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Singlet Oxygen Quenching by Deoxygadusol and Related Mycosporine-Like Amino Acids from Phytoplankton *Prorocentrum micans*

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Deoxygadusol (DG) and structurally related mycosporine-like amino acids, i.e. mycosporine glycine (MG) and mycosporine taurine (MT), were isolated from phytoplankton *Prorocentrum micans* and studied for the reactivity toward singlet oxygen. These water-soluble compounds with a cyclohexenone chromophore were all shown to be highly effective in quenching singlet oxygen ($^1O_2$), with the efficiencies being significantly larger compared with histidine, a well-known $^1O_2$ quencher. The $^1O_2$ reaction rate constant (k_Q) of DG was determined to be $5.4 \times 10^7 M^{-1}s^{-1}$ by a steady state method based on competitive inhibition of rubrene oxidation. The feasibility of this method was confirmed by estimating the k_Q values for MG and two other quenchers, furfuryl alcohol and 1,4-diazabicyclo [2,2,2]octane, and comparing with those values determined by the time-resolved $^1O_2$ decay method in the previous work.

key words: singlet oxygen ($^1O_2$) quenching, $^1O_2$ reaction rate constant, photooxidation, mycosporine-like amino acid, deoxygadusol

INTRODUCTION

Mycosporine-like amino acids (MAA) are found in all of the major divisions of marine algae including cyanobacteria as secondary metabolites. These water-soluble compounds are also found in tissues of marine arthropods, invertebrates and fishes that feed on algae or contain dinoflagellates and cyanobacteria as symbionts [1]. Despite wide distribution and abundance across marine organisms, their physiological function remains still unclear. Because MAA strongly absorb UV, numbers of papers have concerned their function as a UV screen to protect the organisms against sunlight damage [2-5]. Some MAA have been shown to inhibit lipid peroxidation induced by radicals [1, 6], implying that they may act as radical-scavenging antioxidants in cells. Previously, we have approached to the problem related to the primary role of MAA from a different viewpoint. That is, MAA could be an active defense against photooxidative sunlight effects as a singlet oxygen ($^1O_2$) quencher rather than, or in addition to, a passive role as a simple UV screen [7, 8].

About twenty MAA including biosynthetically related deoxygadusol have thus far been identified in marine organisms [1]. Although most of them have in common a cyclohexenimine chromophore and show the absorption peaks at wavelengths ranging from 320 to 360 nm, several members, such as mycosporine glycine (MG), mycosporine taurine (MT), and deoxygadusol (DG), have a cyclohexenone moiety (Fig. 1) and exhibit the maximum absorption at rather short wavelengths, such as 310, 309 and 268 nm, respectively. In this work, we found that dinoflagellate *Prorocentrum micans* contained these three cyclohexenone-type MAA together with five other MAA that are believed to be of cyclohexenimine type and that the former are all highly active in scavenging $^1O_2$ while the latter are either far less effective or virtually ineffective. The $^1O_2$-quenching rate constants were measured for the MAA of cyclohexenone type.

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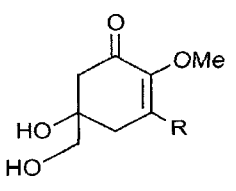
MATERIALS AND METHODS

MAA preparation

Harvested cells of *P. micans* were obtained from National Fisheries Research and Development, Korea. MAA were prepared from the algal cells following essentially the same procedures as described earlier for MAA preparation from ascidian *Lissolinum patella* [7]. The procedures comprised four major steps, i.e. homogenization and extraction in 80% aqueous methanol, removal of lipids and pigments by washing with chloroform, chromatographic separation of total MAA in a column packed with Bakers Bakerbond Amino Sorbent in methanol, and reverse-phase HPLC in a YMC-Pack ODS-A



Deoxygadusol, (R – OH)
Mycosporine-glycine, (R – CH_2COOH)
Mycosporine-taurine, (R = $CH_2CH_2SO_3H$)

Figure 1. Deoxygadusol and structurally related MAA.

column for isolation of MAA components. For MG, MT and DG preparations, further purification was done by use of a Dowex 50W (H^+ form) column (30 × 5 mm id) with water as the eluent. Molecular identities of the MAA of cyclohexenone type from *P. micans* were confirmed by spectral (UV, ESI-MS and NMR) data.

