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Peroxynitrite-scavenging Constituents from the Brown Alga *Sargassum thunbergii*

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Abstract Peroxynitrite formation *in vivo* is implicated in numerous human diseases and there is considerable interest in the use of antioxidants and natural products for their treatment. The three components (**1-3**) isolated from *Sargassum thunbergii* as well as the organic solvent-soluble fractions and the aqueous layer of *S. thunbergii* were evaluated for their potential to scavenge authentic $ONOO^-$ and $ONOO^-$ derived from 3-morpholinosydnonimine (SIN-1). The antioxidant activity of the individual fractions was in the order of 85% aqueous (aq.) MeOH > *n*-BuOH > *n*-hexane > H_2O. The three known compounds, sargahydroquinoic acid (**1**), sargaquinoic acid (**2**) and sargachromenol (**3**) showed peroxynitrite-scavenging activities comparable to those of L-ascorbic acid and penicillamine. These results showed a possible antioxidant activity in major constituents of *S. thunbergii.*

Keywords: Sargassum thunbergii, peroxynitrite, 3-morpholinosydnonimine (SIN-1), plastoquinone, NMR assignment

INTRODUCTION

While there are a number of theories to explain degenerative diseases, several of them involve molecular damage that results from continuous damage by reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1-3].

Peroxynitrite (oxoperoxonitrate; $ONOO^-$), one of the reactive nitrogen species has been implicated in numerous human disease pathologies. It is generated *in vivo* from the rapid reaction of nitrogen monoxide (NO·) and superoxide ($O_2\cdot^-$) at near diffusion controlled rates. The most powerful cellular antioxidant system protecting against the harmful effects of the superoxide is embodied in the superoxide dismutases (SOD). However, it has been shown that NO· efficiently competes with SOD for superoxide. It was proposed that under conditions of increased NO· production, NO· can compete with SOD for superoxide resulting in peroxynitrite ($ONOO^-$) formation. As either excess NO· or excess superoxide decreases the bioavailability of peroxynitrite, equimolar concentrations of the radicals are ideal for peroxynitrite formation [4-6].

Under physiological conditions, $ONOO^-$ has a half life of under 1 second and is converted into its protonated form, peroxynitrous acid (ONOOH), which in turn decays to generate multiple toxic products after reaction with the nitryl cation (NO_2^+), the nitrogen dioxide radical($\cdot NO_2$) and the hydroxyl radical (·OH). Peroxynitrite and species derived from it can oxidize and nitrate lipids, proteins, DNA and carbohydrates [7-9]

Hence there is considerable interest in potential therapeutic peroxynitrite scavengers to treat oxidative damage. Many researchers have long sought a powerful and non-toxic natural antioxidant from edible and/or medicinal plants in order to prevent these reactive species from causing disorders in humans as well as to replace synthetic compounds [10-13]. Marine organisms are a virtually untapped source of novel natural products, and many of them are biologically active and potentially useful. Also the significance of seaweed as a source of bioactive natural products is already well-known [14-15]. Seaweed has been used from ancient as a source of medicinal drugs. The brown alga *S. thunbergii* is widely distributed in the southern coastal area of the Korean Peninsula [16]. Previously we reported that the crude extracts of *S. thunbergii* exert *in vitro* scavenging activities on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and authentic peroxynitrite as well as peroxynitrite derived from the peroxynitrite donor, 3-morpholinosydnonimine hydrochloride (SIN-1) [17,18]. In this study, the crude extract of the brown alga *S. thunbergii* was partitioned with organic solvents. This paper describes an evaluation of the organic solvent fractions and compounds isolated from the *S. thunbergii* for their ability to scavenge authentic peroxynitrite and SIN-1.

