Enhancing effects of a high fat diet on 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline-induced lung tumorigenesis in female A/J mice

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Abstract. Both heterocyclic amines and a high fat diet are associated with an increased risk of cancer in many organs. Female A/J mice were fed a diet supplemented with 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and a high fat diet to test for the development of lung tumors. In experiment 1, the mice were divided into 6 groups. Groups 1, 2, 3 and 4 were fed a diet supplemented with MeIQx at a concentration of 600 ppm for 0-12 weeks. A high fat diet containing 20% corn oil was given to Groups 1 and 5 for 0-32 weeks, Group 2 for 12-32 weeks and Group 3 for 0-12 weeks. Group 6 was fed a basal diet without supplements. MeIQx-treated groups (Groups 1, 2, 3 and 4) showed a significant increase in macroscopic and microscopic lung nodules compared with the control (Group 6). Areas of adenomas were increased dependent on the duration of exposure to the high fat diet. In experiment 2, Group 1 mice were fed MeIQx and a high fat diet, Group 2 a MeIQx alone diet, Group 3 a high fat alone diet, and Group 4 a basal diet without supplements. CYP1A2 mRNA in the liver was significantly decreased by a high fat diet (Group 3). The MeIQx alone group (Group 2) showed a tendency towards increased CYP1A2 expression, which was partially reduced in the MeIQx + high fat-treated group (Group 1). In the lungs, CYP1A2 mRNA expression was at an extremely low level, with no intergroup differences. In conclusion, MeIQx exerts tumorigenic potential in the lungs, and a high fat diet increases the size of induced lesions. The expression level of CYP1A2 in relation to MeIQx and a high fat diet may be associated with lung carcinogenesis.

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Introduction

Lung cancer is one of the most common causes of mortality and morbidity in the world (1,2). Though the main risk factor for the disease is tobacco smoking, non-smokers are also at risk. Nevertheless, risk factors other than tobacco use have yet to be fully elucidated. It has been suggested that diet exerts an effect, possibly interacting with smoking and genetic susceptibility (3).

Previous studies have demonstrated that heterocyclic amines generated in cooked meat and fish are highly mutagenic and carcinogenic in rodents (4-6). Case control studies in humans have further provided evidence that high-temperature-cooked meat is associated with the risk of colon (7,8), breast (9,10), gastric (11) and lung (12,13) cancer. MeIQx, one of the major amines contained in cooked meat, is activated by cytochrome P450 (CYP)1A2 after being ingested, and is reported to induce liver and lung tumors as well as lymphomas and leukemias in CDF_1 mice (14). Although the incidence of MeIQx-induced liver tumors was high, the values for lung and hematopoietic system tumors were much lower, and only females were affected among the lung cases. Therefore, whether the lung is indeed a target organ for MeIQx is controversial.

Fat consumption is associated with the risk of cancer in several organs (15-19), including the lungs (13,16,20-22). One epidemiological study linked a high fat diet to an increased risk of human lung adenocarcinomas, but specific fats were found to lack significant influence after adjustment for total fat intake (13). Experimental studies in rats have shown that fats containing ω-6 fatty acids (for example, corn oil) enhance, while ω-3 fatty acids (for example, fish oil and mustard oil) reduce, the development of chemically-induced colon tumors (23,24). Previously, we reported that a high fat diet (20% corn oil) enhanced 4-nitroquinoline 1-oxide (4NQO)-induced pulmonary tumorigenesis in male ICR mice (25). Therefore, in the present study, female A/J mice, which are highly susceptible to lung carcinogens, were fed a diet supplemented with MeIQx

and a high concentration of corn oil, to test for the development of lung tumors. The influence of cytochrome P450 (CYP)1A2 induction was also examined in a separate experiment.

Materials and methods

Chemicals and animals. MeIQx was purchased from the Nard Institute (Osaka, Japan). Female A/J mice (5 weeks of age) were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan) and maintained at the Kagawa University Animal Facility according to the institutional animal care guidelines. The animals were housed for feeding in polycarbonate cages with white wood chips, and were given free access to a powdered basal diet (CE-2; Clea Japan Inc., Tokyo, Japan) and tap water under controlled conditions of humidity (60±10%), lighting (12-h light/dark cycle) and temperature (24±2˚C). Experimentation was commenced when the mice were 7 weeks of age.

Experiment 1

Protocol. The experimental protocol is shown in Fig. 1. Starting at 7 weeks of age, mice in Groups 1, 2, 3 and 4 were fed a diet supplemented with MeIQx at a concentration of 600 ppm for 0-12 weeks, while Groups 5 and 6 received a diet lacking the carcinogen. A high fat diet containing 20% corn oil was fed to Groups 1 and 5 for 0-32 weeks, Group 2 for 12-32 weeks, and Group 3 for 0-12 weeks. Group 6 was fed the basal diet CE-2 without supplements. The mice were maintained on their respective diets under controlled conditions of humidity (60±0%), lighting (12-h light/dark cycle) and temperature (24±2˚C) until termination of the experiment at week 32. All surviving mice were then sacrificed under ether anesthesia. At autopsy, the lungs, livers and kidneys were excised and weighed. Lungs were infused through the bronchi with 10% neutral buffered formalin and carefully inspected grossly to determine the numbers of macroscopic lung nodules using a stereomicroscope.

Histopathological analysis. The excised tissues were routinely processed for embedding in paraffin, serially sectioned (3.5 μm) and stained with hematoxylin and eosin for histopathological examination. Lung nodules, alveolar hyperplasias and adenomas were diagnosed according to established criteria (26), and their numbers were counted under a microscope.

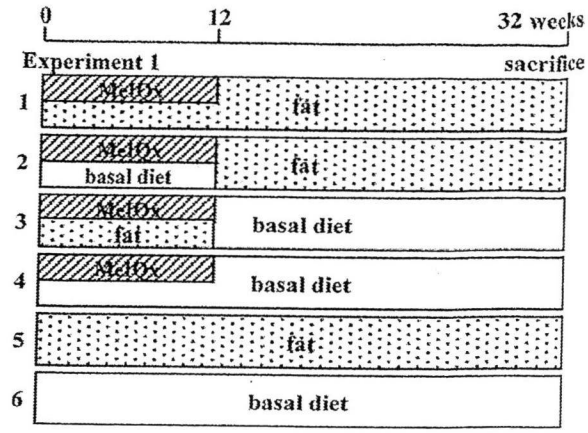

Figure 1. Protocol for experiment 1. Animals were 7-week-old female A/J mice. MeIQx (600 ppm) or fat (corn oil 20%) was added to the pelleted diet. CE-2 was used as the basal diet.

Areas of lung proliferative lesions were measured with the assistance of an image analyzer (IPAP, image processor for analytical pathology; Sumika Technoservice Co., Hyogo, Japan). The total area (mm^2) of lung proliferative lesions per mouse was used for statistical analysis.

