BPB Reports

Report

Oleanolic Acid-3-(1′2′Orthoacetate-Glucoside)-28-Glucoside Alleviates Methylmercury Toxicity *in Vitro* and *in Vivo*

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Received July 27, 2019; Accepted August 14, 2019

Methylmercury (MeHg) is one of the most toxic environmental pollutants and causes serious health hazards worldwide. Recently, we demonstrated that oleanolic acid (OA) 3-glucoside (OA3Glu), a saponin derivative in which glucose is bound to the C3 position of OA, has anti-MeHg activity by suppressing Hg accumulation in organs of mice. In this study, we examined the anti-MeHg activity of OA-3-(1′2′orthoacetate-Glu)-28-Glu in which glucose is bound to the C3 position of OA in a different binding form from that in OA3Glu. We found that OA-3-(1′2′orthoacetate-Glu)-28-Glu suppressed cellular MeHg uptake and improved cell viability upon exposure to MeHg in Caco-2 cells. To verify the *in vivo* anti-MeHg activities of OA-3-(1′2′orthoacetate-Glu)-28-Glu, mice were orally administered MeHg (0.02, 1.0, or 5.0 mg·kg^{-1}·d^{-1}), with or without OA-3-(1′2′orthoacetate-Glu)-28-Glu. The mice cotreated with 0.02 mg·kg^{-1}·d^{-1} MeHg and OA-3-(1′2′orthoacetate-Glu)-28-Glu showed significantly lower Hg content in the liver and kidney than those treated with MeHg alone. In addition, interleukin (IL)-1β and IL-6 levels in the brain of mice cotreated with 5.0 mg·kg^{-1}·d^{-1} MeHg and OA-3-(1′2′orthoacetate-Glu)-28-Glu were significantly lower than those of mice treated with MeHg alone. These results suggested that OA-3-(1′2′orthoacetate-Glu)-28-Glu had potential as an anti-MeHg accumulation compound, owing to its ability to suppress MeHg distribution into organs especially under low-level MeHg exposure condition. Taken together, it was suggested that glucose binding to the C3 position of OA is important for anti-MeHg activity of OA saponin derivatives.

Key words anti-methylmercury activity, distribution, oleanane-type triterpene saponin, low-level methylmercury exposure

INTRODUCTION

Methylmercury (MeHg) is a widely distributed compound in nature and one of the most toxic environmental pollutants that causes neurotoxic effects.[1,2] MeHg is absorbed in the intestines, distributed to all tissues in the body, and accumulated in various organs such as brain, liver, kidney, and developing fetus.[3,4] It is important to understand the effects of low-dose MeHg to which humans are exposed to through food intake as well as those of high-dose exposure. Recently, we have suggested that the immunotoxic effect on T helper 2 (Th2) responses to low-dose MeHg is negligible in ovalbumin- or mite-induced Th2 allergy mouse models.[5] Moreover, we have shown that a low concentration of MeHg activated autophagy, which functions as protective response for cell survival, in various cell types.[6,7] To date, very little is understood about the effects of low-dose MeHg exposure on animals and humans, especially through daily food intake. Furthermore, anti-MeHg medicines are in demand.

Saponins are natural glycosides of steroid or triterpene, which exhibit many different biological and pharmacological actions such as immunomodulatory, antitumor, and anti-inflammatory.[8–11] Saponins have a diverse range of characteristics, which include sweetness, bitterness,[12–14] foaming, and hemolytic properties.[15,16] Wide application of saponins in beverages and confectionery as well as in pharmaceutical products is not uncommon.[8–11] It is believed that saponins form the main constituents of many phototherapies and folk medicines and they are considered to be responsible for numerous pharmacological properties.[16]

Onjisaponins, which are derived from the roots of *Polygala tenuifolia* Willd., are saponin derivatives with OA as an aglycon and have various pharmacological activities such as induction of nerve growth factor (NGF) synthesis and enhancement of autophagy.[17,18] Since NGF and autophagy contribute to cell protection against MeHg toxicity,[6,19] these activities of onjisaponins suggest the potential of OA saponin derivatives to have anti-MeHg poisoning properties. However, it is difficult to isolate enough target saponin compound from plant material for clinical and animal experiments due to the structural similarity of onjisaponins. Therefore, in the previous study, we synthesized OA saponin derivatives and compared their anti-MeHg activity; as a result, we demonstrated that OA 3-glucoside (OA3Glu) in which glucose is bound to the C3 position of OA exhibits anti-MeHg activity.[20] In the process of OA saponin derivative synthesis, we obtained OA-3-(1′2′orthoacetate-glucoside)-28-glucoside [OA-3-(1′2′orthoacetate-Glu)-28-Glu], an orthoester saponin that differs from OA3Glu in the glucose binding form to the C3 position of OA, as a by-product. In this study, to further understand how anti-MeHg activ-