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Table 1. NMR spectral data for compounds **1**-**3**[a]

no	1		2		3	
	H	C	H	C	H	C
1	3.27 (2H, d, 7.2)	30.0 t	3.12 (2H, d, 7.2)	27.6 t	6.21 (1H, d, 9.6)	122.8 d
2	5.25 (1H, t, 7.2)	121.7 d	5.13 (1H, t, 7.4)	117.9 d	5.54 (1H, d, 9.6)	130.5 d
3		138.0 s		139.7 s		77.7 s
4	2.08 (2H, m)	39.5 t	2.08 (2H, m)	39.6 t	1.65 (2H, m)	40.8 t
5	2.12 (2H, m)	26.1 t	2.09 (2H, m)	26.4 t	2.11 (2H, m)	22.7 t
6	5.13 (1H, t, 6.9)	124.2 d	5.11 (1H, m)	124.4 d	5.12 (1H, t, 6.9)	124.8 d
7		134.6 s		134.5 s		134.2 s
8	2.08 (2H, m)	39.1 t	2.09 (2H, m)	39.1 t	2.05 (2H, t, 7.5)	39.1 t
9	2.57 (2H, dt, 7.0, 7.0)	28.4 t	2.59 (2H, q, 7.2)	28.3 t	2.58 (2H, dt, 7.0, 7.5)	28.2 t
10	5.97 (1H, t, 7.0)	145.3 d	5.99 (1H, t, 7.2)	145.3 d	5.96 (1H, t, 7.0)	145.4 d
11		130.5 s		130.5 s		130.4 s
12	2.25 (2H, t, 7.7)	34.6 t	2.26 (2H, t, 7.2)	34.6 t	2.25 (2H, t, 6.9)	34.6 t
13	2.12 (2H, m)	27.9 t	2.12 (2H, m)	27.9 t	2.11 (2H, m)	27.9 t
14	5.07 (1H, t, 6.9)	123.4 d	5.07 (1H, m)	123.4 d	5.07 (1H, t, 6.9)	123.3 d
15		132.1 s		132.1 s		132.1 s
16	1.67 (3H, s)	25.7 q	1.67 (3H, s)	25.7 q	1.66 (3H, s)	25.7 q
17	1.58 (3H, s)	17.8 q	1.58 (3H, s)	17.8 q	1.57 (3H, s)	17.8 q
18		172.5 s		172.7 s		172.9 s
19	1.59 (3H, s)	16.1 q	1.60 (3H, s)	16.1 q	1.56 (3H, s)	15.6 q
20	1.74 (3H, s)	16.2 q	1.62 (3H, s)	16.2 q	1.35 (3H, s)	26.0 q
1'		146.2 s		187.9 s		144.6 s
2'		127.5 s		148.4 s		121.2 s
3'	6.45 (1H, br s)	113.9 d	6.45 (1H, m)	132.1 d	6.30 (1H, d, 2.2)	110.3 d
4'		148.6 s		187.8 s		148.5 s
5'	6.47 (1H, br s)	115.4 d	6.53 (1H, quin, 1.4)	133.0 d	6.45 (1H, d, 2.2)	117.0 d
6'		125.4 s		145.8 s		126.2 s
Ar-Me	2.17 (3H, s)	16.2 q	2.05 (3H, d, 1.4)	16.0 q	2.11 (3H, s)	15.8 q

[a] ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solution at 300 and 75 MHz, respectively. Assignments were based upon ^{1}H COSY, TOCSY, HSQC, and HMBC experiments. Abbreviations br s, s, d, t, dt, m, q, and quin represent broad singlet, singlet, doublet, triplet, doublet triplet, multiplet, quartet, and quintet, respectively.

MATERIALS AND METHODS

Plant Materials

S. thunbergii was collected by hand in December, 2001 on the coast of Youngdo Island, South Sea, Korea. The collected samples were briefly dried under shade and kept at -25°C until chemically investigated.

