ was purchased from Tokyo Kasei (Tokyo, Japan). All solvents were of the highest grade commercially available. POPC, PLPC, PAPC, and SQ were used after purification using HPLC equipped with a UV detector monitored at 215 nm using CAPCELPAK C18 column (20×250 mm, 5 μm; Shiseido, Tokyo, Japan) and methanol containing 0.02% triethylamine (methanol for purification of SQ) as an eluent (flow rate: 10.0 ml/min). The concentrations of POPC, PLPC, and PAPC were determined using a Phospholipid B test (Wako, Osaka, Japan) which is a choline-containing phospholipid-specific analytical kit. The concentration of SQ was determined by weighing the dried sample and from its molecular weight. POPC, PLPC, and PAPC were dissolved in methanol. SQ was dissolved in chloroform. They were stored at −80°C prior to use.

Lipid samples were dissolved in chloroform or methanol and then the solvent was removed under reduced pressure. The residue obtained was redissolved in the reaction solvent to obtain an adequate concentration of lipid. One mM lipid was incubated with or without 1, 2, or 3 mM NEPO at 37°C under aerobic conditions. The concentration of lipid hydroperoxide, the primary oxidation product of lipid, was measured by using a hydroperoxide-specific, isoluminol chemiluminescence assay [11]. Formation of phosphatidylcholine hydroperoxide (PC-OOH) was monitored by HPLC equipped with a silica gel column (4.6×250 mm, 5 μm; Supelco) using methanol/40 mM monobasic sodium phosphate (9:1, v/v) as the mobile phase (flow rate: 1.0 ml/min) and 1 mM isoluminol, 5 mg/l microperoxidase-11 in methanol/0.1 M borate buffer, pH 10 (1:1, v/v) as the chemiluminescence reagent (flow rate: 1.5 ml/min). Formation of SQ hydroperoxide (SQ-OOH) was monitored by HPLC equipped with a CAPCELPAK C18 column (4.6×250 mm, 5 μm; Shiseido) using methanol as the mobile phase (2.0 ml/min) and 1 mM isoluminol, 5 mg/ml microperoxidase-11 in methanol/0.1 M borate buffer, pH 10 (1:1, v/v) as the chemiluminescence reagent (1.5 ml/min). 50 μl of reaction mixture was injected onto the HPLC. Results are shown as mean ± standard deviation of three independent experiments and were analyzed statistically by Bonferroni comparisons.

2.3. Cytotoxicity studies on human umbilical vein endothelial cells

Deep-frozen normal human umbilical vein endothelial cells (HUVEC) were purchased from Kurabo (Osaka, Japan) and subcultured according to the protocol of Kurabo Cell Culture Kit when subconfluent monolayers were reached. The medium, reagents and buffer used were of Kurabo's Cell Culture Kit. The frozen sample of HUVEC in a test tube was thawed in 50% ethanol at 37°C and was immediately suspended in HuMedia-EG2 culture medium. The inoculated viable cell number was determined using trypan-blue exclusion test [12]. The cells were incubated at 37°C in 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY, USA) in a humidified atmosphere containing 5% CO_2 in air. At subconfluent monolayer stage, the cells were harvested according to Kurabo's Cell Culture Kit protocol. The harvested cells were resuspended in HuMedia-EG2 medium at approximately 1×10^5 cells/ml. Then 250-μl aliquots of cell suspensions were transferred into the wells of a flat-bottom 24-well microtiter tissue culture plate (Corning) and incubated for 24 h at 37°C under 5% CO_2 in air. These cell plates were used for cytotoxicity assay of $^1O_2$. Cytotoxicity of $^1O_2$ on HUVEC was measured by the lactate dehydrogenase (LDH) release assay together with determining the decrease in the number of viable cells after exposure to $^1O_2$. After 24 h incubation, the culture medium was removed by a pipette, attached cells were washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) at pH 7.3 and then 180 μl of PBS was added to each well. 20 μl of 1 mM NEPO buffer solution was added to each well and incubated at 37°C. After the desired time period, the cell-free upper layer (buffer) was carefuly removed from each well. LDH released in the buffer was measured using an LDH-Cytotoxic Test Kit (Wako). The viability of cells in each well was also determined by trypan-blue exclusion method [12]. To determine maximum and negative control cell membrane damage, cells were incubated with 0.2% Tween 20 (Sigma) in PBS or in PBS alone.

