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# Structure−Activity Relationship of Neuroprotective and Reactive Oxygen Species Scavenging Activities for Allium Organosulfur Compounds

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The neuroprotective and antioxidative activities of five organosulfur compounds with a thioallyl structure (−S−$CH_2CH$═$CH_2$) were characterized in terms of structure−activity relationships. Among five organosulfur compounds, only S-allyl-L-cysteine (SAC) having the alanyl group (−$CH_2CH$−$NH_2$−COOH) and lacking the oxo (O═) group with in between molecular properties, was effective in protecting cell death induced by both oxygen glucose deprivation and global cerebral ischemia. Conversely, lipophillic organosulfur compounds including diallyl sulfide, diallyl disulfide, and diallyl trisulfide were devoid of in vitro and in vivo neuroprotective activities. Furthermore, a significant correlation was only found between the in vivo neuroprotective activity and the $OH^-$ scavenging activity ($\gamma = 0.55$ and $p = 0.032$) among reactive oxygen species scavenging activities. These results indicate that the presence of the alanyl group and the absence of the oxo group are essential for the manifestation of neuroprotective activity against ischemic insults and scavenging of OH radical, with SAC surfacing as a potent neuroprotectant.

**KEYWORDS: Organosulfur compounds; S-allyl-L-cysteine; neuroprotective; antioxidative; structure−activity relationship**

## INTRODUCTION

There is accumulating evidence to suggest that oxidative stress is involved in neuronal loss after cerebral ischemia, thereby resulting in the dysfunction of cellular machinery (*1*, *2*). Many antioxidants and free radical scavengers are reported to have potent neuroprotective activities against brain ischemic damage (*3*−*9*). Various thiol compounds including organosulfur compounds derived from garlic (*Allium sativum* L.) are also known to have antioxidant activities (*10*) in some in vitro and in vivo studies (*11*−*14*).

All organosulfur compounds with a thioallyl structure (−S−$CH_2CH$═$CH_2$) have been shown to possess neurotrophic activities in cultured rat hippocampal neurons (*15*). S-Allyl-L-cysteine (SAC), one of the water-soluble organosulfur compounds containing the thioallyl (−S−$CH_2CH$═$CH_2$) group, has been shown to reduce edema formation in the ischemic brain by inhibiting free radical-mediated lipid peroxidation (*16*, *17*). Recently, in our previous experiments, we found that SAC prevented neuronal cell death in cerebral ischemic insult by specifically scavenging peroxynitrite ($ONOO^-$) rather than other oxidants such as hydrogen peroxide ($H_2O_2$), nitric oxide (NO), and superoxide radical ($O_2^-$) (*18*). Diallyl sulfide (DAS) and diallyl disulfide (DADS), lipid-soluble organosulfur compounds containing thioallyl groups, have also been shown to have reactive oxygen species (ROS) scavenging and neurotrophic activities in many in vitro and in vivo experimental models. In contrast, DAS and DADS, which differ from SAC only in the absence of the alanyl group (−$CH_2CH$−$NH_2$−COOH), did not ameliorate ischemic brain injury (*16*, *17*). These findings suggest that the relationship between their structural features and neuroprotective activities is complicated and is not clearly defined. Additionally, the antioxidative activities of organosulfur compounds in connection with their roles in the area of neuroprotection are not yet fully characterized.

Therefore, in the present study, we aimed to characterize the neuroprotective and antioxidative activities of five organosulfur compounds with a thioallyl structure in terms of structure−activity relationships.

## MATERIALS AND METHODS

**Chemicals.** (+)-S-Allyl-L-cysteine sulfoxide (ALI) and SAC were obtained from Nopex Ltd. (East Sussex, United Kingdom) and TCI

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Chemical Co. (Tokyo, Japan), respectively. Both DAS and DADS were obtained from Aldrich Chemical Co. (Milwaukee, WI), and diallyl trisulfide (DATS) was provided by LKT Laboratory (St. Paul, MN). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), deoxyribose, dihydrorhodamine-123 (DHR-123), dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), guaiacol, horseradish peroxidase, hydroxypropyl-$\beta$-cyclodextrin (HP-$\beta$-CD), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 3-morpholinosydnonimine (SIN-1), $\beta$-nicotinamide adenine dinucleotide (reduced form, NADH), phenazine methosulfate (PMS), and 2-thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO). L-Ascorbic acid was obtained from Fluka Chemical Co. (Buchs, Switzerland). Fetal bovine serum (FBS), minimum essential media (MEM), and antibiotics were purchased from Life Technologies, Inc. (Rockville, MD). All other chemicals were of the highest purity available. Unless otherwise stated, lipid-soluble compounds were dissolved in DMSO and diluted with phosphate-buffered saline (PBS, pH 7.4) to give a final concentration for in vitro and in vivo testing.

**Generation of Molecular Property.** Chemical structures of five organosulfur compounds were constructed using Chemdraw Ultra 8.0 software (Cambridgesoft Corp., Cambridge, MA) and were imported into TSAR (TSAR 3D version 3.3 for Windows, 2000, San Diego, CA). The Cosmic 3D analysis software converted two-dimensional structures into appropriate low-energy three-dimensional structures. These structures were energy-minimized using the AM1 Hamiltonian in the VAMP molecular orbital package (Oxford Molecular Ltd., Oxford, United Kingdom), implemented in TSAR. To describe the molecular properties of five organosulfur compounds, eight molecular and two absorption properties were selected as follows: molecular weight (MW) and two structural substituents (R1 and R2) as constitutional descriptors; octanol/water partition coefficient (Clog *P*) and water solubility as chemical descriptors; polar surface area (PSA) and Balaban index, a relative electronegativity, as topological descriptors; total negative charge and total positive charge, as electrostatic descriptors; and blood−brain barrier (BBB) and human intestinal absorption (HIA) as absorption predictors. MW, Clog *P*, PSA, and Balaban index were obtained by means of TSAR software. ASlog S, BBB, and HIA were further generated using PreADME software (version 1.0, Research Institute of Bioinformatics & Molecular Design, Seoul, Korea) (*19*).