$^1O_2$ assay by electron spin resonance

$^1O_2$ produced by a photosensitizer eosin Y (EY) was trapped with 2,2,6,6-tetramethyl-4-piperidone (TMPD), and the resulting formation of TMPD-N-oxyl radical was monitored by ESR in a Jeol T200 spectrometer, as described previously[8]. Air-saturated, buffered (0.1 m*M* K-phosphate, pH 7.0) solutions of TMPD (30 m*M*) plus EY (3 m*M*) with and without MAA (ca. 60 m*M*) in a flat cell were irradiated *in situ* with UV-filtered light (>430 nm, 400 W m^{-2}) from a halogen lamp through a condenser lens placed in front of the ESR cavity. Increases in the amplitude of ESR signal at a fixed magnetic field of 336.92 mT were recorded during photolysis.

Measurement of kinetic rate constants

The rate constant of rubrene photooxidation (k_R) was determined using the relationship

$$K_R = K_D/\beta \quad (1)$$

where k_D is the rate constant for $^1O_2$ decay that is defined as the reciprocal of the lifetime of $^1O_2$, and β is an index of rubrene reactivity toward $^1O_2$ [9].

The β value was measured by plotting the reciprocal of the amount of rubrene photooxidized against the reciprocal of the rubrene concentration; β is given by the ratio of the slope of the straight line to the intercept [10]. Five samples (2 mL each) of rubrene at different concentrations in the range of 0.304 - 0.506 m*M* were placed in a glass cuvette (1 cm light path) with a water jacket (25°C) and irradiated with monochromatic light (529 nm, 65 W m^{-2}), obtained from a 3 KW Osram Xe-lamp by using a grating monochromator (f/3.4, Applied Photophysics), until about 20% of the rubrene was consumed. The rubrene concentrations were determined by reading the OD of samples at 440 nm (ε = 2300) before and after the irradiation. During photolysis, the intensity of light source was continuously monitored with a radiometer (International light Inc., model IL 1700) so as to ensure that all the samples were irradiated at the same fluence rate.

The rate constants of $^1O_2$ reaction with MG and DG were measured according to Monroe [10], based on inhibition of the self-sensitized photooxidation of rubrene by the added substrates. Two-milliliter samples of rubrene (*ca.* 0.38 m*M*, OD_{529} = *ca.* 4.3) were irradiated with monochromatic light as above until approximately half the rubrene was consumed in the sample without MG or DG so that about 99% of the incident light was still being absorbed by rubrene at the end of irradiation. The concentrations of the added quenchers, i.e. MG and DG, were adjusted to 0.12 – 0.17 m*M* so that the amount of rubrene photooxidation was about 80% of the amount of the reaction in the unquenched solution. Considering not only the solubility of both rubrene and MAA but also the lifetime of $^1O_2$, we prepared rubrene samples in 85% chloroform/15% methanol (v/v).

RESULTS

TMPD-*N*-oxyl formation in a solution containing TMPD and a dye under illumination has long been taken as an indication that the dye acts as type II photosensitizer generating $^1O_2$, because the production of the nitroxyl radical results from the reaction of $^1O_2$ with TMPD [11]. When small amounts of MAA fractions (F1-F8) from the reverse-phase HPLC were added to aqueous solutions of TMPD plus EY, the mixtures showed decreased rates of the nitroxyl formation. Among eight MAA fractions, F1, F6, and F8 were found to be highly active in inhibiting the TMPD-$^1O_2$ reaction, whereas the rest were far less effective (Fig. 2 and Table 1). The active fractions were subjected to further purification to obtain DG, MG and MT, respectively. Estimated on an equal concentration basis from the degrees of decreases in the nitroxyl formation rate, $^1O_2$-quenching

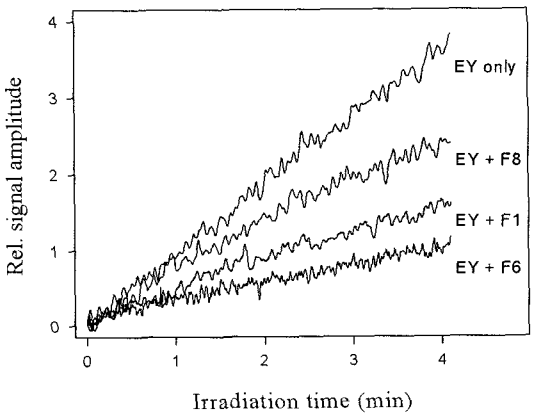


Figure 2. EY-sensitized TMPD-*N*-oxyl formation in air-saturated solutions of TMPD. The aqueous samples were irradiated with visible light (>430 nm, 400 W m^{-2}) in the presence of ca. 60 μ*M* MAA fractions (F1, F6, F8) and their absence at ambient temperature. The concentrations of EY and TMPD were 3 μ*M* and 30 m*M*, respectively.