Experiment 2

Protocol. The second experiment was conducted to determine the effects of MeIQx and a high fat diet on CYP1A2 mRNA expression. MeIQx and a high fat diet were administered by the same routes as those used in experiment 1. Mice in Groups 1 and 2 were fed a diet supplemented with MeIQx for 2 weeks, while Groups 1 and 3 received a high fat diet for 2 weeks. Group 4 was fed the basal diet CE-2 without supplements. The surviving mice were sacrificed at the end of week 2, and their lungs, livers and kidneys were excised for RNA isolation and the quantitative analysis of CYP1A2. Total RNA was isolated from 30 mg of whole lung and liver tissues using RNAlater RNA Stabilization Reagent and an RNeasy Mini Kit (both from Qiagen Corp., Hilden, Germany). The concentration of RNA was measured at an absorbance of 260 nm. First-strand cDNA was synthesized from 400 ng of total RNA using TaqMan Reverse Transcription Reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

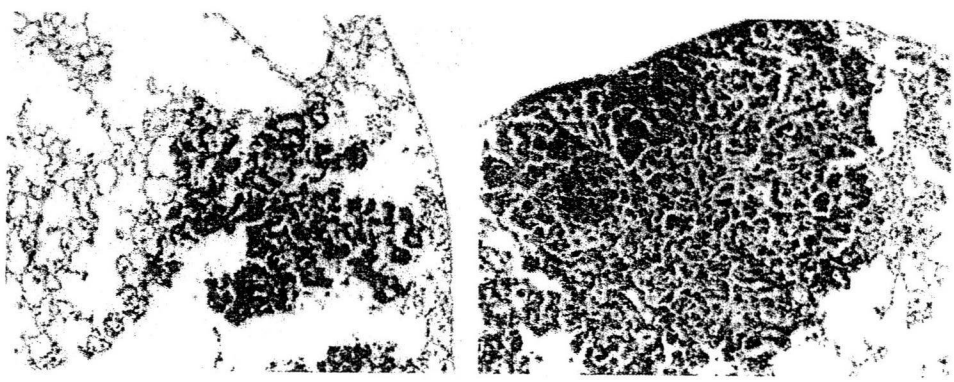

Figure 2. Representative images of lung proliferative lesions. Left, hyperplasia; right, adenoma.

Quantitative real-time RT-PCR. Optimal primers and probes were purchased from the Assays-on-Demand System of ABI (Applied Biosystems). TaqMan Rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Control Reagent (Applied Biosystems) was used for PCR to measure GAPDH mRNA levels as an internal control.

Quantitative real-time RT-PCR was performed with the ABI PRISM 7000 Sequence Detection System using specific primers and a TaqMan probe for CYP1A2. PCR was carried out in 50-μl reaction mixtures containing 25 μl of 2X TaqMan Universal PCR Master Mix, 50 ng of cDNA, 100 nM of each primer and 200 nM of TaqMan probe. Cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 sec at 95°C followed by 1 min at 60°C. TaqMan PCR products were detected as an increase in fluorescence from cycle to cycle. The amplification plots of the PCR reaction were used to determine the threshold cycle (Ct). The Ct value represented the PCR cycle at which an increase in reporter fluorescence (ΔRn) above the line of the optimal value was first detected. The initial copy number of the target mRNA was calculated from plots of the Ct against the input target quantity.

Since the precise amount and quality of total RNA are difficult to assess, we quantified transcripts of the GAPDH gene as an internal control according to a quantitative RT-PCR assay. Normalization of the data was achieved by quantitating the cycle number at an arbitrary fluorescence intensity in the linear exponential phase using the ratio of the cycle number of each enzyme relative to that of GAPDH.

Statistical analysis. Data for final body and organ weights were analyzed by the Student's t-test. The incidence of lung proliferative lesions was analyzed by Fisher's exact probability test, and data for multiplicity and area by the Student's t-test. CYP1A2 mRNA levels were analyzed by the Mann-Whitney U test. Areas of lung lesions were also analyzed by the Spearman's rank correlation coefficient.

Results

Final body and organ weights are shown in Table I. Final body weight was significantly increased in the high fat alone group (Group 5) compared to the control (Group 6). MeIQx + high fat-treated groups (Groups 1 and 2) also showed an increase in final body weight compared to the MeIQx alone group (Group 4).

The relative organ weight of the lungs, liver and kidneys in the high fat alone group (Group 5) and the MeIQx + high fat groups (Group 1, 0-32 weeks and Group 2, 12-32 weeks) was significantly decreased. These changes in relative organ weight were mainly due to an increase in body weight in Groups 1, 2 and 5. The MeIQx alone group (Group 4) showed no significant change in organ weight. Absolute organ weight showed no treatment-related change.

Results for the lung lesions are shown in Table II. Whitish nodules were macroscopically detected in all groups, including the basal diet controls. The MeIQx-treated groups (Groups 1, 2, 3 and 4) exhibited a significant increase in macroscopic lung nodules compared to the control (Group 6), while only a few lung nodules were observed in the high fat alone group (Group 5). Microscopically, the lung nodules were hyperplasias

Table I. Final body and organ weights of mice in experiment 1.

Group	No.[a]	Body weight (g)	Lung weight		Liver weight		Kidney weight	
			Absolute (g)	Relative (%)	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
1 MeIQx + fat (0-32 weeks)	20	33.00±4.79[bc]	0.19±0.03	0.59±0.11[bc]	0.96±0.11	2.94±0.40[bc]	0.29±0.02	0.90±0.10[bc]
2 MeIQx + fat (12-32 weeks)	20	31.74±3.53[bc]	0.20±0.03	0.63±0.12[c]	0.98±0.08	3.11±0.47[bc]	0.30±0.02	0.95±0.13[bc]
3 MeIQx + fat (0-12 weeks)	20	27.16±2.35	0.20±0.02	0.74±0.09	0.96±0.10	3.53±0.34	0.29±0.02	1.07±0.11[b]
4 MeIQx	20	26.46±3.19	0.20±0.02	0.78±0.11	0.94±0.09	3.56±0.33	0.29±0.02	1.12±0.12
5 Fat (0-32 weeks)	14	36.10±4.60[bc]	0.20±0.02	0.56±0.12[bc]	0.99±0.11	2.78±0.57[bc]	0.20±0.03	0.82±0.10[bc]
6 Control	10	25.62±2.24	0.18±0.02	0.71±0.06	0.95±0.11	3.71±0.15	0.31±0.03	1.20±0.01

Data are presented as the mean ± SD values. [a]No. of animals. [b]P<0.05 compared with Group 6. [c]P<0.05 compared with Group 4.

Table II. Macroscopic and microscopic results for lung lesions of mice in experiment 1.

Group	No.[a]	Macroscopic incidence of nodules (%)	Macroscopic lung nodules/mouse	Microscopic lung nodules/mouse			Area of hyperplasia (mm^2)	Area of adenoma (mm^2)	Area of hyperplasia + adenoma (mm^2)
				Hyperplasia	Adenoma	Total (hyperplasia + adenoma)			
1 MeIQx + fat (0-32 weeks)	20	15/20 (75.0)[b]	1.30±0.98[b]	0.15±0.37	0.70±0.80[b]	0.85±0.81[b]	0.02±0.05	0.35±0.45[bc]	0.37±0.45[b]
2 MeIQx + fat (12-32 weeks)	20	14/20 (73.7)[b]	1.79±1.44[b]	0.37±0.50	0.95±0.91[b]	1.32±1.00[b]	0.08±0.16	0.34±0.37[bc]	0.42±0.39[b]
3 MeIQx + fat (0-12 weeks)	20	17/20 (85.0)[b]	1.80±1.24[b]	0.55±0.61	0.85±0.99[b]	1.40±1.10[b]	0.11±0.20	0.30±0.34[bc]	0.41±0.35[b]
4 MeIQx	20	15/20 (78.9)[b]	1.32±1.11[b]	0.37±0.68	0.79±0.54[b]	1.16±0.90[b]	0.09±0.21	0.24±0.22[bc]	0.34±0.31[b]
5 Fat (0-32 weeks)	14	3/14 (21.4)	0.21±0.43	0.14±0.36	0.07±0.27	0.21±0.58	0.01±0.04	0.02±0.09	0.04±0.13
6 Control	10	2/10 (25.0)	0.38±0.74	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Data are presented as the mean ± SD values. [a]No. of animals. [b]P<0.05 compared with Group 6. [c]P<0.02, R=0.36, Spearman's rank correlation coefficient.