**Cell Cultures and Oxygen−Glucose Deprivation (OGD).** The human neuroblastoma cell line, SK-N-SH, was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in MEM with phenol red supplemented with 10% heat-inactivated FBS and antibiotics. The culture medium was changed every 2−3 days, and cells were trypsinized and subcultured at near confluence every 5−7 days at a split ratio of 1:4−1:6. OGD was performed by placing the glucose-deprived cells inside an anaerobic chamber (Coy Laboratory Products Inc., MI) maintaining a 90% $N_2$/5% $CO_2$/5% $H_2$ condition. After 6 h of OGD, cells were returned to their normoglycemic (5.5 mM glucose) and normoxic conditions for 24 h (reperfusion). To test organosulfur compounds with protective activity against ischemic insult, cells were preincubated with varying concentrations of organosulfur compounds for 48 h and then were subjected to ischemic insult for 6 h in the presence of test samples. For testing volatile DAS, DADS, or DATS, cells were incubated with an inclusion vehicle, HP-$\beta$-CD, to minimize the loss of the highly volatile DAS, DADS, and DATS in culture medium, and consequently to increase their bioavailability, at the molar ratio of 13:1, as described previously (*20*). After incubation in the normoglycemic and normoxic conditions for another 24 h, cell viability was assessed by the MTT assay (*21*). The percentage of neuroprotection against ischemic insult was then expressed in terms of cell viability as follows: neuroprotection (%) $= 100 - [(x - z)/(x - y) \times 100]$, where $x$ is the absorbance read in nonischemic cells, $y$ is the absorbance read in sample-untreated (solvent alone) ischemic cells, and $z$ is the absorbance read in sample-treated ischemic cells. All experiments were performed a minimum of three times.

**Global Cerebral Ischemia Model.** Male Mongolian gerbils (*Meriones unguiculatus*) weighing 60−80 g were purchased from Charles River Laboratories (Seoul, Korea) and kept on a 12 h light/dark cycle with ad libitum access to food and water. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee. Male Mongolian gerbils were placed under general anesthesia with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. A midline ventral incision was made in the neck. Both common carotid arteries were isolated, freed of nerve fibers, and occluded using nontraumatic aneurysm clips. Complete interruption of blood flow was confirmed by observing the central artery in the eyeballs using an ophthalmoscope. After 5 min of occlusion, the aneurysm clips were removed from both common carotid arteries. Restoration of blood flow (reperfusion) was observed directly under the microscope. Sham-operated controls ($n = 7$) were subjected to the same surgical procedures except that common carotid arteries were not occluded. The body temperature was monitored and maintained at 37 ± 0.3 °C during surgery and during the immediate postoperative period until the animals recovered fully from the anesthesia. The gerbils were treated three times (30 min before occlusion, immediately following reperfusion, and 2 h after reperfusion) with ALI (300 mg/kg, i.p.), SAC (300 mg/kg, i.p.), DAS (100 mg/kg, i.p.), DADS (20 mg/kg, i.p.), DATS (5 mg/kg, i.p.), or vehicle (PBS or DMSO). Seven days after the operation, the gerbils were anesthetized with chloral hydrate and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). The brains were removed and placed in 30% sucrose at 4 °C for 2 days. Transverse sections 30 $\mu$m thick were cut through the hippocampus with a cryostat and stained with cresyl violet. The extent of neuronal damage in the hippocampal CA1 region was evaluated with a light microscope, and a count was made of the number of surviving neurons per 1 mm length in the middle of the CA1 region.

**Trolox Equivalent Antioxidant Capacity (TEAC).** The total antioxidant capacity was measured by the TEAC assay as described by Re et al. (*22*), with a slight modification. The TEAC assay is based on the ability of the antioxidant to scavenge the blue-green-colored $ABTS^{\bullet+}$ radical cation relative to the $ABTS^{\bullet+}$ scavenging activity of the water-soluble vitamin E analogue, Trolox. A 7 mM concentration of ABTS was allowed to react with a 2.45 mM concentration of potassium persulfate solution for 16 h in the dark, in order to generate $ABTS^{\bullet+}$. The $ABTS^{\bullet+}$ solution was diluted in PBS to an absorbance of 0.7 at 734 nm. Ten microliters of sample or Trolox solution was added to 990 $\mu$L of $ABTS^{\bullet+}$ solution. The mixture was incubated at room temperature for 20 min, and the absorbance at 734 nm was measured in time. This was compared to a blank where 10 $\mu$L of the solvent was added to 990 $\mu$L of the $ABTS^{\bullet+}$ solution. The decrease in absorbance at 734 nm observed 20 min after the addition of each sample was used to calculate the TEAC value, which represents the concentration of a Trolox solution that has the same antioxidant capacity as the analyzed sample.

**Scavenging of $O_2^-$.** $O_2^-$ was generated in a nonenzymatic system using PMS and NADH and detected through the reduction of nitroblue tetrazolium (NBT) as described by Robak and Gryglewsk (*23*). The reaction mixtures contained 190 $\mu$L of 78 $\mu$M NADH/50 $\mu$M NBT, 10 $\mu$L of sample, and 200 $\mu$L of 20 $\mu$M PMS. The mixtures were incubated in a 96 well plate at room temperature for 3 min. Absorbances at 540 nm against the blank sample were measured using a microplate reader. Ascorbic acid was used as a positive control. The percentage inhibition of absorbance at 540 nm was calculated.

**Scavenging of Hydroxyl Radicals ($OH^-$).** The deoxyribose method was used for determining the scavenging activity on $OH^-$ (*24*). $OH^-$ was generated by a Fenton system (ascorbic acid/$FeSO_4$−EDTA/$H_2O_2$), and the deoxyribose exposed to $OH^-$ was degraded to malonaldehyde, which generated a pink chromogen on heating with TBA at low pH. The reaction mixtures contained 1.5 mL of 3.74 mM deoxyribose, 100 $\mu$L of 28.4 mM $H_2O_2$, 100 $\mu$L of 2 mM EDTA, 100 $\mu$L of 2 mM ascorbic acid, 100 $\mu$L of 400 $\mu$M $FeSO_4$, and 100 $\mu$L of sample. After incubation at 37 °C for 1 h, 1 mL of 1% (w/v) TBA and 1 mL of 2.8% (w/v) TCA were added, and then, the mixture was heated in a water bath at 100 °C for 12 min followed by cooling down on ice for 10 min. Absorbances at 532 nm against the blank sample were measured using a spectrophotometer. Ascorbic acid was used as a positive control. The percentage inhibition of absorbance at 532 nm was calculated.

