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In Vivo Evaluation of 4-Methyl-2, 4-bis(4-hydroxyphenyl) pent-1-ene (MBP), a Bisphenol A Metabolite, Using the Nematode *Caenorhabditis elegans*

Shinya KOHRA*,Daisuke INOUE**, Akemi YAMAGUCHI***, Yuji TAKAO*, Nobuaki TOMINAGA*, Koji ARIZONO**** and Kazuo UEDA*

Abstract: In this study, we investigated the lethal and sublethal effects of 4-methyl-2 ,4-bis(4-hydroxyphenyl)-pent-1-ene (MBP), a bisphenol A (BPA) metabolite, using the nematode *Caenorhabditis eleganas* (*C. elegans*) grown on Nematode Growth Medium (NGM) 1.7% agar plates. The bioconcentration factor (BCF) was also determined. The 24 hr-median lethal concentration (24 hr-LC_{50}) value of MBP was 0.49 mM (134 mg/l), and the acute toxicity of MBP against *C. elegans* was slightly stronger than that of BPA. In contrast to BPA, the sublethal toxicity of MBP in a multi-generation test, as determined by reproduction and fecundity, was slightly lower. The growth of animals, as determined by body length, was enhanced by 3–8% at 0.1 mM MBP, after 48–72 hr. The BCF value after exposure for 48 hr was determined to be approximately 19–105 as compared with the nominal concentration.

Key words : *Caenorhabditis elegans*, metabolite, bisphenol A, bioconcentration factor

INTRODUCTION

2,2-Bis(4-hydroxyphenyl)propane (Bisphenol A, BPA) is an endocrine disrupting chemical (EDC) that is routinely ingested by humans.[1] Recently, Yoshihara *et al.* reported that a BPA metabolite, namely 4-methyl-2, 4-bis (4-hydroxyphenyl) pent-1-ene (MBP), displayed stronger estrogenic activity in an *in vitro* assay than BPA itself.[2] Therefore, MBP has become one of the most prominent chemicals in studies of EDCs. However, only a few attempts have been made so far for testing MBP in an *in vivo* assay. In this study, we investigated the lethal and sublethal effects of MBP in *Caenorhabditis eleganas* (*C. elegans*), which is an organism used by many researchers in aquatic, sediment, and soil toxicity tests.[4-9]

MATERIALS AND METHODS

Chemicals ——— The sources of chemicals used in this study were as follows: Agar, cholesterol, dimethyl sulfoxide (DMSO), and sodium chloride were purchased from Wako Pure Chem. (Japan). Bacto yeast extract and bacto-tryptone were supplied by Becton Dickinson (MD, USA). *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) obtained from Spelco Ltd., (USA) was used as the chemical reagent for silylation. Unless stated otherwise, the other reagents for analyzing pesticide residues were of analytical reagent grade (Kanto Chemical, Japan). MBP was synthesized according to the method described by Dai *et al.*[10]

Animals and Culture Conditions ——— The wild-type N2 strain of *C. elegans* was used in this study. Worms were maintained and handled as described by Brenner.[11] Briefly, five to ten worms were grown on a Nematode Growth Medium (NGM) agar plate with a lawn of *Escherichia coli* (*E. coli*) as a food source, and incubated at 20 °C. Every four or five days, the worms were sub-cultured to new plates.

Synchronized Cultures of *C. elegans* ——— Worms were washed out from the NGM agar plates using M9 buffer and were subsequently washed twice with this buffer. They were then synchronized by the alkali-bleaching method. An appropriate number of eggs was transferred on a agar plate without food and incubated at 20 °C for 20 hr. Over 90% of these eggs hatched, and *C.*

* Faculty of Environmental Sciences, Nagasaki University
** Graduate School of Science and Technology, Nagasaki University
*** Department of Chemical and Biochemical Engineering, Ariake National College of Technology
****Faculty of Environmental and Symbiotic Sciences, Kumamoto Prefectural University
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elegans larvae were synchronized at the first instar (L1) larvae stage. This larval culture was used for the acute toxicity assay.

MBP Analysis ———

All the samples were prepared in the dark. Agar-extract samples were prepared as follows: Two grams of agar medium was taken in a vial with 2 g of Milli Q water, and 190 µl of 5 ppm BPA-d16 acetone solution was added as an internal standard. The mixture was vigorously shaken for 5 min. These samples were extracted with hexane (2 ml), and the organic layers were removed after centrifugation. One milliliter of 1 M hydrochloric acid solution was added to the aqueous layer, and MBP was extracted from the test solution by adding dichloromethane (3 × 5 ml) and vigorously shaking the sample for 30 min. Dichloromethane extracts were dried with anhydrous Na_2SO_4 and the eluent was then evaporated down to a volume of approximately 0.5 ml, under a stream of high-purity nitrogen.