Table 1. Inhibition of TMPD-*N*-oxyl formation rate by crude MAA preparations from *p. micans*

MAA fraction* (λ_{max} in nm)	degree of inhibition (%)	major MAA (λ_{max} in nm)
F1 (276)	55.0	DG (268)
F2 (330)	6.2	
F3 (334)	2.9	
F4 (320)	12.5	
F5 (333)	5.4	
F6 (310)	67.5	MG (310)
F7 (334)	14.1	
F8 (310)	32.2	MT (310)

*HPLC fractions eluted from an YMC-Pack ODS-A column

efficiencies of purified DG, MG and MT were 1.7, 1.9 and 1.2 times larger than the efficiency of histidine, respectively.

Two types of methods have been used to determine the rate constants of $^1O_2$ quenching by various compounds; one is based on the time-resolved decay of $^1O_2$ phosphorescence in the presence and absence of quenchers, and the other type involves steady state methods that make use of competitive inhibition by quenchers of $^1O_2$-caused acceptor oxidation. We have previously determined the $^1O_2$-quenching rate constant for MG [8] by the time-resolved method that requires an elaborate instrument to obtain an intense pumped-laser beam. For the present study, however, a steady state method based on rubrene photooxidation that needs only a simple spectrophotometer was used instead. In the presence of a certain acceptor R, which reacts with but does not physically quenches $^1O_2$, and a $^1O_2$ quencher Q, which removes $^1O_2$ through chemical and/or physical processes, $^1O_2$ disappears by three different routes.

$$^1O_2 \xrightarrow{k_D} O_2 \quad (2)$$

$$^1O_2 + R \xrightarrow{k_R} RO_2 \quad (3)$$

$$^1O_2 + Q \xrightarrow{k_Q} QO_2 \text{ and/or } Q + O_2 \quad (4)$$

According to Carlsson et al. (12), the rate constant for $^1O_2$ reaction with Q (Equation 4) can be calculated from the following equation, if two solutions of equal volume, one containing Q and the other without Q, and each having the same initial concentration of R, are each exposed to the same amount of $^1O_2$.

$$k_Q = \frac{k_R([R]_F^Q - [R]_F^0) + k_D \ln([R]_F^Q/[R]_F^0)}{[Q]\ln([R]/[R]_F^Q)} \quad (5)$$

where $[R]$ is the initial concentration of acceptor, $[Q]$ is an average concentration of quencher, and $[R]_F^Q$ and $[R]_F^0$ are the final concentrations of acceptor in the solutions with and without Q, respectively.

Rubrene has been used not only as an acceptor but also as a photodynamic generator of $^1O_2$ *in situ*, simplifying the procedure for k_Q determination by the steady state method [10]. In order for rubrene to be used as a $^1O_2$ generator, however, no significant quenching of the rubrene excited (singlet and triplet) states by a $^1O_2$ quencher itself should take place. Measurements of fluorescence from dilute rubrene solutions containing MG, MT or DG at various concentrations (0.05 – 0.3 mM) revealed that rubrene fluorescence intensity was unchanged by the presence of these MAA (data not shown), indicating that the presence of the cyclohexenone-type MAA did not affect the singlet excited state of rubrene. Furthermore, a solvent system (85% $CHCl_3$/15% CH_3OH), in which $^1O_2$ has been shown to have relatively long lifetime, i.e. 50.2 ms, as reported earlier [8], was used so as to keep the MAA concentrations to the lowest feasible level and thereby to minimize the quenching of rubrene triplet by the substrates, if any.

From 5 determinations, $\beta = 3.28 \times 10^{-4}$ M was found for rubrene in chloroform. This value agrees reasonably well with a β value of 3.12×10^{-4} M in chloroform reported by Monroe who used a 'merry-go-round' apparatus to insure the exposure of each sample to the same amount of light [10], supporting the reliability of our irradiation method for kinetic measurements. In our solvent system of 85% $CHCl_3$/15% CH_3OH, $\beta = 5.02 \times 10^{-4}$ M was found for rubrene (Fig. 3). The rate constants, k_R and k_Q, were calculated from Equation 1 for the $^1O_2$ reaction with rubrene and Equation 5 for the $^1O_2$ reactions with two most active members of MAA from *P. Micans*, MG and DG, respectively. A $^1O_2$ lifetime of 50.2 μs was used for the calculations. We also performed the experiment for k_Q measurement in both oxygen-saturated and air-saturated solutions, obtaining the same values of k_Q within experimental error (data not shown). This may indicate that no significant quenching of rubrene triplet by MG and DG took place during photolysis.