**Scavenging of $H_2O_2$.** $H_2O_2$ was measured using the Guaiacol method as described by Aruoma et al. (*25*). The reaction mixtures contained 880 $\mu$L of 150 mM potassium PB (pH 7.4), 50 $\mu$L of 10 mM $H_2O_2$, 50

**Table 1.** Molecular Descriptors and Absorption Predictors Generated for Organosulfur Compounds

| | constitutional | | | physicochemical | | topological | | electrostatic | | absorptional | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | substituent[a] | | | | | | | | | |
| compound | MW | R1 | R2 | Clog $P$[b] | solubility[c] (mg/L) | PSA (Å)[d] | Balaban index[e] | total negative charge | total positive charge | BBB ($C_{brain}/C_{blood}$) | HIA (%) |
| ALI | 177.22 | O← | $-CH_2-CH(NH_2)-COOH$ | −3.871 | 75006.2 | 104.05 | 3.469 | 0.240 | 0.264 | 1.305 | 79.23 |
| SAC | 162.22 | | $-CH_2-CH(NH_2)-COOH$ | −2.156 | 11277.3 | 93.07 | 3.107 | 0.300 | 0.181 | 1.112 | 81.69 |
| DAS | 114.21 | | $-CH_2-CH=CH_2$ | 2.330 | 994.7 | 25.30 | 2.575 | 0.235 | 0.120 | 1.997 | 99.31 |
| DADS | 146.21 | | $-S-CH_2-CH=CH_2$ | 2.760 | 170.9 | 50.60 | 2.531 | 0.221 | 0.120 | 1.760 | 97.66 |
| DATS | 178.34 | | $-S-S-CH_2-CH=CH_2$ | 2.877 | 18.4 | 75.90 | 2.505 | 0.220 | 0.120 | 1.703 | 96.45 |

[a] Chemical basic structure $CH_2=CH-CH_2-S-R2$ (R1). [b] Calculated logarithm of octanol/water partition coefficient. [c] Calculated water solubility. [d] Topological PSA. [e] The relative electronegativity.

$\mu$L of 0.2% (v/v) guaiacol solution, 10 $\mu$L of horseradish peroxidase (1450 U/mL), and 10 $\mu$L of sample. The reaction mixtures were incubated for 30 min at room temperature. Absorbances at 436 nm against the blank sample were measured using a spectrophotometer. Ascorbic acid was used as a positive control. The percentage inhibition of absorbance at 436 nm was calculated.

**Scavenging of $ONOO^-$.** $ONOO^-$ formed from decomposition of SIN-1 was determined by measuring the $ONOO^-$-induced oxidation of DHR-123 to rhodamine-123 (RH-123) by fluorimetry (*26*). The reaction mixtures contained 60 $\mu$L of PBS (pH 7.4), 10 $\mu$L of catalase (600 U/mL), 10 $\mu$L of 2 mM SIN-1, 10 $\mu$L of 25 mM DHR-123, and 10 $\mu$L of sample. The formation of RH-123 was measured fluorimetrically (excitation, 485 nm; emission, 538 nm) using a fluorescence microplate reader (FL600, Bio-Tek Instruments Inc., Winooski, VT). Ascorbic acid was used as a positive control. The percentage inhibition of fluorescence was calculated.

**Statistical Analysis.** A Statistical Analysis Systems program (version 8.2; SAS Institute Inc., Cary, NC) was used for statistical analysis. Unless otherwise stated, all of the results were calculated as means ± standard errors of the mean (SEM). Statistical differences between the two mean values were determined by Student's $t$-test. The Pearson correlation coefficient ($\gamma$) was used to evaluate the relationship between the neuroprotective activity, the ROS scavenging activity, and the molecular property, and $p < 0.05$ was considered significant.

## RESULTS

**Molecular Properties of Five Organosulfur Compounds.** As summarized in **Table 1**, molecular and absorption properties, which include constitutional, chemical, topological descriptors, and the absorption predictor of five organosulfur compounds, were calculated using TSAR and PreADME software. Five organosulfur compounds of thioallyl structure exhibited a narrow range of constitutional properties: MW 114−178 and substitutions on the S-allyl skeleton including the allyl or oxo group (R1) and the alanyl group (R2). A chemical descriptor, Clog $P$, representing hydrophobicity or lipophilicity, ranged from −3.871 to 2.877 in five organosulfur compounds. ALI and DATS showed the lowest and the highest Clog $P$ values, respectively. ASlog S, representing water solubility, had a broad range of values from 18 (DATS) to 75006 mg/L (ALI). PSA, a topological descriptor that means sum of polar atom surfaces, ranged from 25 to 104, indicating that five organosulfur compounds were predicted to be well-absorbed (*27*). Another topological descriptor, the relative electronegativity expressed as Balaban index, indicated that all compounds ranged from 2.51 to 3.47. With regard to BBB permeability, five organosulfur compounds manifested over 1.0, implying that all may permeate across the BBB (*28*). As another absorption prediction, HIA showed a different tendency in comparison with BBB. ALI and SAC presented the lower HIA value ranging from 79 to 82, as compared with oil-soluble organosulfur compounds, DAS, DADS, and DATS.