Table III. Relative quantification of CYP1A2 mRNA in the liver and lungs of A/J mice in experiment 2.

Group	No.[a]	CYP1A2 mRNA in the liver	CYP1A2 mRNA in the lung ($x10^{-3}$)
1 MeIQx + fat	5	1.13±0.23	4.21±6.54
2 MeIQx	5	1.98±0.89	3.06±2.41
3 Fat	5	0.55±0.14[b]	3.38±4.03
4 Control	5	1.08±0.30	5.92±2.70

Data are presented as the mean ± SD values. [a]No. of animals. [b]P<0.05 compared to Groups 1, 2 and 4.

and adenomas (Fig. 2). Values for microscopic hyperplasias and adenomas in the MeIQx-treated groups (Groups 1, 2, 3 and 4) were significantly higher than the respective data for the group without MeIQx treatment (Group 6). Additionally, areas of adenomas and hyperplasias + adenomas were significantly elevated in the MeIQx-treated groups (Groups 1, 2, 3 and 4), with the degree of increase dependent on the duration of the high fat diet. Microscopically, the liver and kidney showed no treatment-related changes.

Data regarding the relative quantification of CYP1A2 mRNA in the liver and lungs of A/J mice are summarized in Table III. Expression levels of CYP1A2 mRNA in the liver were significantly decreased by a high fat diet (Group 3). The MeIQx alone group (Group 2) showed a tendency toward an increase in CYP1A2 mRNA compared to the controls (Group 4). By contrast, the MeIQx + high fat-treated group (Group 1) showed a tendency toward a decrease compared to Group 2, though without statistical significance. In the lungs, CYP1A2 mRNA expression levels were extremely low, with no intergroup differences.

Discussion

In agreement with a previous study (14), this study confirms that MeIQx induces mouse lung tumors, and corroborates the epidemiological finding that MeIQx is associated with the risk of lung cancer. Furthermore, a high concentration of corn oil in the diet significantly increased the areas of MeIQx-induced lung adenomas, without any influence on the incidence and multiplicity of lung tumors. Notably, the enhancing effect of the high fat diet was duration-dependent. In this study, MeIQx-induced lung lesions exhibited almost the same histopathological features as lesions induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (33,34), though with relatively low numbers.

It is well known that MeIQx is converted to genotoxic metabolites by liver CYP1A2 (27,28), resulting in DNA adduct formation. Previous research revealed expression levels of CYP1A2 mRNA in the liver to be increased approximately 1.5- to 2-fold by treatment with MeIQx (29); in the present study, values were elevated 1.83-fold. A high fat diet caused a significant decrease in CYP1A2 mRNA, suggesting that it has the potential to reduce the metabolic activation of MeIQx, consequently inhibiting MeIQx-induced lung tumorigenesis

during the initiation phase. On the other hand, the observed enhancing effect on areas of MeIQx-induced lung adenomas indicates the promotion of lung tumor development. In this study, CYP1A2 mRNA levels in the lungs were very low compared to findings in humans, where CYP1A2 is mainly detected in the liver (30). The results indicate that CYP1A2 in the liver may play an important role in MeIQx-induced lung tumorigenesis.

In a previous study, CDF_1 mice exhibited a high incidence of liver tumor development when fed a diet containing MeIQx at 600 ppm for 84 weeks (14), while in this study no liver tumors were observed in any of the groups. This is in line with the known resistance of the A/J mouse strain to hepatocarcinogenesis (31). By contrast, while male and female CDF_1 mice exhibited a lung tumor incidence of 43% (14), the figure for A/J female mice in this study was 78.9%, confirming that the A/J strain is highly susceptible to lung tumorigenesis (32).

Epidemiological studies suggest a relationship between the risk of cancer and increased consumption of ω-6 fatty acids found in corn oil (15-19). It has been reported that a decreased ω-6/ω-3 fatty acid ratio reduces the invasive potential of human lung cancer cells by downregulating cell adhesion/invasion-related molecules such as MMP-1, integrin-α2 and nm23-H4 (35). The enhancing effect of a corn oil diet on MeIQx-induced lung tumors in the present study might therefore be due to the high levels of ω-6 included.

In conclusion, MeIQx shows tumorigenic potential in female A/J mouse lungs, and a high fat diet increases the areas of MeIQx-induced lung adenomas, implying a promotion potential. Our results also suggest a role for CYP1A2 in relation to MeIQx and the effects of a high fat diet on lung carcinogenesis.

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Induction of SCEs in CHL cells by dichlorobiphenyl derivative water pollutants, 2-phenylbenzotriazole (PBTA) congeners and river water concentrates

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PBTA-2
PBTA-6

ABSTRACT

We recently identified dichlorobiphenyl (DCB) derivatives and 2-phenylbenzotriazole (PBTA) congeners as major mutagenic constituents of the waters of the Waka River and the Yodo River system in Japan, respectively. In this study we examined sister chromatid exchange (SCE) induction by two dichlorobiphenyl derivatives, 3,3′-dichlorobenzidine (DCB, 4,4′-diamino-3,3′-dichlorobiphenyl) and 4,4′-diamino-3,3′-dichloro-5-nitrobiphenyl (5-nitro-DCB); three PBTA congeners, 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1), 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2), and 2-[2-(acetylamino)amino]-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-6); and water concentrates from the Waka River in Chinese hamster lung (CHL) cells. Concentration-dependent induction of SCE was found for all DCBs and PBTAs examined in the presence of S9 mix, and statistically significant increases of SCEs were detected at 2 μg per ml of medium or higher concentrations. SCE induction of MeIQx was examined to compare genotoxic activities of these water pollutants. According to the results, a ranking of the SCE-inducing potency of these compounds is the following: 5-nitro-DCB ≈ MeIQx > PBTA6 > PBTA-1 ≈ PBTA-2 > DCB.

Water samples collected at a site at the Waka River showed concentration-related increases in SCEs at 6.25–18.75 ml-equivalent of river water per ml of medium with S9 mix. The concentrations of 5-nitro-DCB and DCB in the river water samples were from 2.5 to 19.4 ng/l and from 4100 to 18,900 ng/l, respectively. However, these chemicals showed only small contribution to SCE induction by the Waka River water.

1. Introduction

Genotoxic compounds are detected in many surface waters in the world. These compounds are often released directly from industrial discharges as a result of insufficient treatment of wastewater or unintentional formation in the environment after discharge of effluents [1–5]. In the previous studies, we found two novel chemical classes, dichlorobiphenyl derivatives and 2-phenylbenzotriazole (PBTA) congeners, as major mutagenic constituents in the water of rivers flowing through several industrial areas in Japan [6–20].

Among dichlorobiphenyl derivatives, 3,3′-dichlorobenzidine (DCB, 4,4′-diamino-3,3′-dichlorobiphenyl), 4,4′-diamino-3,3′-dichloro-5-nitrobiphenyl (5-nitro-DCB), and so forth were identified as major mutagens in the water of the Waka River flowing through an industrial area in Wakayama, where a number of large chemical plants are found [6–9]. 5-Nitro-DCB is a novel chemical and is presumed to be formed unintentionally by the process of wastewater treatment of drainage water containing DCB discharged from chemical plants [6]. DCB is a raw material in the manufacture of polymers and dye intermediates, and there are large-scale chemical plants producing DCB in this industrial area. 5-Nitro-DCB is highly mutagenic in the Ames assay using *Salmonella typhimurium* YG1024, which is an *O*-acetyltransferase-overproducing derivative of TA98, with S9 mix, and its activity was ~7 times higher than that of DCB.