Worm-extract samples were prepared as follows: The worms were washed out from the NGM plates using M9 buffer and were subsequently washed thrice with this buffer. These worms were crushed in an agate bowl, and the crushed worms were transferred into a new vial containing 30 ml of Milli-Q water using small amount of methanol (ca. 2 ml). An internal standard, 190 µl of 5 ppm BPA-d16 acetone solution, was added to these samples, and the mixture was vigorously shaken for 5 min. These samples were extracted with hexane (10 ml) and the organic layers were removed after centrifugation. One milliliter of 1 M hydrochloric acid solution was added to the aqueous layer, and MBP was extracted from it by adding dichloromethane (3 × 10 ml) and vigorously shaking the sample for 30 min. Dichloromethane extracts were dried with anhydrous Na_2SO_4, and the eluent was evaporated down to a volume of approximately 0.5 ml under a stream of high-purity nitrogen.

Two hundred microliters of BSTFA was added to each of these MBP extracts for the silylation of MBP and BPA-d16. The resultant sample was sealed and left to stand for 1 hr in the dark, at room temperature, to allow the reaction to proceed. The conditions of gas chromatography mass analysis equipment were performed as Takao reported.[3] The initial and final MBP concentrations of test plates under the experimental conditions were measured. The concentrations were expressed as mean value of initial concentration and final concentration of MBP in agar.

Acute Toxicity Test ——— The L1 larvae were transferred to NGM agar plates containing six concentrations (1 mM, 0.75 mM, 0.5 mM, 0.25 mM, 0.1 mM, and 0 mM as control) of MBP. Approximately fifty worms were dispensed on each plate. After 1, 2, 3, 4, 24, and 48 hr of exposure, the total number of worms and the number of moving worms in each plate were counted under a dissecting microscope, to estimate the viability ratios. All experiments were carried out at 20 °C in triplicate at least three different times. The median lethal concentration (LC_{50}) was calculated using the PROBIT method.

Multi-Generation Test ——— *E. coli* DH5α was distributed on the test plates containing four concentrations (0.1 mM, 0.01 mM, 0.001 mM, and 0 mM as control) of MBP prior to the multi-generation test. The first generation L1 larvae were poured onto each plate in order to begin the test. When worms grew to the L4 larvae stage, one worm was transferred to a new plate of the same composition and incubated at 16 °C. The number of worms and eggs on the plates were counted under a dissecting microscope at 24-hr intervals. The first day was defined as the day when the first offspring was identified. The second generation worms were allowed to grow to the L4 larvae stage on the same plate as the first generation; then, one worm was transferred and sub-cultured to a new plate of the same composition. The above steps were repeated until the fourth generation was cultured. These experiments were performed in quintuplicate.

Growth Test ———The L1 larvae (mean body length: 289 ± 25 µm standard deviation; n = 105) were transferred to NGM agar plates containing four concentrations (0.2 mM, 0.1 mM, 0.01 mM, and 0 mM as control) of MBP. After 24, 48, and 72 hr at 20 °C, the L1 larvae were evaluated by determining the body length of the worms under a microscope.

Statistical Analyses ——— An analysis of variance (ANOVA) was used for the statistical evaluation of the data. Values of $p < 0.01$ and 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The initial measured MBP concentration was lower than the nominal concentration and the MBP concentration decreased during experiments. The plate with nominal concentration of 0.1 mM (26.8 mg/l) contained 0.069 mM (0.47-0.078, n=4) (18.5 mg/l) at beginning of the exposure experiment and 0.055 mM (0.038-0.063, n=4)(0.14.7 mg/l) after exposure for 48 hr.

For calculations of effects, average concentrations

of the measured beginning and end concentrations were used. The acute lethal toxicity test was examined, and the time-response plots and concentration-response plots showing the viability of *C. elegans* exposed to MBP are shown in Figs. 1 and 2. The viability rates at 8 hr and 24 hr were comparable (Fig. 1). Ura *et al.* reported a 24 hr-median lethal concentration (24 hr-LC_{50}) value of 325 mg/l for BPA.[12] In our study, the 24 hr-LC_{50} value for the worms was 0.49 mM (134 mg/l), corresponding to almost one-third that of BPA (Fig. 2). After metabolism, BPA was changed to a substance that has a stronger acute lethal toxicity against *C. elegans*. However, this observation was different between MBP and BPA with respect to the sublethal toxicity in a multi-generation test.