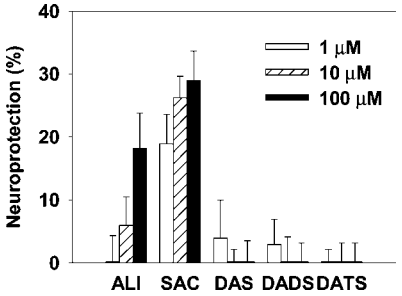


**Figure 1.** Neuroprotective effect of organosulfur compounds in the in vitro ischemia model. Results were expressed means ± SEM from three to five experiments, each done at least in triplicate. Neuroprotection (%) = 100 − [($x - z$)/($x - y$) × 100], where $x$ is the absorbance read in nonischemic cells, $y$ is the absorbance read in sample-untreated (solvent alone) ischemic cells, and $z$ is the absorbance read in sample-treated ischemic cells.

**Effects of Organosulfur Compounds on Neuroprotection in SK-N-SH Cells.** Neuroprotective effects of five organosulfur compounds were assessed by comparing cell viabilities in SK-N-SH cultures with exposure to OGD at various concentrations ranging from 0.1 to 1000 $\mu$M (**Figure 1**). The ALI [$CH_2=CHCH_2S(O)CH_2CH(NH_3^+)CO_2^-$], a hydrophilic allylsulfinothiolated derivative of cysteine, increased the cell viabilities against ischemia by 5−18% at 10−100 $\mu$M concentrations. SAC, which differs from ALI only in the absence of the oxo (O=) group, increased the cell viabilities against OGD by 18−30% at 1−100 $\mu$M concentrations, in a dose-dependent manner. On the other hand, lipophilic allyl sulfides, DAS, DADS, and DATS, did not increase cell viabilities at all. Rather, these three compounds were cytotoxic at >100 $\mu$M.

**Effect of Organosulfur Compounds on Reduction of Ischemic Injury in Global Ischemia Model.** To test whether five organosulfur compounds reduce the in vivo ischemic damage, they were intraperitoneally administered three times 30 min before, immediately, and 2 h after ischemic insult. Because DAS, DADS, and DATS are toxic in high concentrations, the different doses ranging from 5 to 300 mg/kg were chosen for testing. The survived neuronal densities and microphotographs of the hippocampal CA1 subfield in each group are shown in **Figure 2**. In sham-operated animals, CA1 pyramidal neurons were laid in 3−4 layers and presented round and large nuclei and clear nucleoli with neuronal densities of 241.8 ± 10.4/mm$^2$. Widespread damage was evident in ischemic animals treated with the vehicle (PBS or DMSO) with neuronal

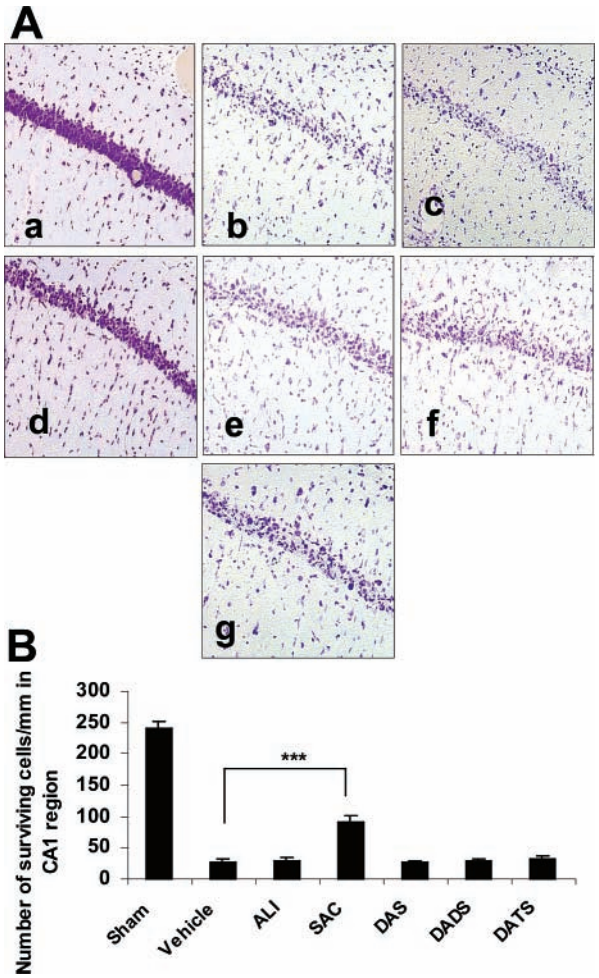


**Figure 2.** Effect of organosulfur compounds on neuroprotection in the in vivo global ischemia model. (**A**) Representative photomicrograph of the dorsal hippocampus in gerbils. CA1 region in (a) sham control, (b) ischemic control (PBS), (c) ALI, (d) SAC, (e) DAS, (f) DADS, and (g) DATS. (**B**) The number of surviving cells in the hippocampal CA1 region. No significant effect of PBS and DMSO on the number of survival cells was observed as compared with the ischemic control. Values are means ± SEM. ***$p$ < 0.01, a significant difference from ischemic control. Bar = 100 $\mu$M.

densities of 27.7 ± 4.0/mm$^2$. Pyramidal neurons either presented a densely stained shrunken appearance with minimal cytoplasm or had disappeared. Such neuronal damage was suppressed by only SAC treatment showing a significant increase in the neuronal densities of 91.2 ± 10.3/mm$^2$ ($p$ < 0.01, vs vehicle-treated ischemic animals). In contrast, other organosulfur compounds, ALI, DAS, DADS, and DATS, did not affect neuronal damage induced by ischemic insult.