5-Nitro-DCB was detected in river water concentrates at the maximum level of 6.9 μg/g of blue rayon. DCB was also detected in the concentrates at 13.2–104 μg/g of blue rayon. The percent contributions of 5-nitro-DCB and DCB to the mutagenicity of the water concentrates in YG1024 with S9 mix were 11% and 28%, respectively,

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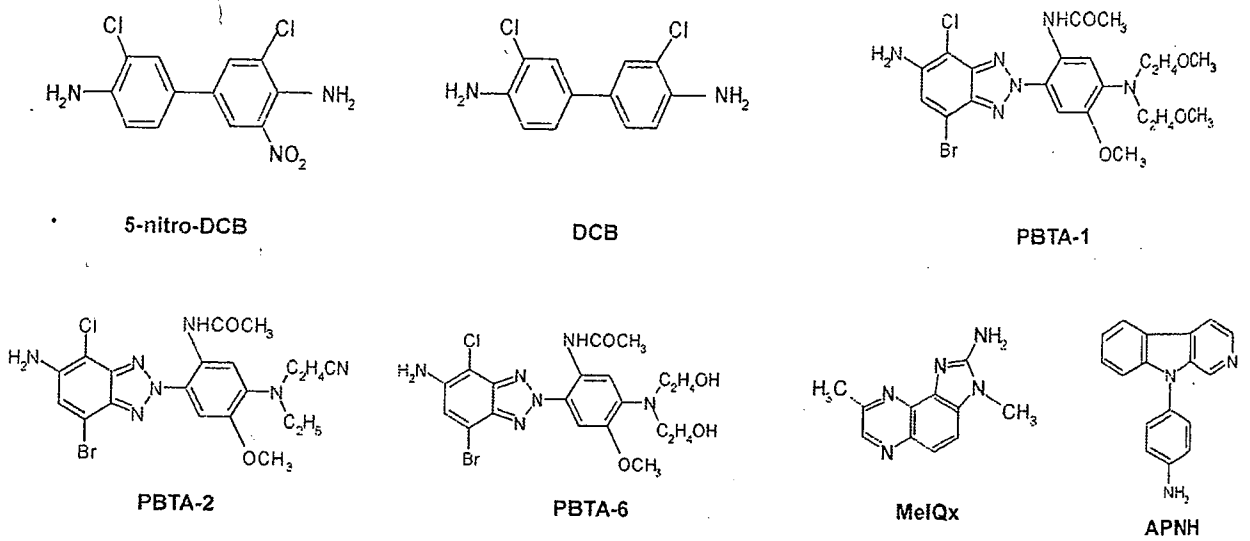

Fig. 1. Chemical structures used in the present study.

on average. 5-Nitro-DCB is a new chemical, and it has no biological activity data except for mutagenicity in the Ames assay.

PBTA congeners were identified as major indirect-acting river water mutagens, and seven kinds of such compounds were detected in highly mutagenic river waters, e.g., the Yodo River system, the Asuwa River, the Nikko River and so on, flowing through areas of textile dyeing industries [10–20]. PBTA congeners were suggested to be formed from corresponding dinitrophenylazo dyes via reduction with sodium hydrosulfide during the industrial dyeing process and following chlorination in the disinfection process at sewage plants. PBTA congeners show potent mutagenicity in *S. typhimurium* YG1024 in the presence of S9 mix. 2-[2-(Acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1), 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2), and 2-[2-(acetylamino)amino]-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzo-triazole (PBTA-6) were detected in many river water concentrates [10–15]. However, there are few reports on the genotoxicity of PBTA congeners in mammalian cells.

In this study, we investigated the induction of sister chromatid exchanges (SCEs) by DCB, 5-nitro-DCB, PBTA-1, PBTA-2, PBTA-6, and water concentrates from the Waka River in Chinese hamster lung (CHL) cells to evaluate the genotoxic effect of water pollutants and environmental samples contaminated with DCB and 5-nitro-DCBs. DCB and 5-nitro-DCB in the river water concentrates were quantitatively analyzed, the mutagenicity of the water concentrates were evaluated in YG1024, and the contribution of 5-nitro-DCB and DCB to the mutagenicity of the river water concentrates are also estimated.

2. Materials and methods

2.1. Materials

5-Nitro-DCB (CAS 1073239-90-3) was synthesized according to the method reported previously [6]. Dichlorobenzidine dihydrochloride (CAS 612-83-9) was purchased from Sigma–Aldrich Co. Ltd. (MO, USA). PBTA-1 (CAS 194590-84-6), PBTA-2 (CAS 215245-16-2), and PBTA-6 (CAS 392274-07-6) were synthesized as described previously [11,13,16]. 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx, CAS 77500-04-0) was obtained from Wako Pure Chemical Industries (Osaka, Japan). The chemical structures of six compounds used in the present study are shown in Fig. 1. All other chemicals and reagents were of analytical grade.

2.2. Preparation of river water concentrates and analysis of DCBs

Each 10 l water sample was collected at a site where wastewater was discharged from chemical plants and a sewage treatment plant into the Waka River in Wakayama, Japan from September 2006 to March 2007. Collected water samples were passed through Supelpak2 columns (SUPELCO, PA, USA, 20 mm i.d. × 800 mm), and adsorbed materials were then extracted with methanol (300 ml) according to our previous paper [8]. Each extract was used for SCE assay, the Ames assay, and quantification of 5-nitro-DCB and DCB by HPLC. Quantification of 5-nitro-DCB and DCB was performed according to the method reported in the previous paper [6].

2.3. Chemical treatment

All chemicals tested for SCE assay were dissolved in dimethyl sulfoxide (DMSO), and freshly prepared solutions were added to cultures in appropriate final concentrations. The final concentration of DMSO in all cultures was 0.5% (v/v). Aminophenyl-norharman (APNH, Fig. 1) was used as the positive control [22] and was dissolved in DMSO before use.

2.4. SCE assay

CHL cells, obtained from Health Science Research Bank (HSRRB), Japan, were subcultured at a cell density of 1.5×10^5 cells per 60-mm dish and cultivated in 4 ml of Eagle's minimum medium (MEM, Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Trace Scientific Ltd., Melbourne, Australia). In the system with metabolic activation, cells were treated with each chemical for 6 h in the presence of S9 mix. The S9 mix was prepared immediately before use by mixing 1 ml of phenobarbital- and 5,6-benzoflavone-induced rat liver S9 (Oriental Yeast Co. Ltd., Tokyo, Japan) with 0.1 ml of 0.5 M glucose-6-phosphate, 0.4 ml of 0.1 M NADP, 0.2 ml of 1.65 M KCl solution, 0.2 ml of 0.4 M $MgCl_2$ solution, 4 ml of 0.25 M phosphate buffer (pH 7.4), and 4.1 ml of distilled water. The final concentration of S9 used was 1.25% (v/v). After treatment, cells were washed with phosphate buffered saline (PBS) and cultured in fresh medium for a recovery period of 24 h. In an experiment designed to detect SCEs, 5-bromodeoxyuridine (BrdU; final concentration 5 μg/ml) was added to the cultures just after addition of the test chemical.

Colcemid (final concentration 0.2 μg/ml; Gibco) was added to each culture 2 h prior to harvest. Harvested cells were then treated with a hypotonic solution of 75 mM KCl and fixed with cold methanol:acetic acid (3:1, v/v). Solvent-treated cells served as the negative control. Air-dried chromosome preparations prepared for SCEs were stained with the fluorescence plus Giemsa method described by Perry and Wolff [23]. SCEs were scored in 25 well-spread metaphases for each treatment. The results were expressed as the frequency of SCEs per metaphase. The significance between mean SCE in treated versus control groups were determined using the Student's *t*-test.