Extraction, Fractionation, and Isolation

Shade-dried samples (250 g) were chopped into small pieces and repeatedly extracted for 2 days with acetone-CH_2Cl_2 (1:1) (1.5 L × 2) and MeOH (1.5 L × 2). The combined crude extracts (12.5 g) were evaporated under vacuum to give a dark brown oil that was partitioned between CH_2Cl_2 and water. The organic layer was repartitioned with 85% aqueous MeOH and *n*-hexane. The aqueous fraction was also further fractionated with *n*-BuOH and H_2O, successively, to produce the *n*-hexane (3.6 g), 85% aqueous MeOH (8.8 g), *n*-BuOH (1.2 g) and H_2O (8.0 g) fraction. A portion of the 85% aqueous MeOH fraction (4.3 g) was subjected to C_{18} reversed-phase vacuum flash chromatography eluting with stepwise gradient mixtures of MeOH and water (50%, 60%, 70%, 80%, 90% aqueous MeOH, and 100% MeOH, A-F). Purification of fraction E by reversed-phase HPLC (YMC ODS column, 87% aqueous MeOH) yielded 15.3 and 14.1 mg of a mixture and compound **3**, respectively. The mixture was further separated by HPLC under different solvent condition (65% aqueous MeCN) to provide 6.0 mg of pure compound **1**. Fraction F was subjected to reversed-phase HPLC (YMC ODS column, 95% aqueous MeOH) to yield 52.3 mg of impure **2**. Final purification was made by another reversed-phase HPLC (YMC ODS, 20% aqueous MeCN) to give 13.2 mg of compound **2**.

Measurement of $ONOO^-$ Scavenging Activity

$ONOO^-$ scavenging ability was measured by monitoring the oxidation of dihydrorhodamine 123 with a modified version of Kooy's method [19]. A stock solution of DHR 123 (5 mM) purged with nitrogen was prepared in advance and stored at -80°C. A working solution of DHR

123 [final concentration (f.c.), 5 μM] diluted from the stock solution was placed on ice in the dark immediately prior to the study. The buffer of 90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4) and 5 mM potassium chloride with 100 μM (f.c.) diethylenetriaminepentaacetic acid (DTPA) was purged with nitrogen and placed on ice before use. $ONOO^-$ scavenging ability, measured by the oxidation of DHR 123 was determined at room temperature with a microplate flurescence spectrophotometer FL 500 (Bio-Tek instruments, USA) using excitation and emission wavelengths of 485 and 530 nm. The background and final fluorescent intensities were measured 5 min after treatment with or without SIN-1 (f.c. 10 μM) or with authentic $ONOO^-$ (f.c. 10 μM) in 0.3 M sodium hydroxide. Oxidation of DHR 123 by decomposition of SIN-1 gradually increased whereas authentic $ONOO^-$ rapidly oxidized DHR 123 with its final fluorescent intensity being stable over time. Penicillamine was used as a positive control.

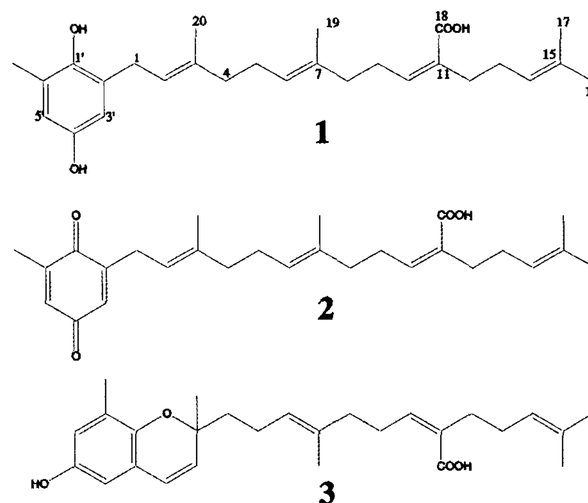

Fig. 1. Chemical structure of compounds **1-3**.

RESULTS AND DISCUSSION

Isolation and Structural Identification

Bioassay-guided separation of organic materials from seaweed using peroxynitrite as a bioassay tool yielded three compounds of the plastoquinone structural class.

Compound **1**, sargahydroquinoic acid was isolated as a colorless gum which was analyzed for $C_{27}H_{38}O_4$ by a combination of HRFABMS and ^{13}C NMR spectrometry. Several carbon signals in the region of δ 150-110 in the ^{13}C NMR spectrum and proton signals at δ 6.47 (1H, br s), and 6.45 (1H, br s) in the ^{1}H NMR spectrum indicated the existence of an aromatic ring. A signal at δ 172.5 in the ^{13}C NMR spectrum and a strong absorption band at ν_{max} 1,680 cm^{-1} and a broad band at 3,400-2,500 cm^{-1} in the IR spectrum also showed the presence of a carboxylic acid functionality.