**Effects of Organosulfur Compounds on Antioxidant Activity.** To investigate the antioxidant activity of organosulfur compounds, the TEAC values of these compounds were estimated and compared with that of ascorbic acid. The TEAC values of five organosulfur compounds ranged from 0.0017 to 0.0048 and were lower than that of ascorbic acid (**Figure 3**). Also, the scavenging activity of these organosulfur compounds was measured in $O_2^-$, $OH^-$, $H_2O_2$, and $ONOO^-$ generating systems and compared with those of SOD and/or ascorbic acid. Although two hydrophilic organosulfur compounds, ALI and SAC, did not scavenge $O_2^-$ and $H_2O_2$ at concentrations used in the present study, they efficaciously scavenged $OH^-$ more than ascorbic acid. The scavenging activity of $ONOO^-$ was shown by SAC but not ALI. In contrast, at 100 $\mu$M concentrations, DADS and DATS effectively inhibited $O_2^-$ as much as SOD

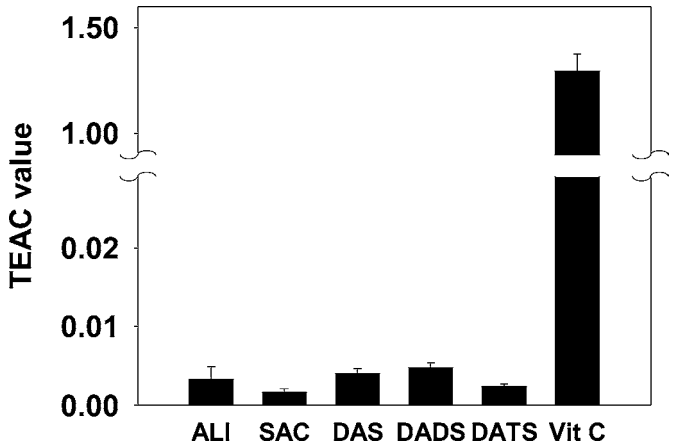


**Figure 3.** Antioxidant activity of organosulfur compounds (TEAC values). TEAC values were examined as described in the Materials and Methods. Each value represents the means ± SEM for three different experiments. TEAC values were calculated from the slope, obtained from $A$ plot ($A_0/A_i$) − 1 vs the compound concentration at ($A_0/A_i$) − 1 = 1, $A_0$ = the absorbance in the absence of tested compound, and $A_i$ = the absorbance in the present of tested compounds. The data are normalized to 1 mM Trolox.

and/or ascorbic acid did (**Figure 4**). While the scavenging effect of three lipophilic organosulfur compounds on $OH^-$ and $H_2O_2$ was negligible, these compounds inhibited $ONOO^-$ more or less at 10 $\mu$M concentrations. To gain a better understanding of the relationship between radical scavenging activity and neuroprotective activity, correlation analysis was performed as presented in **Table 2**. The $OH^-$ scavenging activity of organosulfur compounds was significantly correlated with neuroprotective activity ($\gamma$ = 0.555 and $p$ = 0.032). As shown in **Figure 4**, all of the sulfur compounds except ALI showed a high correlation between neuroprotection and $OH^-$ radical scavenging activity. However, there were no significant correlations between TEAC, $O_2^-$, $H_2O_2$, and $ONOO^-$ scavenging activities and neuroprotective activity.

## DISCUSSION

The neuroprotective and antioxidative activities of five organosulfur compounds with a thioallyl structure (−S−$CH_2$-CH=$CH_2$) that originated from the same precursor, $\gamma$-glutamyl-S-allyl-L-cysteine in garlic bulb, were characterized in terms of structure−activity relationships. The present study clearly indicates that the presence of the alanyl group and lack of the oxo group are essential structural features for the manifestation of their neuroprotective activities against ischemic insults. In addition, in vivo neuroprotective activity exerted by five organosulfur compounds is closely associated with their $OH^-$ scavenging activities.

Efforts to relate the molecular/structural difference of five organosulfur chemicals with the observed neuroprotective data support the following viewpoints. First, among many aspects of molecular properties, the presence of the alanyl group at the R2 position in organosulfur compounds is the major attribute in protecting neuronal damage induced by ischemic insult. Because the alanyl moeity linking to sulfur has three potential nucleophiles, sulfur, nitrogen, and oxygen, the biochemical interactions of these moieties with redox sensitive signals and/or transcription factors are presumably involved in exerting their neuroprotective activity. Consistent with this hypothesis, ALI and SAC showed increased in vitro neuroprotective activity. Both ALI and SAC tended to have relatively low Clog $P$ ($\gamma$ =

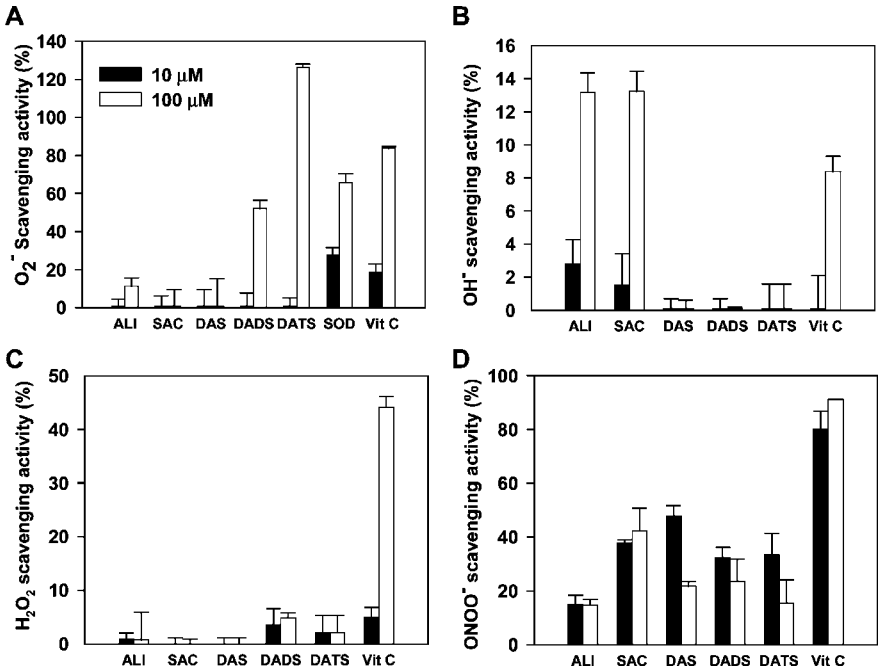


**Figure 4.** Effect of organosulfur compounds on scavenging of radicals. (**A**) $O_2^-$ scavenging activity, (**B**) $OH^-$ scavenging activity, (**C**) $H_2O_2$ scavenging activity, and (**D**) $ONOO^-$ scavenging activity. Values are means ± SEM.