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ity of OA derivatives differing in the binding form of glucose at the C3 position of OA varies, the anti-MeHg activity of OA-3-(1′2′orthoacetate-Glu)-28-Glu was examined *in vitro* and *in vivo.*

MATERIALS AND METHODS

Cell Culture The human colon carcinoma (Caco-2) cell line was obtained from the European Collection of Cell Cultures (ECACC number 86010202; UK). The cells were maintained in minimum essential medium supplemented with 20% (v/v) fetal bovine serum, 0.1 mM non-essential amino acids, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were incubated at 37 °C in an atmosphere with 95% relative humidity and 5% CO_2.

Cell Viability Assay Caco-2 cells (1×10^4 cells/well) were seeded in 96-well plates. MeHg (Tokyo Chemical Industry, Tokyo, Japan) and OA-3-(1′2′orthoacetate-Glu)-28-Glu were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the samples was 0.1%. After 24 h of incubation, the cells were pretreated for 1 h with OA-3-(1′2′orthoacetate-Glu)-28-Glu (0.032, 0.32, or 3.2 μM), and then exposed to 5 μM MeHg for 24 h at 37 °C. Then, a cell counting kit (Nacalai Tesque, Kyoto, Japan) was used to measure cell viability according to the manufacturer's instructions. Briefly, MTT solution, including tetrazolium salt was added to each well and the cells were incubated at 37 °C for 2 h, and then solubilization solution (isopropanol with 0.04 mol/L HCl) was added to each well. The number of viable cells was quantified by measuring the absorbance of solution at 450 nm using an iMark microplate reader (Bio-Rad, Hercules, CA, USA).

Measurement of Incorporated Radiolabeled MeHg [^{14}C]-CH_3HgCl was obtained from Amacham (Bucks, UK). Caco-2 cells (2×10^5 cells/well) were seeded in a 24-well plate and cultured to confluence. The cells were then treated with OA-3-(1′2′orthoacetate-Glu)-28-Glu (0.032, 0.32, or 3.2 μM) for 20 min, followed by exposure to 5 μM [^{14}C]-CH_3HgCl (2.11 GBq/mmol) for 30 min at 37 °C. The cells were washed three times with Hanks' balanced salt solution (Nacalai Tesque), and then lysed with 1 M sodium hydroxide. The lysates were neutralized with hydrochloric acid, and their radioactivity was measured using a liquid scintillation spectrometer A310001 (PerkinElmer, Waltham, MA, USA).

Animal Experiment Specific pathogen-free, 8-week-old male NC/Nga mice were purchased from Japan SLC (Shizuoka, Japan). The mice were allowed to acclimatize for 7 d upon arrival and allocated randomly to experimental groups while being housed in an animal facility that was maintained at 24 to 26°C with 55 to 75% humidity and a 12-h light/dark cycle and provided with food (Japan Crea, Tokyo, Japan) and water. After the acclimation period, the mice were randomly divided into eight experimental groups of 7–8 animals each: (1) vehicle control (0.5% DMSO); (2) OA-3-(1′2′orthoacetate-Glu)-28-Glu (4 $mg \cdot kg^{-1} \cdot d^{-1}$); (3) MeHg (0.02 $mg \cdot kg^{-1} \cdot d^{-1}$); (4) MeHg (0.02 $mg \cdot kg^{-1} \cdot d^{-1}$) + OA-3-(1′2′orthoacetate-Glu)-28-Glu (4 $mg \cdot kg^{-1} \cdot d^{-1}$); (5) MeHg (1 $mg \cdot kg^{-1} \cdot d^{-1}$); (6) MeHg (1 $mg \cdot kg^{-1} \cdot d^{-1}$) + OA-3-(1′2′orthoacetate-Glu)-28-Glu (4 $mg \cdot kg^{-1} \cdot d^{-1}$); (7) MeHg (5 $mg \cdot kg^{-1} \cdot d^{-1}$); and (8) MeHg (5 $mg \cdot kg^{-1} \cdot d^{-1}$) + OA-3-(1′2′orthoacetate-Glu)-28-Glu (4 $mg \cdot kg^{-1} \cdot d^{-1}$). The mice were administered OA-3-(1′2′orthoacetate-Glu)-28-Glu and after 1 h, received the corresponding dose of MeHg (0.02, 1 or 5 $mg \cdot kg^{-1} \cdot d^{-1}$). Each solution was orally administered at a volume of 4 mL/kg five times a week for 4 weeks. Twenty-four hours after the last MeHg treatment, the mice were anesthetized with somnopentyl (pentobarbital sodium; Kyoritu Seiyaku, Tokyo, Japan), and then euthanized by exsanguination. Subsequently the brain, kidney, and liver were collected and stored at -80 °C. All experimental procedures followed in this study conformed to the Procedures for Animal Experiments and the Guiding Principles for the Care and Use of Laboratory Animals and were approved by the Committee for Animal Experiments at the Kitasato University.