From the comparative standpoint, $^1O_2$-quenching rate constants were determined for other known quenchers, such as furfuryl alcohol and 1,4-diazabicyclo[2,2,2]octane, by the rubrene photooxidation (steady state) method and compared with the rate constants previously determined in the same solvent by the time-resolved $^1O_2$ luminescence technique [8]. As it turned out (Table 2), both methods yielded practically identical results, ensuring the feasibility of the steady state method for the kinetic measurements.

DISCUSSION

There is clear evidence that MG efficiently protects biological systems at various levels from microorganisms to biomolcules against photosensitized damage and that such MG effect results from direct removal of $^1O_2$ generated from the systems

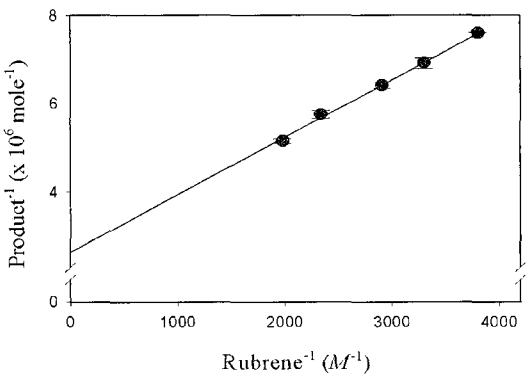


Figure 3. Determination of β for rubrene in 85% chloroform/15% methanol.

Table 2. Rate constants of singlet oxygen reactions in MeOH-$CHCl_3$ (15:85)

Compound	Rate constant ($\times 10^7 M^{-1}S^{-1}$)	Method*	Reference
Furfuryl alcohol	2.3	S	this work
	2.0	T	8
1,4-Diazabicyclo[2,2,2,]octane	2.4	S	this work
	2.2	T	8
MG	6.7	S	this work
	5.7	T	8
DG	5.4	S	this work

*S, steady state method based on competitive inhibition of rubrene oxidation; T, time-resolved method based on decay kinetics of $^1O_2$ luminescence

under high light conditions [8]. In the present investigation, DG and MT are found to be almost as effective as MG in quenching $^1O_2$. Intriguingly enough, these three MAA have in common a cyclohexenone moiety in their molecular structures (Fig. 1), while five other MAA from *P. micans* (Table 1) and two MAA from *Lissoclinum patella* [7], which are all far less effective in scavenging $^1O_2$, are apparently of cyclohexeimine type.

The techniques employed here for the kinetic study only measure the rate at which a quencher removes $^1O_2$ from the system, not how it removes $^1O_2$ [10, 12]. Therefore, the measured rate constants for DG and MG are not necessarily for substrate oxidation corresponding to chemical quenching of $^1O_2$. It is actually for total reaction including physical $^1O_2$ quenching, if any, in addition to the chemical quenching. The rate constants have been reported for total reaction of $^1O_2$ with a wide variety of compounds belonging to many different families [13]. Except for a handful of compounds including diamagnetic nickel complexes and conjugated polyenes with large numbers of double bond, the reported values are in the range of 10^4–10^7 M^{-1} s^{-1} [13]. The k_Q values of $5\sim7 \times 10^7$ M^{-1} s^{-1} found for DG and MG in 85% $CHCl_3$/15% CH_3OH (Table 2) are larger than, or at least comparable to, the values for well-known $^1O_2$ scavengers, such as furfuryl alcohol, DABCO and cyclopentadiene etc. [8].

There are some water-soluble, biological compounds whose rate constants for reaction with $^1O_2$ are known. The representative examples are oxidation-prone amino acids, such as cystein, histidine, methionine, tryptophan and tyrosine. Among these, histidine and methionine undergo photooxidation by the presence of most photosensitizers primarily via the $^1O_2$ mechanism [14]. The measured k_Q values for DG and MG are larger by factors of 2~7 than the values for histidine (3.2×10^7 M^{-1} s^{-1}) and methionine (0.86×10^7 M^{-1} s^{-1}) reported by Kraljic and Sharpatyi [15]. Because different solvents have been used for the kinetic measurements, i.e. a chloroform-methanol mixture for the MAA in this work and aqueous phosphate buffer (pH 7.1) for the amino acids, it might be unfeasible to directly compare the k_Q values for quantitatively evaluating the $^1O_2$ quenching activities of different compounds. Nevertheless, it has been shown that, in aqueous solutions, the efficiency of MG for removing $^1O_2$ and thereby protecting biological systems is larger than histidine by a factor of about 2 when estimated on an equal concentration basis [7]. This agrees fairly well with the ratio of the k_Q values for MG to histidine, likely suggesting that the measured rate constants for MAA may also be useful for assessing the relative $^1O_2$ quenching efficiency in aqueous solutions.

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