2.5. Ames assay

Ames assay was performed for DMSO solutions of chemicals and water concentrates described above using *S. typhimurium* YG1024 according to the method reported previously [24–26]. *S. typhimurium* YG1024 were kindly provided by Dr. T. Nohmi from the National Institute of Health Sciences, Tokyo. The S9 mix contained

Table 1
SCEs induced by 5-nitro-DCB, DCB, PBTA-1, PBTA-2 and PBTA-6 in CHL cells in the presence of S9 mix.

Sample	Dose (μg/ml)	SCEs per metaphase		
		MI (%)[c]	Mean ± S.D.	Range
5-Nitro-DCB[a]	1.25	2.0	13.32 ± 6.27	4–29
	2.5	2.5	15.92 ± 7.43	6–31
	5	2.1	18.58 ± 6.01	7–29
	10	2.2	25.04 ± 9.50	11–48
DCB[a]	1	2.9	11.16 ± 4.14	4–20
	2	2.2	14.76 ± 4.35	7–23
	10	2.9	16.96 ± 5.95	8–35
	20	2.5	19.16 ± 7.69	7–37
PBTA-1[b]	1.25	2.6	17.88 ± 5.21	11–29
	2.5	2.6	18.60 ± 8.29	9–34
	5	2.0	19.06 ± 6.27	7–29
	10	1.7	20.04 ± 7.37	10–43
PBTA-2[b]	1.25	2.8	14.28 ± 3.60	8–23
	2.5	2.9	14.56 ± 6.58	6–38
	5	2.7	17.84 ± 5.74	8–28
	10	2.0	20.96 ± 7.81	9–34
PBTA-6[b]	1.25	2.9	15.08 ± 4.95	8–26
	2.5	2.7	16.28 ± 6.13	8–26
	5	2.9	19.40 ± 5.22	12–31
	10	1.8	22.76 ± 7.04	10–36
MeIQx[b]	1.25	3.5	15.97 ± 8.20	6–39
	2.5	3.9	18.20 ± 5.12	9–35
	5	3.7	23.96 ± 9.20	9–43
	10	2.7	26.68 ± 9.02	13–53

SCE frequency for positive control (APNH, 0.005 μg/ml) was 21.44 ± 5.04 (mean ± S.D.).

[a] SCE frequency for Control (DMSO) was 8.68 ± 4.28 (mean ± S.D.).

[b] SCE frequency for Control (DMSO) was 10.16 ± 2.65 (mean ± S.D.).

[c] MI; mitotic index. MI (%) was calculated by counting the number of mitotic cells among 1000 round nuclei.

* Significantly different from control, $p < 0.05$.

** Significantly different from control, $p < 0.01$.

25 μl of S9 (25 mg of protein/ml) at a total volume of 500 μl. Mutagenic activities of test samples were calculated from the linear portions of the dose-response curves obtained with four doses with duplicate plates in two independent experiments, and the results were the average of two independent experiments. The positive controls were 2-aminoanthracene (0.1 μg/plate) and Trp-P-1 (0.01 μg/plate) with S9 mix. The mutagenic potencies were expressed as revertants/l of river water.

3. Results

3.1. SCE induction by DCBs and PBTAs

We evaluated the genotoxic effect of 5-nitro-DCB, DCB, three PBTA congeners, and MeIQx using SCEs in cultured CHL cells in the presence of S9 mix. These chemicals are indirect-acting mutagens for bacteria and show potent mutagenicity with S9 mix [6,7,10,11,13,27]. As shown in Table 1, statistically significant increases in SCEs were found for DCBs and PBTAs at doses used in this study (up to 10 or 20 μg/ml). Among DCBs and PBTAs, the highest SCE frequency, 25.04 ± 9.50 (mean ± S.D.), was detected for 5-nitro-DCB at the dose of 10 μg/ml. MeIQx is a well-known mutagenic and carcinogenic heterocyclic amine [28], and it shares structural features common to those of DCBs and PBTAs. Dose-related increases in the frequencies of SCEs were found for MeIQx between the concentration of 1.25 and 10 μg/ml. The highest SCE frequency, 26.68 ± 9.02, was detected at 10 μg/ml. Concentrations of DCBs, PBTAs, and MeIQx leading to two-fold increases of SCE frequencies relative to that of control were shown as SCE-inducing activity in Table 2, with mutagenicity data by Ames test. SCE-inducing activities of DCBs and PBTAs were from 4.5 to 13.9 μg/ml. The SCE-inducing activity of 5-nitro-DCB, 4.5 μg/ml was as high as that of MeIQx, 4.6 μg/ml. APNH, which was used as a positive control, significantly increased SCE induction at a dose of 0.005 μg/ml in the presence of S9 mix, and this result was consistent with that of our previous report [22].

Table 2
SCE-inducing activity and mutagenicity data of 5-nitro-DCB, DCB, PBTA-1, PBTA-2, PBTA-6 and MeIQx in the presence of S9 mix.

Sample	SCE-inducing activity (μg/ml)[a]	Mutagenicity (revertants/μg)[b]	
		TA98	YG1024
5-Nitro-DCB	4.5	8,700	24,200
DCB	13.9	100	3,400
PBTA-1	8.4	88,000	3,000,000
PBTA-2	8.4	93,000	3,200,000
PBTA-6	6.9	17,900	485,000
MeIQx	4.6	117,000	1,400,000

[a] Concentration leading to a two-fold increase relative to control level.

[b] Data from reference [6,9,12,17,38].

3.2. SCE induction by the water concentrates from the Waka River

The dose-response effects of SCE induction for three water concentrates obtained from the Waka River are shown in Table 3. For all water concentrates, dose-related increases in the frequencies were found between the concentration of 6.25 and 18.75 ml eq/ml of medium in the presence of S9 mix. The highest SCE frequency, 20.84 ± 5.08, was detected for river water concentrate No. 2 at the dose of 18.75 ml/ml, but this concentrate showed toxicity at a higher dose.

3.3. Concentrations of DCBs in the river water and mutagenicity in Salmonella

Table 4 shows amounts of 5-nitro-DCB and DCB in the three water samples from the Waka River and mutagenicity of water concentrates toward *S. typhimurium* YG1024 in the presence of S9 mix. 5-Nitro-DCB and DCB were detected in all the water samples. The concentrations of 5-nitro-DCB and DCB in the river water samples were from 2.5 to 19.4 ng/l and from 4100 to 18,900 ng/l, respectively. The three water concentrates showed potent mutagenicity in YG1024, and the highest activities was detected for the water concentrate No.2. The percent contributions of DCB to the mutagenicity of the river water concentrates were from 8% to 26%, but those of 5-nitro-DCB were less than 1%.

4. Discussion

In the present study, we evaluated the genotoxic effect of the water pollutants 5-nitro-DCB, DCB, PBTA-1, PBTA-2, and PBTA-6, and river water samples, which included 5-nitro-DCB and DCB as constituents, using SCEs in CHL cells. In addition, SCE induction of MeIQx was examined to compare genotoxic activities of these water pollutants. MeIQx was deduced to be possibly carcinogenic to human (Group 2B) by the International Agency for Research on Cancer (IARC) [28], and it has the structural features of an aromatic amine similar to those of DCBs and PBTAs. MeIQx was reported to induce SCEs in human lymphocyte cultures and to show mutagenicity in CHL cells for diphtheria toxin resistance in the presence of S9 mix [29,30]. As shown in Table 1, the dose-response effects of SCE induction were detected for DCBs, PBTAs, and MeIQx at doses from 1.25 to 10 μg/ml or from 1 to 20 μg/ml with S9 mix, and the increases of SCEs were statistically significant at almost all doses tested. All chemicals tested in the present study induced SCE induction in cultured mammalian CHL cells in the presence of S9 mix. Among the composites tested, 5-nitro-DCB was found to have

Table 3
SCEs induced by river water concentrates in CHL cells in the presence of S9 mix.