Measurement of Hg Content in Animal Organs The kidneys and liver were excised, weighed, and homogenized in 3 mL of 10 mM potassium phosphate buffer (pH 7.4). The homogenates were digested with a concentrated acid mixture (nitric acid:perchloric acid = 4:1) for 2 h at 160 °C, and the total Hg content was measured using an atomic absorption spectrometry analyzer HG-310 (Hiranuma, Ibaraki, Japan). All results are expressed as ng mg^{-1} (ppm) dry weight.

Quantification of Brain Cytokine Levels by Enzyme Linked Immunosorbent Assay (ELISA) Brains were excised, weighed, flash frozen in liquid nitrogen, and stored at −80°C until required for further processing. Frozen brain tissues were homogenized in 3 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (Sigma Aldrich, St. Louis, MO), 0.1 mM phenylmethanesulfonyl fluoride (Nacalai Tesque), 1 μM pepstatin A (Peptide Institute, Osaka, Japan), and 2 μM leupeptin (Peptide Institute). The homogenates were then centrifuged at 105,000 × g for 1 h. The supernatants were stored at −80°C until analysis by ELISA. This analysis was done to measure interleukin (IL)-1β (R&D Systems, Minneapolis, MN, USA) and IL-6 (Biolegend, San Diego, CA, USA) in brain homogenates, according to the manufacturer's instructions. The absorbance was read in a microplate reader at 450 nm. The control reading at 550 nm was then subtracted. The ELISA results were converted to pg/mL, using values obtained from standard curves generated by assay of varying concentrations of recombinant IL-1β and IL-6.

Statistical Analyses The data are presented as mean ± standard error of the mean (SE). Differences between groups were evaluated by one-way analysis of variance (ANOVA). If the differences were significant, Tukey–Kramer post hoc test was used to compare each treatment group with the vehicle control. The results with a p-value of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

OA-3-(1′2′orthoacetate-Glu)-28-Glu was synthesized by deprotection of the C3-TES group in C28-acetylated glucoside as previously reported,[21] followed by preparation of orthoacetate under usual glycosylation condition with acetylated imidate donor, deprotection, and careful purification (Supplemental information).

To evaluate the effects of OA-3-(1′2′orthoacetate-Glu)-28-Glu on MeHg tolerance of cells, we conducted the cell viability assay. We used Caco-2 cells derived from the intestinal tract, which is the major route of exposure to MeHg in humans and animals. After MeHg exposure, cell viability was reduced to 60.8% compared with that in the control group. Treatment with 0.03 and 0.3 μM OA-3-(1′2′orthoacetate-Glu)-28-Glu significantly improved cell viability compared with OA-3-

(1′2′orthoacetate-Glu)-28-Glu untreated group (Fig. 1B). Furthermore, MeHg accumulation was measured in Caco-2 cells to elucidate whether OA-3-(1′2′orthoacetate-Glu)-28-Glu affected cellular MeHg uptake. Treatment with 3 μM OA-3-(1′2′orthoacetate-Glu)-28-Glu significantly reduced intracellular MeHg accumulation to 77.7% compared with that of the control values (Fig. 1C). Our previous study demonstrated that OA3Glu, a synthetic OA saponin derivative, attenuates MeHg-induced cell death by suppressing cellular MeHg accumulation. In this study, OA-3-(1′2′orthoacetate-Glu)-28-Glu showed similar effects as OA3Glu. As both compounds have glucose in common at the C3 position of OA, glucose bound to the C3 position of OA might be important for suppressing MeHg-induced cell death by diminishing intracellular accumulation of MeHg *in vitro*.