2.4. Determination of viability of E. coli strains in aqueous medium

E. coli mutants carrying the lycopene producing genes (pACCCRT-EIB, Misawa et al. [13]) were used to determine the bactericidal effect of NEPO. The amount of lycopene in lycopene-producing *E. coli* was estimated to be about 0.02 fmol/cell. Wild-type and lycopene-producing *E. coli* were cultured in LB medium [14] containing ampicillin (150 μg/ml) and/or chloramphenicol (30 μg/ml) at 27°C with shaking (80 shakes/min) in test tubes. Growth was monitored by measuring the optical density of the test at 600 nm. Cells at early stationary phase were harvested, washed with and suspended in the minimal medium [15] without Vitamin B12. The cell suspensions were dispensed in polypropylene vials (∅ 17×51 mm) and mixed with the $^1O_2$ generating medium. The reaction mixture contained 10^8 cells/ml, 0.1 mM NEPO and 0.1 M acetate buffer, pH 4.5 or phosphate buffer, pH 7.4 in a total volume of 2 ml. The reaction was initiated by the addition of 200 μl of NEPO in water at 25°C and maintained at 37°C. After the specified time period, samples were taken, washed and the viable cells were counted by spreading in triplicate on LB agar plate after appropriate dilutions. Colonies were counted after a 24-h aerobic incubation at 37°C.

3. Results

3.1. Stoichiometry and kinetics of thermolysis of NEPO

NEPO provides $^1O_2$ ($^1\Delta_g$) quantitatively at 37°C in sodium phosphate buffer (pH 7.2), acetate buffer (pH 4.5), methanol or chloroform, through the retro-Diels-Alder reaction shown in Fig. 1; the time course of NPA production in acetate buffer is shown in Fig. 2. It can be seen that the peak absorbance of NPA at 288 nm at time 0 is not zero. Since NEPO has no absorption band at 288 nm, the non-zero-intercept (A_0) is attributable to NPA formation during preparation of the sol-

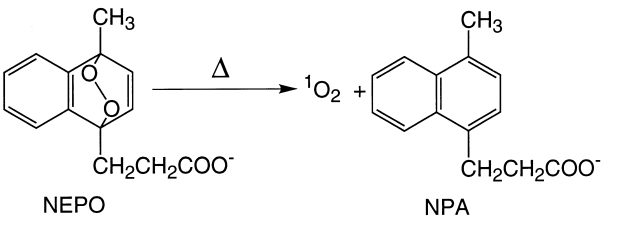

Fig. 1. Decomposition of NEPO to yield $^1O_2$ and NPA.

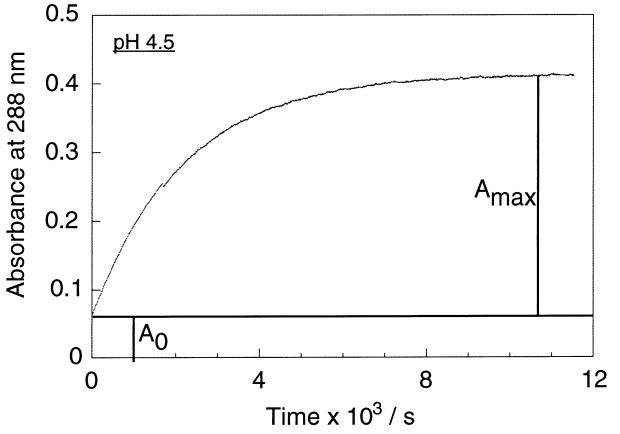

Fig. 2. Time course of NPA formation from NEPO at pH 4.5. About 50 μM NEPO in 3 ml of 0.1 M acetate buffer at pH 4.5 was incubated at 37°C and absorbance of NPA at 288 nm was followed during the time cited. A_0 and A_{max} represent initial and maximum absorbance at 288 nm.