Sample	Sampling date	Dose (ml/ml)[a]	SCEs per metaphase		
			MI (%)[b]	Mean ± S.D.	Range
Concentrate No. 1	14 September 2006	6.25	2.4	10.12 ± 3.50	3–18
		12.5	2.9	12.12 ± 3.94	5–22
		18.75	2.5	16.16 ± 6.08	6–28
		25	1.2	16.12 ± 6.35	4–31
Concentrate No. 2	19 December 2006	6.25	3.2	12.44 ± 5.08	5–25
		12.5	2.2	18.72 ± 5.95	9–35
		18.75	1.5	20.84 ± 5.08	13–37
		25	0	Toxic	Toxic
Concentrate No. 3	29 March 2007	6.25	2.8	10.64 ± 2.90	6–16
		12.5	2.4	14.00 ± 5.42	6–28
		18.75	2.6	17.44 ± 6.87	9–38
		25	2.1	16.36 ± 5.18	10–29

SCE frequency for Control (DMSO) was 8.68 ± 4.28 (mean ± S.D.). SCE frequency for positive control: APNH (0.005 μg/ml) 21.44 ± 5.04 (mean ± S.D.).
[a] Dose is expressed as ml eq of river water per 1 ml of medium.
[b] MI; mitotic index. MI (%) was calculated by counting the number of mitotic cells among 1000 round nuclei.
** Significantly different from control, $p < 0.01$.

the most pronounced frequency of SCEs, showing the same level of MeIQx, although mutagenicity with *Salmonella* TA 98 and YG1024 of 5-nitro-DCB was relatively low compared with those of PBTA congeners and MeIQx as shown in Table 2. Moreover, SCE-inducing activity of 5-nitro-DCB was 1.5–2 times higher than those of PBTA congeners and was 3 times higher than that of DCB. A ranking of the SCE-inducing potency of these compounds is the following: 5-nitro-DCB ≈ MeIQx > PBTA6 > PBTA-1 ≈ PBTA-2 > DCB.

All water concentrates from the Waka River also showed dose-related increases in SCEs between the concentration of 6.25 and 18.75 ml eq/ml of medium in CHL cells with S9 mix (Table 3). SCE-inducing activities of water concentrates were from 13 to 24 ml eq/ml and a ranking of the water concentrates for SCE induction was concentrate No. 2 > 3 > 1. Both 5-nitro-DCB and DCB were detected in all water concentrates, but amounts of DCB in the water samples were about 1000-fold or higher than those of 5-nitro-DCB. Since DCB and 5-nitro-DCB showed similar SCE-inducing activity, and the amounts of DCB in the water samples were much higher than those of 5-nitro-DCB, the contribution of DCB to SCE-induction of river water concentrates may be larger than that of 5-nitro-DCB. However, contribution ratios of SCE activities based on the concentration of DCB and 5-nitro-DCB, respectively, to the total SCE activities by the river water concentrates were <3% and <0.01%, respectively. Some unknown compounds may be affecting SCE induction of the river water.

Mutagenicity of these river water concentrates was also examined by the Ames assay using YG1024 with S9 mix (Table 4). These concentrate showed potent activities, and percent contributions of DCB, i.e., 8–20%, was much higher than those of 5-nitro-DCB, which was <1%. These high percent contributions of DCB were caused by an abundance of DCB in the river water examined in this study. In a previous study, we quantified 5-nitro-DCB and DCB in blue rayon extracts from the Waka River water and detected relatively high amounts of 5-nitro-DCB [6]. The concentration of 5-nitro-DCB in the river water likely differs on sampling days. More quantitative investigations are necessary to estimate the effect of DCB and 5-nitro-DCB to the genotoxicity of the Waka River water.

In a previous study, we reported that blue rayon extracts from the water of the Yodo River system, Japan, collected in October and December, 1991, showed SCE induction in CHL cells with S9 mix [21]. PBTA-1, PBTA-2, and PBTA-6 were detected in the water samples collected from the same river system in 1994, 1995, and 1999, respectively [10,11,13]. Besides these three PBTAs, other PBTA congeners were continually detected in the water samples collected from this river system from 1994 to 2005 [15]. From synthesis studies, PBTAs are thought to be formed from corresponding dinitrophenylazo dyes used in textile dyeing factories and released into the river system. The SCE induction by the blue rayon extracts from the Yodo River system might be due to PBTA congers.

Table 4
Mutagenicity of water concentrates from the Waka River and amounts of 5-nitro-DCB and DCB.

Sample	Mutagenicity (revertants/l)[a]	Amount (ng/l)		Contribution ratio (%)[b]	
		5-Nitro-DCB	DCB	5-Nitro-DCB	DCB
Concentrate No. 1	246,200	4.8	18,900	<1	26.1
Concentrate No. 2	374,800	19.4	18,200	<1	16.5
Concentrate No. 3	179,300	2.5	4,100	<1	7.8

[a] Mutagenicity was examined in *S.typhimurium* YG1024 with S9 mix.
[b] The mutagenic potencies of 5-nitro-DCB and DCB used to calculate the contribution ratios were 24,200 and 3400 revertants/μg, respectively [6,7].

Besides 5-nitro-DCB and DCB, three dichlorobiphenyl derivatives, i.e., 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl (ADDB), 3,3′-dichloro-4,4′-dinitrobiphenyl, and 4-amino-3,3′-dichloro-4′-nitrobiphenyl, which were mutagenic in YG1024, were detected in water samples collected from the Waka River in 2003–2004 [8,9]. These DCB derivatives are thought to be formed from DCB, like 5-nitro-DCB. DCB was positive in some *in vivo* genotoxicity assays, e.g., the chromosomal aberration test [31], the micronucleus assay [32], and the alkaline single cell gel electrophoresis assay (comet assay) [33]. Moreover, DCB is carcinogenic in mice, rats, hamsters, and dogs [34], and it has been designated a probable human carcinogen (Group 2B) by IARC [35]. PBTA-1 and PBTA-2 induced micronuclei in Chinese hamster cell line V79-NZ [36]. PBTA-6 and ADDB induced micronuclei in gill cells by i.p. injection into goldfish [37]. Furthermore, DNA damaging activity was detected for PBTA-6 and ADDB in peripheral erythrocytes of goldfish *in vivo* by the comet assay [37].

Our results indicate that various dichlorobiphenyl derivatives and PBTA congeners were detected in the water of the Waka River and the Yodo River system, respectively. Except for DCB, biological activities of these water pollutants have been evaluated mostly by the Ames assay, and data on biological effects of these compounds, including genotoxicity in mammalian cells and *in vivo*, are quite limited. To estimate risks of these compounds to aquatic biota and human health, further investigations on their biological activities to aquatic organisms and experimental animals are necessary. In addition, quantitative studies on these compounds in these rivers are important, and exposure levels of aquatic organisms and human

to those compounds need to be determined. Because aquatic organisms inhabiting in these rivers may be exposed chronically to these genotoxic chemicals, ecological studies, including the incidence of cancer in fish and aquatic animals, are also needed.

Conflicts of interest

None.

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Synthesis of a New Mutagenic Benzoazepinoquinolinone Derivative

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Abstract: A novel mutagenic compound **1**, isolated as a Maillard product from tryptophan and glucose, was synthesized using Larock's quinoline formation, where addition of iodonium cation to an acetylene moiety of *N*-propargylaniline triggers subsequent intramolecular electrophilic aromatic substitution to afford quinolines. The key synthetic intermediate **14** was obtained in a good yield when iodonium chloride was employed as an initiator of Larock's method. Conversion of **14** with another six steps, including annulation of a lactam ring and Curtius rearrangement, furnished the target molecule **1**. The synthesized and isolated **1** were identical in comparison of physical and spectral data.