To evaluate the therapeutic effects of OA-3-(1′2′orthoacetate-Glu)-28-Glu *in vivo*, we treated the mice in our test groups with OA-3-(1′2′orthoacetate-Glu)-28-Glu, and then exposed them to low dose (0.02 $mg \cdot kg^{-1} \cdot d^{-1}$: a dose that is at human exposure levels in Hg-contaminated areas), intermediate dose (1 $mg \cdot kg^{-1} \cdot d^{-1}$: a dose that did not exhibit apparent toxicity in NC/Nga mice in a previous study),[5] or high dose (5 $mg \cdot kg^{-1} \cdot d^{-1}$: a dose at which toxicity appears in this line of mice) of MeHg. After the experimental period, the total Hg content was measured in the liver and kidneys. At all MeHg exposure concentrations, the total Hg accumulation in the OA-3-(1′2′orthoacetate-Glu)-28-Glu-cotreated group was lower than that in the MeHg group in both organs. Especially, under the low-dose MeHg exposure condition, Hg accumulation in the OA-3-(1′2′orthoacetate-Glu)-28-Glu-cotreated group was significantly lower than that in the OA-3-(1′2′orthoacetate-Glu)-28-Glu-untreated group in both organs. In the OA-3-(1′2′orthoacetate-Glu)-28-Glu-cotreated group, the total Hg content in the liver and kidney was 2.6% and 11.8% compared with that in the OA-3-(1′2′orthoacetate-Glu)-28-Glu-untreated group, respectively (Table 1). These results indicate a possibility that OA-3-(1′2′orthoacetate-Glu)-28-Glu alleviates low-dose MeHg toxicity by suppressing Hg accumulation. We previously reported that OA3Glu suppressed Hg accumulation in organs under intermediate-dose MeHg exposure condition, while OA3Glu hardly affected Hg levels in organs under low-dose MeHg exposure condition.[20] The results suggested that OA-3-(1′2′orthoacetate-Glu)-28-Glu is more effective than OA3Glu against the toxicity of low concentration MeHg exposure to which humans are exposed through daily food intake.

As MeHg has been reported to cause neuropathy by inducing inflammatory cytokine production in the brain,[22,23] we examined the effect of OA-3-(1′2′orthoacetate-Glu)-28-Glu on pro-inflammatory cytokine production, such as IL-1β and IL-6, in the brain. In OA-3-(1′2′orthoacetate-Glu)-28-Glu untreated mice, the levels of both pro-inflammatory cytokines were elevated with increasing MeHg exposure level, suggesting that inflammation occurred in the brain due to exposure to high concentrations of MeHg. Under 5 $mg \cdot kg^{-1} \cdot d^{-1}$ MeHg exposure condition, the levels of IL-1β and IL-6 in the brain of OA-3-(1′2′orthoacetate-Glu)-28-Glu-treated mice were significantly lower than those of OA-3-(1′2′orthoacetate-Glu)-28-Glu untreated mice (Fig. 2). The results suggested that OA-3-(1′2′orthoacetate-Glu)-28-Glu attenuated MeHg-induced neurotoxicity by suppressing the production of pro-inflammatory cytokines such as IL-1β and IL-6. These results indicate that OA-3-(1′2′orthoacetate-Glu)-28-Glu alleviates low-dose MeHg toxicity by suppressing Hg accumulation and mitigates neuropathy caused by high-dose MeHg exposure. As OA-3-(1′2′orthoacetate-Glu)-28-Glu inhibited Hg accumulation in organs under high- and low-concentration MeHg exposure conditions (Table 1), it was hypothesized that the major anti-MeHg action mechanism of OA-3-(1′2′orthoacetate-Glu)-28-Glu was the inhibition of MeHg uptake in the evaluated organs.