ution and the thermoequilibrium in the cell. The experimental parameters fit well with a first order kinetic equation, consistent with those reported by Saito et al. [4]. The $^1O_2$ flux from NEPO and the total amount of $^1O_2$ derived from NEPO during the time t (in seconds) were calculated from Eqs. 1 and 2, respectively.

$$v = k[\mathrm{NEPO}]_0 \ [\mathrm{M/s}] \quad (1)$$

$$[^1O_2] = [\mathrm{NEPO}]_0\{1-\exp^{-kt}\} \ [\mathrm{M}] \quad (2)$$

The initial concentration of NEPO, $[\mathrm{NEPO}]_0$, can be calculated from A_{max} and A_0 in Fig. 2 using Eq. 3

$$[\mathrm{NEPO}]_0 =$$

$$(A_{max}-A_0)/\text{molar extinction coefficient } (\varepsilon) \text{ of NPA } [\mathrm{M}] \quad (3)$$

where A_{max} and A_0 are absorbance (A_{288nm}) at 4 h or 8 h and 0 time, respectively. The parameters obtained in the present study are shown in Table 1 and can be used in Eqs. 1–3. The yield of $^1O_2$ in the thermal decomposition of NEPO at 37°C in water at pH 7.2 was found to be 98 ± 3% [16]. Other investigators have reported a maximum yield of 80% $^1O_2$ after thermal decomposition of NEPO [17].

3.2. Dioxygenation of lipids in organic solvents

Fig. 3 shows representative chromatograms of SQ analyses. One mM SQ was incubated with 3 mM NEPO at 37°C in chloroform under aerobic conditions. Immediately after NEPO addition, no hydroperoxide formation was observed. However, three peaks were obtained after 90 min oxidation of SQ using NEPO. These peaks correspond to mono-hydroperoxide, di-hydroperoxide and tri-hydroperoxide [7]. Since there is no conjugated diene in SQ, these hydroperoxides should have been formed via $^1O_2$-mediated oxidation (ene reaction) of SQ. In the present work, only mono-SQ-OOH was taken as the major dioxygenated product of SQ. When lipids were oxidized in chloroform instead of methanol, comparable results were obtained (Table 2); i.e. the number of ene in lipids influences their oxidizability by $^1O_2$ (POPC < PAPC ≪ SQ). However, the amount of lipid hydroperoxide formed is much higher in chloroform than in methanol. This may be due to the fact that the lifetime of $^1O_2$ is longer in chloroform (60 μs) than in methanol (5–11.4 μs) [18].

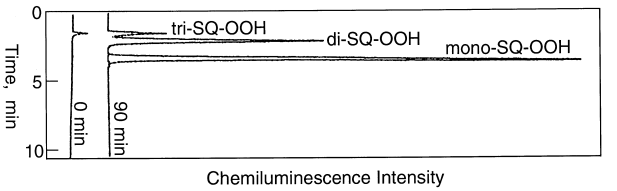

Fig. 3. Representative chromatograms of three squalene hydroperoxides. One mM squalene was incubated with 3 mM NEPO in chloroform at 37°C for 0 min and 90 min under aerobic conditions.

Of all lipids used in this study, SQ was the most susceptible to oxidation by $^1O_2$. However, the oxidizability of SQ cannot be explained only by the number of double bonds in the molecule. The oxidizability of SQ by $^1O_2$ may be attributable to the small ionization potential of SQ [19] which is a measure of electron donating capacity.

The results obtained in this study suggest that in the skin $^1O_2$ generated by sunlight and a photosensitizer can be trapped by SQ in the epidermis, thereby protecting the skin against $^1O_2$ toxicity.