Key words: Larock's quinoline formation, Curtius rearrangement, benzoazepinoquinolinone derivative

The browning reaction that produces melanoidine on heating reducing sugar and amino compounds, such as amino acids and peptides, is referred to the Maillard reaction. The reaction is important for making appetite-stimulating flavors during baking or heating; however, it sometimes yields undesirable compounds, for example, neurotoxic acrylamide produced from asparagine and glucose. Since the Maillard reaction can also produce carcinogenic or mutagenic fused-aromatic compounds as well as neurotoxic amines, a huge number of its products have been identified and assayed to date. Recently, one of the authors and his colleagues isolated a novel mutagenic compound from a Maillard reaction involving L-tryptophan and D-glucose as an amino acid and a sugar, respectively, and initiated by Fenton's reagent. The compound was presumed to be a benzoazepinoquinolinone derivative **1** based on spectroscopic data;[1] however, this was not confirmed with chemical evidence (Figure 1). Therefore, we embarked on the synthesis of **1** to confirm its structure and develop a method to provide enough amounts of **1** for further biological analysis.

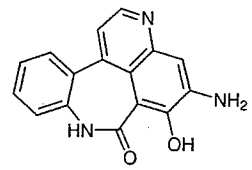

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Figure 1 Structure of compound **1**

Initially, Skraup's quinoline formation[2] was employed in the synthesis of **1** (Scheme 1). Namely, starting with 2-nitrophenyl vinyl ketone (**2**)[3] and 3,5-dimethyl-4-methoxyaniline (**3a**), easily produced from commercially available 2-nitrobenzaldehyde (**4**) and 2,6-dimethyl-4-nitrophenol (**5**), respectively, the quinoline derivative **6** was prepared according to Skraup's protocol. Although the yield was low, **6** was obtained; however, subsequent oxidation of the methyl groups resulted in unfertilized under all conditions tested.

Moreover, neither 3,5-dimethoxycarbonyl-4-methoxytoluene (**3b**) nor 3,5-dimethoxymethyl-4-methoxytoluene (**3c**) was a good substrate for Skraup's method. Since these difficulties could not be overcome, another strategy was needed.

After several attempts, our retrosynthetic analysis of **1** finally reached a promising strategy that employs Larock's quinoline formation, that is, the annulation reaction of *N*-(2-alkynyl)aniline with an appropriate electrophile.[4] Stated it concretely, 2-iodonitrobenzene (**7**) was first treated with propargyl alcohol in the presence of a palladium catalyst to afford 2-nitrophenylpropargyl alcohol (**8**), of which the hydroxyl group was converted to bromide **9** with carbon tetrabromide and triphenylphosphine (Scheme 2). On the other hand, nitration of 2-methoxyisophthalic acid (**10**)[5] and successive methylation of carboxylic acid by a conventional method gave dimethyl 2-methoxy-5-nitroisophthalate (**11**), the nitro group of which was reduced to an amino group by catalytic hydrogenation to afford dimethyl 5-amino-2-methoxyisophthalate (**12**). Next, the reaction of **9** and **12** in the presence of potassium carbonate gave the propargylamine derivative **13**.

The propargylamine **13** was transformed to a quinoline derivative **14**[7] using Larock's method, where addition of iodonium cation to an acetylene moiety of *N*-propargylaniline triggers subsequent intramolecular electrophilic aromatic substitution to furnish quinolines (Scheme 3).[4] Herein, iodonium chloride was preferably employed to other iodonium sources, for example, iodine, as an initiator for giving a good yield of **14**. Subsequent reduction of the iodo group of **14** with palladium in the presence of formic acid, followed by catalytic hydrogenation on Pd/C for

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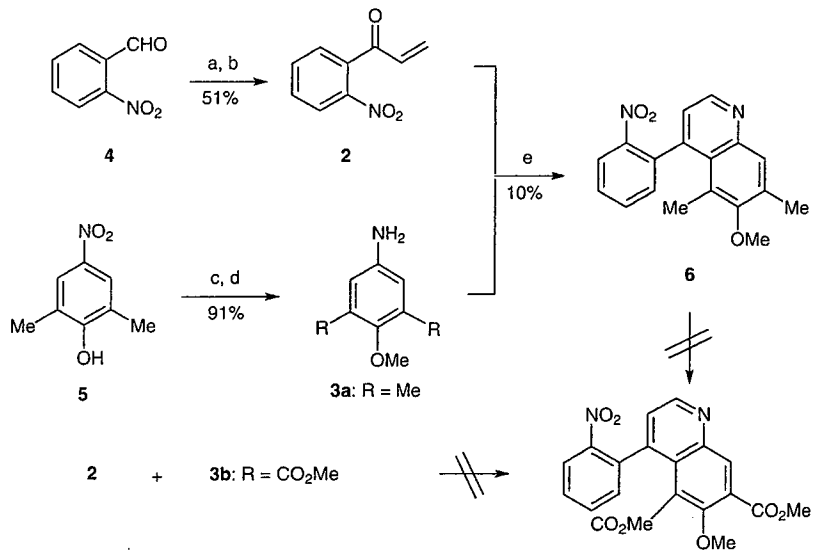

Scheme 1 Early synthetic route to mutagenic compound **1**. *Reagents and conditions*: a: H_2C=CHMgBr (1.5 equiv), THF, –20 °C; b: Dess–Martin periodinane (1.2 equiv), CH_2Cl_2, 0 °C; c: MeI (2.5 equiv), K_2CO_3 (3.0 equiv); acetone, 50 °C; d: Pd/C, H_2, EtOH, r.t.; e: AcOH, sodium 3-nitrobenzenesulfonate (3 equiv).

the reduction of the nitro group yielded amine **15**. Treatment of **15**[8] with methanesulfonic acid at a high temperature provided the ε-lactam derivative **16**,[9] of which the methyl ester was saponified to carboxylic acid and further converted to *tert*-butyl carbamate by Curtius rearrangement with diphenyl phosphoryl azide (DPPA)[6] in *tert*-butyl alcohol to afford compound **17**.[10] In the final step, synchronous cleavage of methyl and Boc protecting groups with boron tribromide furnished the target molecule **1**.[11] The spectral data and physical properties of the synthesized compound were completely consistent with those of the mutagenic component isolated from Maillard medium.

In conclusion, we succeeded in the total synthesis of the mutagenic compound **1**, which was isolated as a Maillard product from tryptophan and glucose, by taking advantage of Larock's quinoline formation. A comparison of spectral data showed that the synthesized and isolated compounds are identical, confirming the structure of **1** shown in Figure 1. Finally, the synthesized form of **1** showed as potent mutagenic activity as **1** isolated from the Maillard medium.