**Table 2.** Correlation Coefficients between in Vivo Neuroprotective Activity and ROS Scavenging Activities of Organosulfur Compounds

| | neuroprotective activity | *p* value |
|---|---|---|
| TEAC value | −0.319[a] | 0.246 |
| $O_2^-$ scavenging activity | −0.418 | 0.121 |
| $OH^-$ scavenging activity | 0.555 | 0.032 |
| $H_2O_2$ scavenging activity | −0.484 | 0.068 |
| $ONOO^-$ scavenging activity | 0.198 | 0.479 |

[a] Values were expressed as a Pearson correlation coefficient ($\gamma$) between in vivo neuroprotective activity and ROS scavenging activity.

$-0.666$) and HIA ($\gamma = -0.612$) values, and high solubility ($\gamma = 0.877$), PSA ($\gamma = 0.569$), Balaban index ($\gamma = 0.725$), and total positive charge ($\gamma = 0.808$) values, which were significantly well-correlated with in vivo neuroprotective activity (data not shown). Conversely, DAS, DADS, and DATS, which are derived from ALI in crushed or minced garlic preparations and lack the alanyl group, were devoid of in vitro neuroprotective activity. These findings are consistent with another study using an in vivo focal ischemia model, in which DAS and DADS, ALI-derived sulfides, exacerbated ischemic brain injury in contrast to SAC (*16*). Quite the opposite, Moriguchi et al. (*15*) have reported that all organosulfur compounds containing the thioallyl group such as SAC, ALI, DAS, and DADS promoted the survival of rat hippocampal neurons in vitro, revealing the necessity of the allyl group being attached to the sulfur atom for manifestation of neurotrophic activity. From this discrepancy, it is reasonable to assume that different structural features may be associated with their neuroprotective and neurotrophic activities. However, more studies are required to clarify this assumption.

Second, the presence of the oxo (O═) group at the R1 position is another important attribute in determining neuroprotective activity and is found to be counteractive. ALI [$CH_2$═$CHCH_2S(O)$-$CH_2CH(NH_3^+)CO_2^-$], which differs from SAC only in the presence of the oxo group, was effective in protecting cell death induced by OGD in cultured SK-N-SH cells but was ineffective in reducing ischemic brain injury by two-vessel occlusion in gerbils. The presence of the oxo group in the structure of allylthio-2-aminopropionate, SAC, resulted in a decrease in Clog *P* and total negative charge and an increase in solubility, PSA, Blaban index, and total positive charge. Additionally, it is well-known that the S═O bond between the sulfur and the oxygen atoms is not a normal double bond, and it is believed that electrostatic interaction between a negatively charged oxygen and a positively charged sulfur accounts for most of the bonding. It thus seems to be a reasonable postulation that such molecular and bond natures may result in different neuroprotection in vivo.

Third, the number of sulfurs linked to allyl moiety is unlikely to affect neuroprotective activity against ischemic insult. None of the organosulfur compounds with different number of sulfurs linked to allyl moiety protected neuronal damage induced by in vitro and in vivo ischemic insult. In contrast to the present results, organosulfur compounds have been shown to modify the activation of chemical carcinogens or to control the growth of pathogenic microorganisms in proportion with the number of sulfurs (*29*, *30*). Recently, compounds containing sulfane sulfur including DADS and DATS have been known to play important roles in the cell regulation processes through activation or inactivation of several enzymes and in modification of intracellular redox potential (*31*).

ROS, such as $ONOO^-$ formed from the $O_2^-$ and NO and its decomposition product, $OH^-$, have been suggested to be imperative causative agents in the pathogenesis of brain ischemic damage (*32*, *33*). Our previous study reported that SAC inhibited cell death induced by $ONOO^-$ through scavenging $ONOO^-$ in glucose-deprived immunostimulated or glucose-deprived SIN-1-treated glial cells (*18*). We investigated the effect of molecular/structural differences in organosulfur compounds with a thioallyl structure on ROS scavenging activities in this study. The present data demonstrated that SAC dose dependently scavenged $ONOO^-$ up to a 100 $\mu$M concentration but DAS, DADS, and DATS scavenged up to a 10 $\mu$M concentration. The present results also indicated that ALI and SAC, representing a much higher water solubility due to the presence of the alanyl group, possess a very convincing $OH^-$ scavenging potential with almost

no ability to scavenge $O_2^-$ and $H_2O_2$ in vitro. In contrast, DAS, DADS, and DATS with higher Clog *P* values of 2.330−2.877 might be toward inactivation of $O_2^-$ rather than $OH^-$ and $H_2O_2$. The $O_2^-$ scavenging potential by allyl sulfides is likely to be related with the number of sulfurs, since the S atom is more polarizable than the O atom and the adjacent allyl group increases the electron density of the S atom providing a more nucleophilic nature to the S atom. These results are supported by some evidence showing that these organosulfur compounds scavenged radicals such as $O_2^-$ (*34*, *35*), $OH^-$ (*35*, *36*), and $ONOO^-$ (*16*). On the contrary, all five organosulfur compounds were ineffective or only slightly effective in scavenging $ABTS^{\bullet+}$, relative to that of the water-soluble vitamin E analogue, Trolox (TEAC value). In accordance with our result, Kosuge et al. (*37*) reported that SAC had an extremely low antioxidant activity expressed as a TEAC value.

The brain is particularly susceptible to radical-mediated neuronal damage because of high levels of oxygen consumption, unsaturated fatty acids, and iron stores, combined with low antioxidant resources. In this regard, the relationship between radical scavenging activity and neuroprotective activity was further investigated in this study. Significant correlation was found only between neuroprotective activity and $OH^-$ scavenging activity ($\gamma = 0.55$ and $p = 0.032$). OH radicals, which are formed nonenzymatically from $H_2O_2$ in a metal-dependent reaction, are the most reactive and toxic radicals known to date. In accordance with this, the neuroprotection was observed by challenging potent hydroxyl radical scavengers as reported (*38*, *39*). Furthermore, it appears that higher both $OH^-$ and $ONOO^-$ scavenging activities of organosulfur chemicals, especially SAC, tended to be associated with higher in vivo neuroprotective activities. In the case of possessing only a convincing $OH^-$ scavenging potential with almost no ability to scavenge $ONOO^-$, ALI did not correlate with the neuroprotective activity. Conversely, no significant correlated relationships between TEAC, $O_2^-$, $H_2O_2$, and $ONOO^-$ scavenging activities and neuroprotective activity were found. Nevertheless, further studies in vivo are needed to delineate the relationship between radical scavenging activity and neuroprotective activity.