Previously, we reported that BPA affected the fecundity rate of *C. elegans* at a dose 10000-fold lower than the LC_{50}, and that the number of worms decreased significantly to approximately 50% of the control value at the fourth generation.[14] In contrast, when exposed to 0.1 mM of MBP a concentration that was one-fifth of the LC_{50}, the worm abundance from the first to the fifth generation did not differ significantly from the controls. After exposure on 0.1 mM MBP plates for 48 hr, the whole-body concentrations of MBP ranged from 0.5 to 1.5 mg/g. Therefore, the bioconcentration factor (BCF) of MBP on *C. elegans* was approximately 40 (19–56, n = 5) as compared with nominal concentration. After 48 hr on 0.01 mM MBP plates, the BCF had a mean of 57 (33–105, n = 3). It was proven that the MBP was incorporated or stored in the worms under this experimental condition. Takao *et al.* reported that MBP was easily degraded in sunlight or even UV light. For example, it was demonstrated that over 90% of the MBP present in water was degraded within 8 hr of exposure to sunlight [3]. These results suggest that the stability of MBP in animal body was higher than in the environment.

The result of the sublethal effects on the first and the forth generation are presented in Fig. 3. A detailed observation of the fourth generation shows that the order of the concentrations in terms of the means number of worms was 0.1 mM > 0.01 mM > 0.001 mM > control (Fig. 3 D). The peak period of ovulation was accelerated to the second day in the case of the fourth generation, as compared with first generation for which it was the third day (Fig. 3 C). It appeared that the reproduction and fecundity of *C. elegans* were enhanced in the presence of MBP.

The effect of MBP on growth is shown in Fig. 4. Nematode body length increased slightly (3–8%) in the presence of 0.1 mM and 0.2 mM MBP. These results indicate that low concentrations of MBP have no toxicity in terms of fecundity of *C. elegans*, but that weak concentrations enhance the growth. There has been a similar report on 4-nonylphenol (NP).[13] Höss *et al.* observed that the growth and reproduction of *C. elegans* were enhanced with NP concentrations of 66 mg/l and 40 mg/l, and these concentrations were 100-fold lower than the LC_{50} value. The maximum increase in body length in the presence of NP was 1.1 fold, and the number of offspring per worm was almost double after 72 hr exposure. In that case, the effects showed dose-response relationships. Some researchers reported that as compared with lethality, sublethal endpoints such as behavioral and reproductive responses are much more sensitive indicators of toxicity in nematodes.[12-14] In the case of MBP, there was no remarkable difference between median lethal and sublethal concentrations.

In conclusion, the biological effects of one of the BPA metabolites, MBP, were evaluated *in vivo*, using *C. elegans* as the model organism. The BCFs

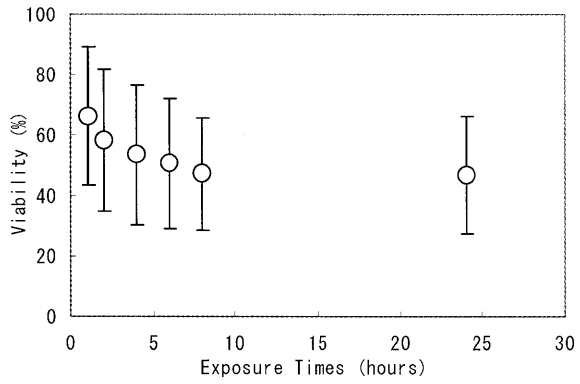

Fig. 1 Time-response Plots Showing the Viability of *C. elegans* Exposed to 0.5 mM MBP. The means ± S. D. are plotted.