The overall structure of **1** was determined by 2-D extentional NMR experiments such as ^{1}H COSY, TOCSY, NOESY, HSQC, and HMBC. The presence of four trisubstituted double bonds including at least one methyl group was revealed by the long-ranged coupling of the olefinic proton signal with those of vinyl methyl protons in the ^{1}H COSY experiment. The HMBC correlations of vinyl methyl protons with neighboring carbons, combined with proton-proton couplings between the signals of olefinic and upfield protons, defined a geranyl chain bearing a carboxyl group at C-11. In addition, a careful examination of the downfield signals in both the ^{1}H and ^{13}C NMR spectra, combined with 2-D NMR analysis exhibited that the aromatic ring was 1,4-dihydroquinone. A small coupling (J=2.0 Hz) between the signals of aromatic protons at δ 6.47 and 6.45 was indicative of the presence of a meta substitution. A long-range coupling of the former with the signal of methyl protons at δ 2.17 (3H, br s) assigned the location of a benzylic methyl group at C-6. Thus, the structure of the aromatic moiety was determined to be 6-alkyl-2-methyl-1,4-hydroquinone. With the aid of this information, a literature survey revealed that compound **1** was previously reported from *S. sagamianum* var. *yezoense*. Comparison of NMR spectral data showed very good correlation with published data for **1** [20].

Compound **2** was isolated as a colorless gum, that was also analyzed by high resolution mass and ^{13}C NMR spectroscopic methods. Spectral data of **2** was very similar to that from **1**. The most significant difference was a downfield shift of the quaternary carbon signals at δ 148.6 and 146.2 to those of δ 187.8 and 187.9 which were assigned to C-1 and C-4, two ketone carbons of quinone ring of **2**. Thus, compound **2** was determined to be 2-methyl-6-[(2E,6E,10Z)-11-carboxyl-3,7,15-trimethyl-2,6,10,14-hexadecatetraenyl]-2,5-cyclohexadien-1,4-ione. This compound was identified as sargaquinoic acid after comparison with published data. Its ^{1}H and ^{13}C NMR spectral data as measured in the same NMR solvent was in good agreement with the reported data [21,22].

Compound **3** was isolated as a colorless gum whose molecular formula was deduced as $C_{27}H_{36}O_4$ by HRFABMS and ^{13}C NMR analyses. Spectral data for this compound was very similar to that obtained for **1**. However, the most significant differences between **3** and **1** were the appearance of an oxygenated tertiary carbon (δ 77.7) in the ^{13}C NMR spectrum and replacement of benzylic methylene protons and their adjacent vinyl proton in **1** by an AB system (δ 6.21 and 5.54 each d, 9.6 Hz), assignable to the olefin protons at C-1 and C-2 in the ^{1}H NMR spectrum, respectively. These changes could be accommodated by introducing a new cyclic ether linkage between 1'-OH and C-3 to form a chromene ring. This was confirmed by the HMBC correlations observed between olefinic proton H-1 with C-3, C-1', C-2', and C-3', while the methyl protons H-20 was correlated with C-2. Thus, the structure of **3** was determined to be 2-[(3E,7Z)-(8-carboxyl-4,12-dimethyl-3,7,11-decatrienyl)-2,8-dimethyl-3-chromen-6-ol which was known as sargachromenol in the literature. Comparison of spectral data showed very

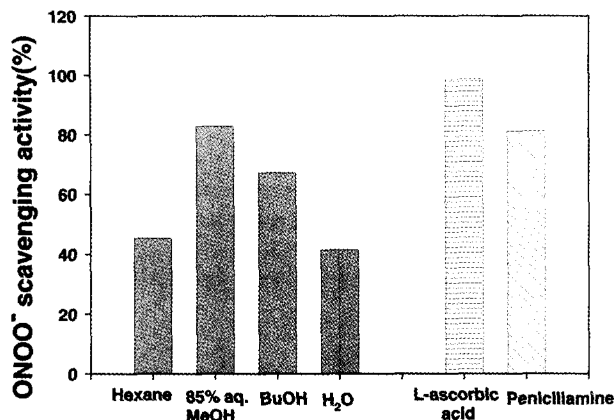

Fig. 2. $ONOO^-$ scavenging activity of several fractions from *Sargassum thunbergii* at 5 μg/mL.

good correlation with published data for this compound [21,22].