Taken together, our results collectively demonstrate that the presence of the alanyl group and absence of the oxo group are essential for the manifestation of neuroprotective activity against ischemic insults and scavenging of OH radicals, with SAC surfacing as a potent neuroprotectant. Particularly, SAC is easily absorbed in the gastrointestinal tract and can be detected with plasma, liver, and kidney after oral intake, with a bioavailability of 103% in mice, 98% in rats, and 87.2% in dogs (*40*). Furthermore, it is reported to have low toxicity in mice and rat ($LD_{50}$ value > 8890 mg/kg, p.o.; 3225 mg/kg, i.p.) and be synthesized in an easy and cost-effective manner (*41*, *42*). Such characteristics of SAC together with its structural congeniality for manifesting the neuroprotection could provide a therapeutic strategy as a dietary supplement.

## LITERATURE CITED


(1) Flamm, E. S.; Demopoulos, H. B.; Seligman, M. L.; Poster, G. R.; Ransohoff, J. Free radical in cerebral ischemia. *Stroke* **1978**, *9*, 445−447.

(2) Sakamoto, A.; Ohnishi, S. T.; Ohnishi, T.; Ogawa, R. Relationship between free radical production and lipid peroxidation during ischemia-reperfusion injury in the rat brain. *Brain Res.* **1991**, *554*, 186−192.

(3) Abe, K.; Yuki, S.; Kogure, K. Strong attenuation of ischemic and postischemic brain edema in rats by a novel free radical scavenger. *Stroke* **1988**, *19*, 480−485.

(4) Carney, J. M.; Floyd, R. A. Protection against oxidative damage to CNS by $\beta$-phenyl-*tert*-butyl nitrone (PBN) and other spin-trapping agents: A novel series of nonlipid free radical scavengers. *J. Mol. Neurosci.* **1991**, *3*, 47−57.

(5) Floyd, R. A.; Garney, J. M. Free radical damage to protein and DNA: Mechanisms involved and relevant observations on brain undergoing oxidative stress. *Ann. Neurol.* **1992**, *32*, S22−S27.

(6) Knuckey, N. W.; Palm, D.; Primiano, M.; Epstein, M. H.; Johanson, C. E. N-Acetylcysteine enhances hippocampal neuronal survival after transient forebrain ischemia in rats. *Stroke* **1995**, *26*, 305−310.

(7) Kurata, M.; Okuda, M.; Muneyuki, M.; Ohnishi, S. T. $^{31P}$MRS study of the protective effects of prostaglandin oligomers on forebrain ischemia in rats. *Brain Res.* **1991**, *545*, 315−318.

(8) Ohnishi, S. T.; Tominaga, T.; Katsuoka, M. Inhibition of ischemic edema formation by post-ischemic administration of a prostaglandin oligomer. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **1989**, *37*, 107−111.

(9) Oishi, R.; Itoh, Y.; Nishibori, M.; Watanabe, T.; Nishi, H.; Saeki, K. Effect of MCI-186 on ischemia-induced changes in monoamine metabolism in rat brain. *Stroke* **1989**, *20*, 1557−1564.

(10) Yamasaki, T.; Ki, L.; Lau, B. H. Garlic compounds protect vascular endothelial cells from hydrogen peroxide-induced oxidant injury. *Phytother. Res.* **1994**, *8*, 408−412.

(11) Horie, T.; Murayama, T.; Mishima, T.; Itoh, F.; Minamide, Y.; Fuwa, T.; Awazu, S. Protection of liver microsomal membranes from lipid peroxidation by garlic extract. *Planta Med.* **1989**, *55*, 506−508.

(12) Horie, T.; Awazu, S.; Itakura, Y.; Fuwa, T. Identified diallyl polysulfides from an aged garlic extract which protects the membranes from lipid peroxidation. *Planta Med.* **1992**, *58*, 468−469.

(13) Kagawa, K.; Matsutaka, H.; Yamaguchi, Y.; Fukuhama, C. Garlic extract inhibits the enhanced peroxidation and production of lipids in carbon tetrachloride-induced liver injury. *Jpn. J. Pharmacol.* **1986**, *42*, 19−26.

(14) Lewin, G.; Popov, I. Antioxidant effects of aqueous garlic extract. Second communication: Inhibition of the Cu (2+)-initiated oxidation of low density lipoproteins. *Arzneim.-Forsch.* **1994**, *44*, 604−607.

(15) Moriguchi, T.; Masuura, H.; Kodera, Y.; Itakura, Y.; Katsuki, H.; Saito, H.; Nishiyama, N. Neurotrophic activity of organosulfur compounds having a thioallyl group on cultured rat hippocampal neurons. *Neurochem. Res.* **1997**, *22*, 1449−1452.

(16) Numagami, Y.; Ohnishi, S. T. S-allylcysteine inhibits free radical production, lipid peroxidation and neuronal damage in rat brain ischemia. *J. Nutr.* **2001**, *131*, 1100S−1105S.

(17) Numagami, Y.; Sato, S.; Ohnishi, S. T. Attenuation of rat ischemic brain damage by aged garlic extracts: A possible protecting mechanism as antioxidants. *Neurochem. Int.* **1996**, *29*, 135−143.

(18) Kim, J. M.; Lee, J. C.; Chang, N. S.; Chun, H. S.; Kim, W. K. S-Allyl-L-cysteine attenuates cerebral ischemic injury by scavenging peroxynitrite and inhibiting the activity of extracellular signal-regulated kinase. *Free Radic. Res.* **2006**, in revision.

(19) Chun, H. S.; Chang, H. J.; Choi, E. H.; Kim, H. J.; Ku, K. H. Molecular and absorption properties of 12 soy isoflavones and their structure-activity relationship with selected biological activities. *Biotechnol. Lett.* **2005**, *27*, 1105−1111.