3.3. Protective effect of lycopene against E. coli killing by $^1O_2$

When wild-type *E. coli* was exposed to 7 μM $^1O_2$ derived from NEPO for 5 min at 37°C, 34% of bacteria were killed (Fig. 4). Under identical conditions, only 3% of lycopene-producing *E. coli* were killed, suggesting that lycopene by scavenging $^1O_2$ protected *E. coli* against $^1O_2$ toxicity. Viability of *E. coli* at pH 4.5 was essentially the same as at pH 7.3. However, under our experimental conditions at pH 7.3 no toxic effect by $^1O_2$ on HUVEC was observed (results not shown). When wild-type *E. coli* were exposed to 1 mM NEPO for

Table 2
Formation of dioxygenated product from lipids exposed to $^1O_2$ in methanol or chloroform

Reaction solvent	Total amount of $^1O_2$ formed for 30 min (μM)	Hydroperoxide formed (μM)			
		POPC-OOH	PLPC-OOH	PAPC-OOH	SQ-OOH
Methanol	256	0.018 ± 0.003	0.031 ± 0.004	0.121 ± 0.046	1.727 ± 0.237*,**,***
Chloroform	243	0.561 ± 0.174	1.780 ± 0.876	5.083 ± 2.348	20.200 ± 6.851!,!!,!!!

One mM lipid and 1 mM NEPO were used. Results are shown as mean ± standard deviation ($n = 3$).
In methanol: *$P < 0.001$, significant difference against POPC-OOH; **$P < 0.001$, significant difference against PLPC-OOH; ***$P < 0.001$, significant difference against PAPC-OOH.
In chloroform: !$P < 0.005$, significant difference against POPC-OOH; !!$P < 0.005$, significant difference against PLPC-OOH; !!!$P < 0.05$, significant difference against PAPC-OOH.

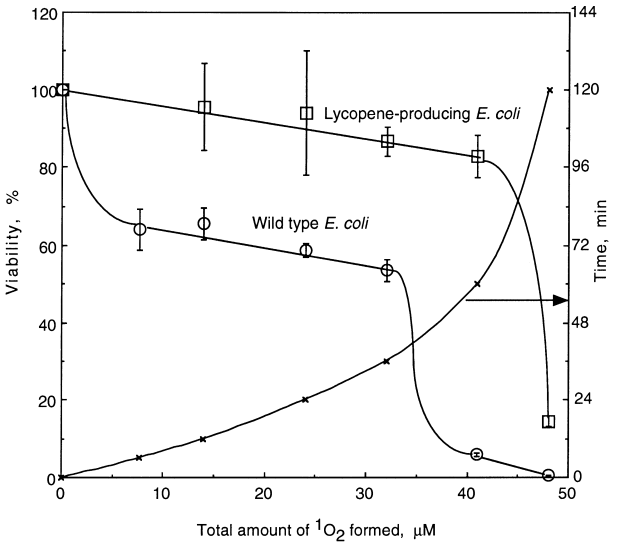

Fig. 4. Viability of wild-type *E. coli* or a lycopene-producing mutant *E. coli* strain exposed to $^1O_2$ during the time indicated. The final pH of the incubation mixture was 4.5. Data are presented as the mean ± S.E.M. ($n = 12$). The statistical analysis was calculated by Bonferroni comparisons. $P < 0.05$ was considered significant.

3 min at pH 4.5 and at a given temperature, the *E. coli* viability at 25°C and 37°C were found to be 97 ± 5.4% (mean ± S.E.M., $n = 6$) and 53 ± 2.6%, respectively. The total amount of $^1O_2$ generated by thermal decomposition of NEPO for 3 min at 25°C was negligible, but was 10 μM at 37°C. Further, exposure of wild-type *E. coli* to 0.1 mM (or 1 mM) NPA for 12 or 60 min, instead of NEPO, had no effect on viability. These observations suggest that bacterial killing is via $^1O_2$ and not by a direct action of NEPO. We have recently found that electron transport systems located on the *E. coli* surface membrane are very vulnerable to short lived $^1O_2$ (unpublished data). Based on these understandings, we believe that the resistance of HUVEC to $^1O_2$ toxicity under our experimental conditions may reflect the absence of oxidatively vulnerable electron transport systems in the HUVEC plasma membrane.

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