These kinds of compounds have been previously reported from brown algae [23-27]. However, NMR spectral full assignment for these compounds have never been reported, although a few partial assignments were previously done. To the best of our knowledge, this is the first case of the correct full assignment for compounds of the plastoquinone structural class using extensive 2-D NMR experiments such as ^1H COSY, TOCSY, HSQC, and HMBC.

Peroxynitrite Scavenging Activity

The combined crude extracts of *S. thunbergii* were fractionated into *n*-hexane, 85% aq. MeOH, *n*-BuOH, and water-soluble fractions and the $ONOO^-$ scavenging activity of each fraction was determined on authentic $ONOO^-$. The 85% aq. MeOH fraction of *S. thunbergii* exhibited the strongest $ONOO^-$ scavenging activity at the 5 μg/mL concentration (Fig. 2). This scavenging effect on peroxynitrite exceeded that of penicillamine which is a well known peroxynitrite scavenger [28]. The BuOH fraction also showed a strong peroxynitrite-scavenging activity, that was slightly lower than that of the 85% aq. MeOH fraction.

The peroxynitrite scavenging effect of compounds **1**-**3** isolated from the 85% aq. MeOH fraction of *S. thunbergii* is shown in Table 2. All three isolated compounds **1**-**3** significantly scavenged peroxynitrite and the resulting activities were compared with those of the reference compounds penicillamine and L-ascorbic acid. The inhibitory activity of sargahydroquinoic acid (**1**), sargaquinoic acid (**2**) and sargachromenol (**3**) showed a potent scavenging effect on peroxynitrite derived from 3-morpholinosydnonimine (SIN-1) and on authentic peroxynitrite. The peroxynitrite scavenging effect of sargachromenol (**3**) exceeded that of penicillamine, a well known peroxinitrite scavenger. Also, the ability of **3** to scavenge $ONOO^-$ formed from the decomposition of SIN-1, which simultaneously generates NO· and $O_2^{\cdot -}$ was

Table 2. $ONOO^-$ scavenging activity of isolated compounds from *Sargassum thunbergii* at 10 μg/mL

Compounds	Authentic $ONOO^-$ (%)	$ONOO^-$ from decomposition of SIN-1 (%)
Sargahydroquinoic acid (1)	78.03	100.00
Sargaquinoic acid (2)	64.18	75.39
Sargachromenol (3)	92.69	99.51
L-ascorbic acid	98.82	97.89
Penicillamine	90.14	84.57

overall similar to its inhibitory effect against authentic $ONOO^-$. These results suggest that the antioxidative activity in the crude extract of *S. thunbergii* were partially attributable to these compounds **1**-**3**.

Compounds **1**-**3** are structurally related to the natural antioxidant vitamin E and were reported to be an antioxidants [29]. Although the DPPH radical-scavenging activities of sargahydroquinoic acid (**1**) and sargachromenol (**3**) were previously reported as greater than that of α-tocopherol, their scavenging activity on peroxynitrite has never been reported. To the best of our knowledge, this is the first report on the peroxynitrite scavenging activity of these compounds. Reactive oxygen species (ROS) are constantly generated under normal conditions as a consequence of aerobic metabolism. ROS include free radicals such as the superoxide anion, hydroxyl radical and the nonradical hydrogen peroxide. The formation of peroxynitrite has been implicated in the pathophysiology of numerous inflammatory diseases, autoimmune disorders, cancer and sepsis. Peroxynitrite ($ONOO^-$) is a potent oxidant formed from nitric oxide (NO·) and superoxide ($O_2^{\cdot -}$) [30,31]. The present work indicates that the peroxynitrite scavenging ability of these compounds can be useful in the prevention of reactive oxygen species and in the treatment of oxidative damage caused by such species. It will be interesting to further investigate the oxidative activity of these natural compounds in preventing various radical-mediated injuries in pathological situations *in vivo*.

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