In this study, OA-3-(1′2′orthoacetate-Glu)-28-Glu reduced the cellular accumulation of MeHg (Fig. 1B). As it has been reported that transporters such as multidrug resistance-associated proteins and the L-type amino acid transporters are involved in the intake or excretion of cellular MeHg,[24–28] it is possible that OA-3-(1′2′orthoacetate-Glu)-28-Glu affects

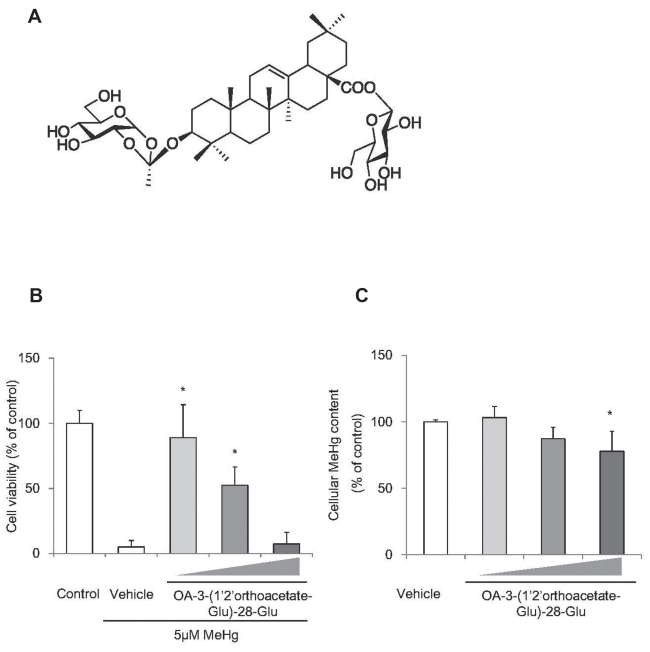

Fig. 1. Effects of OA-3-(1′2′Orthoacetate-Glu)-28-Glu on MeHg Toxicity in Caco-2 Cells

(A) Structure of OA-3-(1′2′orthoacetate-Glu)-28-Glu. (B) Cytotoxicity of MeHg in cells pretreated with OA-3-(1′2′orthoacetate-Glu)-28-Glu (0.032, 0.32, or 3.2 μM) for 1 h and then exposed to MeHg (5 μM) for 24 h and evaluated by MTT assay. (C) Cellular accumulation of Hg in cells pretreated with OA-3-(1′2′orthoacetate-Glu)-28-Glu (0.032, 0.32, or 3.2 μM) for 20 min and then exposed to 1 μM $^{14}CH_3HgCl$ for 30 min. Results are expressed as the mean ± SE of three determinations. *p<0.01 compared with vehicle control, #p<0.05 compared with OA-3-(1′2′orthoacetate-Glu)-28-Glu untreated.

Table 1. Total Hg Content of Liver and Kidney

Experimental group		Total Hg content (ppm)	
MeHg (mg/kg)	OA-3-(1′2′orthoacetate-Glu)-28-Glu	liver	kidney
0	-	0.23 ± 0.22	1.99 ± 1.83
	+	0.00 ± 0.00	0.09 ± 0.01
0.02	-	0.38 ± 0.15	5.65 ± 1.06
	+	0.01$^{\#}$ ± 0.11	0.67$^{\#\#}$ ± 0.06
1.0	-	1.89* ± 0.74	31.3** ± 5.3
	+	1.73* ± 0.37	28.2** ± 3.3
5.0	-	13.4** ± 3.5	77.4** ± 17.0
	+	9.5** ± 5.1	73.7** ± 28.3

*p<0.05, **p<0.01 vs. vehicle control
$^{\#}p$<0.05, $^{\#\#}p$<0.01 vs. OA-3-(1′2′orthoacetate-Glu)-28-Glu untreated