(20) Kim, H. J.; Chun, H. S.; Yang, R. Use of a cyclodextrin for increased protective activity of volatile allyl sulfides against benzo[a]pyrene-induced toxicity in human cell model. *Biotechnol. Lett.* **2001**, *23*, 583−587.

(21) Ka, M. H.; Choi, E. H.; Chun, H. S.; Lee, K. G. Antioxidative activity of volatile extracts isolated from Angelica tenuissimae roots, peppermint leaves, pine needles, and sweet flag leaves. *J. Agric. Food Chem.* **2005**, *53*, 4124−4129.

(22) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying and improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231−1237.

(23) Robak, J.; Gryglewsk, I. R. Flavonoids are scavenger of superoxide anions. *Biochem. Pharmacol.* **1988**, *37*, 837−841.
(24) Halliwell, B.; Gutteridge, J. M. C.; Aruoma, O. I. The deoxyribose method: A simple "test tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Anal. Biochem.* **1987**, *26*, 215−219.
(25) Aruoma, O. I.; Halliwell, B.; Hoey, B. M.; Butler, J. The antioxidant action of N-acetyl cysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide and hypochlorous acid. *Free Radic. Biol. Med.* **1989**, *6*, 593−597.
(26) Thiyagarajanm, M.; Sharma, S. S. Neuroprotective effect of curcumin in middle cerebral artery occlusion induced focal cerebral ischemia in rats. *Life Sci.* **2004**, *74*, 969−985.
(27) Clark, D. E. Rapid calculation of polar molecular surface area and its application to the prediction of transport phenomena. 1. prediction of intestinal absorption. *J. Pharm. Sci.* **1999**, *88*, 807−814.
(28) van de Waterbeemd, H.; Camenisch, G.; Folkers, G.; Chretien, J. R.; Raevsky, O. A. Estimation of blood-brain barrier crossing of drugs using molecular size and shape, and H-bonding descriptors. *J. Drug Target* **1998**, *6*, 151−165.
(29) Kim, H. J.; Chun, H. S. Structure-activity relationships of organosulfur compounds as inhibitors of cytochrome P450 1-mediated activation of benzo[a]pyrene in a human cell model. *Biotechnol. Lett.* **2002**, *24*, 2041−2046.
(30) Chun, H. S.; Kim, H. J.; Choi, E. H. Modulation of cytochrome P4501-mediated bioactivation of benzo[a]pyrene by volatile allyl sulfides in human hepatoma cells. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 2205−2212.
(31) Toohey, J. I. Sulfane sulfur in biological systems: a possible regulatory role. *Biochem. J.* **1989**, *264*, 625−632.
(32) Irving, E. A.; Bamford, M. Role of mitogen- and stress-activated kinases in ischemic injury. *J. Cereb. Blood Flow Metab.* **2002**, *6*, 631−647.
(33) Cuzzocrea, S.; Reiter, R. J. Pharmacological action of melatonin in shock, inflammation and ischemia/reperfusion injury. *Eur. J. Pharmacol.* **2001**, *426*, 1−10.
(34) Ou, C. C.; Tsao, S. M.; Lin, M. C.; Yin, M. C. Protective action on human LDL against oxidation and glycation by four organosulfur compounds derived from garlic. *Lipids* **2003**, *38*, 219−224.
(35) Kim, K. M.; Chun, S. B.; Koo, M. S.; Chio, W. J.; Kim, T. W.; Kwon, Y. G.; Chung, H. T.; Billiar, T. R.; Kim, Y. M. Differential regulation of NO availability from macrophages and endothelial cells by the garlic component S-allyl cysteine. *Free Radic. Biol. Med.* **2001**, *30*, 747−756.
(36) Rabinkov, A.; Miron, T.; Konstantinovski, L.; Willchek, M.; Mirelman, D.; Weiner, L. The mode of action of allicin: trapping of radicals and interaction with thiol containing proteins. *Biochem. Biophys. Acta* **1998**, *1379*, 233−244.
(37) Kosuge, Y.; Koen, Y.; Ishige, K.; Minami, K.; Urasawa, H.; Saito, H.; Ito, Y. S-Allyl-L-cysteine selectively protects cultured rat hippocampal neurons from amyloid $\beta$-protein- and tunicamycin-induced neuronal death. *Neuroscience* **2003**, *122*, 885−895.
(38) Cauquil-Caubère, I.; Kamenka, J. M. New structures able to prevent the inhibition by hydroxyl radicals of glutamate transport in cultured astrocytes. *Eur. J. Med. Chem.* **1998**, *33*, 867−877.
(39) Chen, C.; Wei, T. T.; Gao, Z. H.; Zhao, B. L.; Hou, J. W.; Xu, H. B.; Xin, W. J.; Packer, L. Different effects of the constituents of EGb 761 on apoptosis in rat cerebellar granule cells induced by hydroxyl radicals. *Mol. Biol. Int.* **1999**, *47*, 397−405.
(40) Nagae, S.; Ushijima, M.; Hatono, S.; Imai, J.; Kasuga, S.; Matsuura, H.; Itakura, Y. Pharmacokinetics of the garlic compound *S*-allylcysteine. *Planta Med.* **1994**, *60*, 214−217.
(41) Kodera, Y.; Suzuki, A.; Imada, O.; Kasuga, S.; Sumioka, I.; Kanezawa, A.; Taru, N.; Fujikawa, M.; Nagae, S.; Masamoto, K.; Maeshige, K.; Ono, K. Physical, chemical, and biological properties of *S*-allylcysteine, an amino acid derived from garlic. *J. Agric. Food Chem.* **2002**, *50*, 622−632.
(42) Lawson, L. D.; Wang, Z. J.; Hughes, B. G. $\gamma$-Glutamyl-*S*-alkyl-L-cysteines in garlic and other *Allium* spp.: Precursors of AGE-dependent *trans-1*-propenyl. *J. Nat. Prod.* **1991**, *54*, 436−444.

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