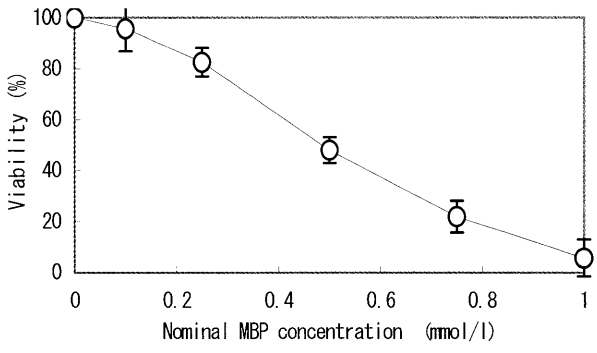

Fig. 2 Concentration-Response Plots Showing the Viability of *C. elegans* after 24 hr Exposure. The means ± standard error (n = 20) are plotted

were in the range of 19–105. The acute lethal toxicity was slightly increased, but the toxicity effect on fecundity was not confirmed. Instead, there were indications that MBP promoted growth and fecundity at a concentration of approximately one-fifth that of the LC_{50}. This study suggests that MBP incorporated in animal body may be stored more stable than outside of animal body. Some studies have been demonstrated the biological activities of MBP. [15] Therefore future research on MBP will focus on the possibility of synthesis of MBP in animal body.