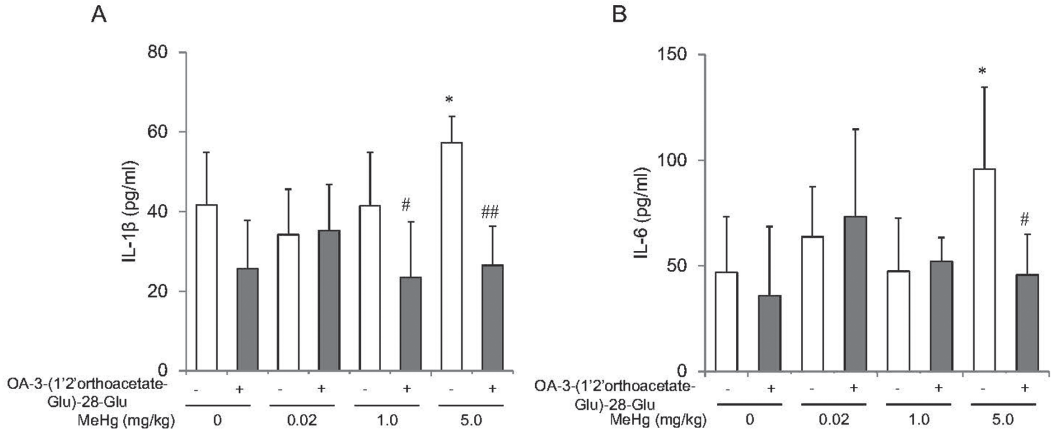

Fig. 2. Effects of OA-3-(1′2′Orthoacetate-Glu)-28-Glu on Levels of Inflammatory Cytokines in the Brain of MeHg Exposed Mice

Brain tissue samples of each mouse were harvested 1 d after the last oral administration of MeHg. The levels of IL-1β (A) and IL-6 (B) in the brain tissue supernatants were measured by ELISA. Results are expressed as the mean ± SE (n = 4–6). *p<0.05 compared with vehicle control, #p<0.05 and ##p<0.01 compared with OA-3-(1′2′orthoacetate-Glu)-28-Glu untreated.

MeHg uptake mediated by these transporters. Because some onjisaponins also reportedly induced NGF production and autophagy in various cell types,[17,18] it is important to examine the effects of OA-3-(1′2′orthoacetate-Glu)-28-Glu on these growth factors to clarify the mechanism of their anti-MeHg accumulation action. In addition, OA-3-(1′2′orthoacetate-Glu)-28-Glu has two glucose units in its structure, which could possibly allow its transfer into cells via glucose transporters and exert anti-MeHg effects. This possibility is supported by our previous results that α-hederin, which has arabinose and rhamnose instead of glucose, showed no anti-MeHg activity.[20] Further investigation is necessary to elucidate the underlying mechanisms of OA-3-(1′2′orthoacetate-Glu)-28-Glu's anti-MeHg activity.

In our previous study, OA 3-glucoside, with glucose at the C3 position of OA exhibited anti-MeHg activity, while OA 3,28-diglucoside, with glucose at the C3 and C28 positions of OA, had no anti-MeHg activity.[20] These results indicate that glucose at the C3 position of OA is important for anti-MeHg activity of OA saponin compounds, while its action may be hindered by glucose at the C28 position of OA. On the contrary, in this study, OA-3-(1′2′orthoacetate-Glu)-28-Glu, with glucose at the C3 and C28 positions of OA, similar to OA 3,28-diglucoside, exhibited anti-MeHg activity. These results indicate that a unique binding form of the orthoester of OA-3-(1′2′orthoacetate-Glu)-28-Glu may prevent the inhibition of anti-MeHg activity of glucose of C28 of OA. It is necessary to study differences in the steric structure of the compound and the direct interaction between the compound and MeHg.

It has been reported that prenatal exposure to MeHg adversely affects neurobehavioral function in humans.[29] In addition, low-level MeHg exposure (an amount corresponding to the provisional tolerable weekly intake (PTWI) from fish consumption in Japan) during adulthood has been reported to induce temporary sympathodominant state.[17] Our results indicate that OA-3-(1′2′orthoacetate-Glu)-28-Glu treatment can effectively suppress MeHg toxicity under conditions of repeated exposure to lower concentrations of MeHg, which corresponds to situations faced in daily life, through regular ingestion of aquatic and marine animals with considerable MeHg accumulation.

Acknowledgments We thank Ms. N. Sato and Dr. K. Nagai at Kitasato University for various instrumental analyses. We kindly thank Dr. Tadashi Adachi (Mitsubishi Chemical Corporation) for providing DIAION™ HP20 and for helpful discussion. We also thank Mr. Y. Kondoh, Mr. R. Nakagawa, Ms. E. Asami, Ms. K. Kojima, Mr. N. Kida and Mr. T. Nishiyama, for technical assistance. This work was supported in part by Grant-in-Aid for Young Scientists (B) (no. 15K18887 and no. 17K15468) to R. N., (no. 20790017 and no. 24790021) to T. S., Challenging Exploratory Research (Grant Number 16K15381) to M. K., Kitasato University Research Grant for Young Researchers to R. N, and the Uehara Memorial Foundation to T. S.

Conflict of interest The authors declare no conflict of interest.

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