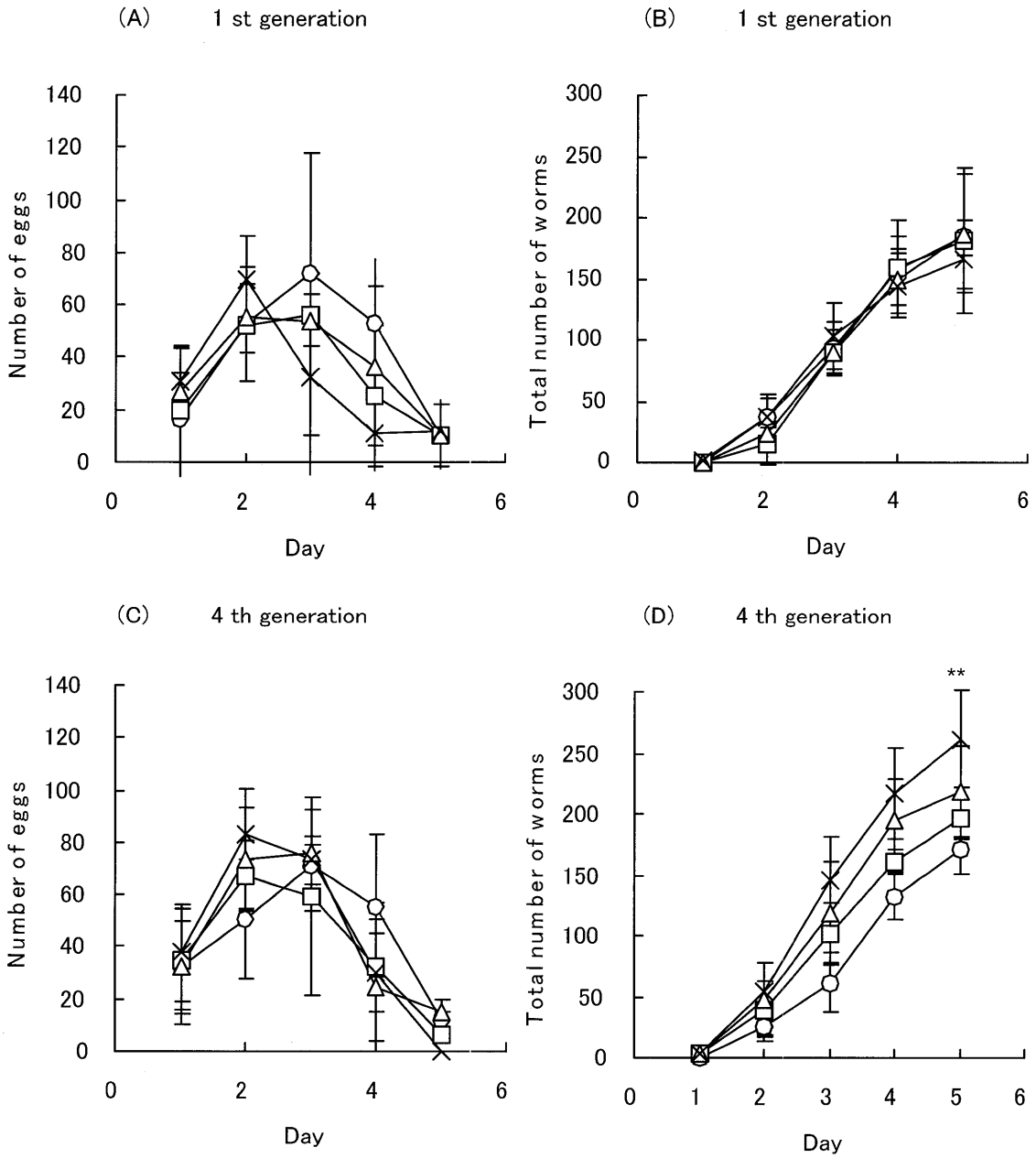

Fig. 3 Effect of MBP on the Fecundity of *C. elegans*.
(A) Egg number at the 1st generation, (B) *C. elegans* number at the 1st generation, (C) Egg number at the 4th generation, (D) *C. elegans* number at the 4th generation
○, Control ; □, 0.001 mM ; △, 0.01 mM ; ×, 0.1 mM. The means ± S. D. (n = 20) are plotted.
＊ : Found to be significantly different from the control group at $p < 0.05$ by ANOVA.
＊＊ : Found to be significantly different from the control group at $p < 0.01$ by ANOVA.

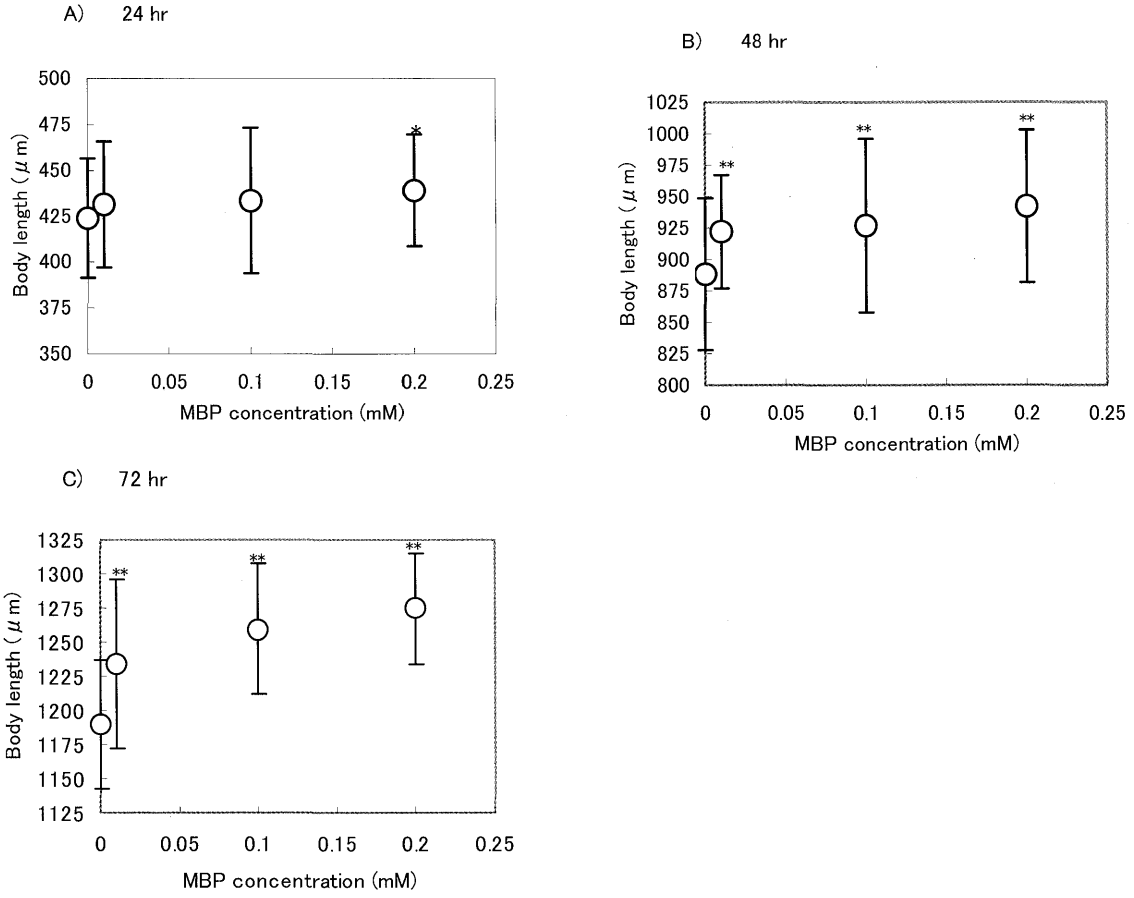

Fig. 4 Effect of MBP on the Growth of *C. elegans*.
(A) 24 hr exposure, (B) 48 hr exposure, (C) 72 hr exposure. The means ± S. D. are plotted.
＊ : Found to be significantly different from the control group at $p < 0.05$ by ANOVA.
＊＊ : Found to be significantly different from the control group at $p < 0.01$ by ANOVA.

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