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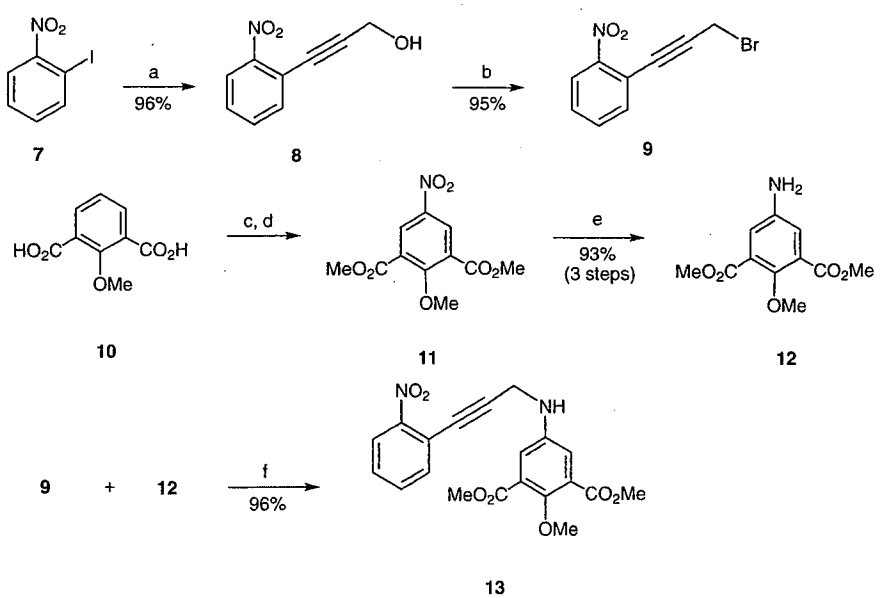
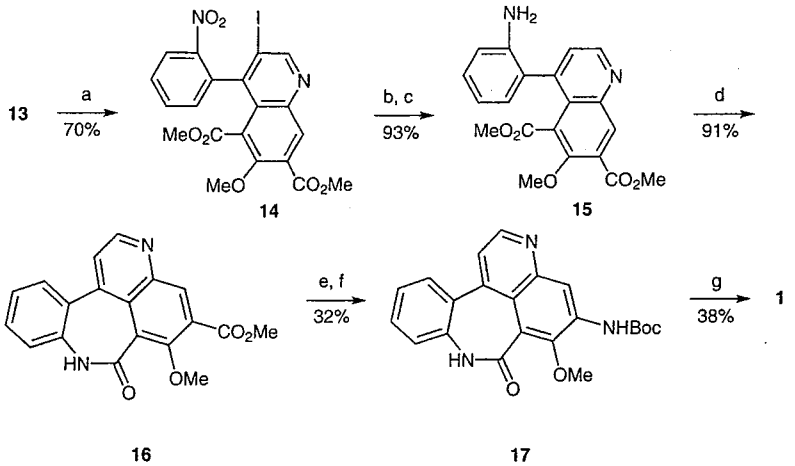


Scheme 2 Synthetic route to propargyl amine (**13**). *Reagents and conditions*: (a) propargyl alcohol (1.8 equiv), $Pd(PPh_3)_2Cl_2$ (2 mol%), CuI (7 mol%), Et_3N, r.t.; (b) CBr_4 (1.2 equiv), Ph_3P (1.2 equiv), CH_2Cl_2, 0 °C; (c) HNO_3, H_2SO_4, r.t.; (d) 5% HCl in MeOH, 70 °C; (e) Pd/C, H_2, *i*-PrOH, 80 °C; (f) K_2CO_3 (1.5 equiv), DMF, r.t.



Scheme 3 Synthetic route of compound **1**. *Reagent and conditions*: (a) ICl (5.6 equiv), $NaHCO_3$ (3.0 equiv), MeCN, 40 °C; (b) $Pd(PPh_3)_4$ (0.2 equiv), HCO_2H (3.0 equiv), Et_3N (3.0 equiv), DMF, 50 °C; (c) Pd/C, H_2, *i*-PrOH, 50–80 °C; (d) MsOH (0.3 equiv), 2-dichlorobenzene, 150 °C; (e) 2 M KOH aq MeOH, r.t.; (f) DPPA (6.3 equiv), Et_3N (9.5 equiv), *t*-BuOH, reflux; (g) BBr_3 (8.7 equiv), CH_2Cl_2, –78 °C.

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(7) **Compound 14**
$NaHCO_3$ (859.8 mg, 10.23 mmol) and ICl (1.0 mL, 19.09 mmol) were added to a soln of **13** (1.35 g, 3.40 mmol) in MeCN (15 mL) at r.t., and the mixture was stirred at 40 °C for 25.5 h. The reaction mixture was quenched with a sat. aq soln of $Na_2S_2O_3$, and diluted with a sat. aq soln of $NaHCO_3$ and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane–EtOAc = 2:1) to afford **14** (1.25 g, 70%). Yellow crystals; mp 194–195 °C (hexane–EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.25 (s, 1 H), 8.75 (s, 1 H), 8.41 (dd, *J* = 8.0, 1.3 Hz, 1 H), 7.82 (dt, *J* = 8.0, 1.3 Hz, 1 H), 7.75 (dt, *J* = 8.0, 1.7 Hz, 1 H), 7.28 (br d, *J* = 8.0 Hz, 1 H), 4.00 (s, 3 H), 3.81 (s, 3 H), 3.23 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.5, 164.7, 156.8, 155.3, 147.9, 143.5, 136.6, 135.2, 133.9, 133.2, 130.7, 127.2, 126.0, 125.8, 124.8, 101.3, 70.9, 64.1, 52.8, 52.2. IR (KBr): 1730, 1608, 1525, 1471, 1438, 1344, 1248, 1213, 1168 cm^{-1}. MS (70 eV): *m/z* (%) = 522 (100) [M$^+$], 491 (16), 400(12), 335 (37), 275 (32). HRMS: *m/z* calcd for $C_{20}H_{15}IN_2O_7$ [M$^+$]: 521.9924; found: 521.9931.

(8) **Compound 15**
Tetrakis(triphenylphosphine) palladium (5.0 mg, 0.004 mmol), Et_3N (12 μL, 0.087 mmol), and formic acid (2.7 μL, 0.065 mmol) were added to a soln of **14** (22.7 mg, 0.043 mmol) in *N*,*N*-dimethylformamide (1 mL) at r.t., and the mixture was stirred at 50 °C for 3 h. The reaction mixture was poured into a sat. aq soln of $NaHCO_3$ and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane–EtOAc = 1:1.5) to afford deiodine compound (16.8 mg, 98%). A suspension of the above compound (15.7 mg, 0.040 mmol) and 10% Pd/C (3.1 mg) in 2-PrOH (1.0 mL) was stirred for 0.5 h at 50 °C and for another 1 h at 80 °C under a hydrogen atmosphere. The mixture was filtered with Celite, and the filtrate was concentrated in vacuo to remove the organic solvent. The residue was purified by column chromatography on silica gel (hexane–EtOAc = 1:1.5) to afford **15** (13.8 mg, 95%). Pale yellow crystals; mp 198–199 °C (hexane–EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.96 (d, *J* = 4.2 Hz, 1 H), 8.76 (s, 1 H), 7.39 (d, *J* = 4.2 Hz, 1 H), 7.24 (ddd, *J* = 8.1, 7.4, 1.3 Hz, 1 H), 6.99 (dd, *J* = 8.1, 1.3 Hz, 1 H), 6.84 (dt, *J* = 7.4, 1.3 Hz, 1 H), 6.80 (dd, *J* = 8.1, 1.3 Hz, 1 H), 4.01 (s, 3 H), 3.89 (s, 3 H), 3.53 (br, 2 H), 3.27 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.5, 165.1, 154.6, 150.4, 145.5, 144.5, 144.3, 136.4, 131.1, 129.9, 127.3, 126.9, 126.5, 126.1, 122.7, 117.9, 115.8, 64.2, 52.7, 52.2. IR (KBr): 3436, 3381, 2950, 1728, 1643, 1606, 1487, 1448, 1248, 1224, 1149 cm^{-1}. MS (70 eV): *m/z* (%) = 366 (44) [M$^+$], 306 (100), 291 (67), 275 (52), 259 (85), 219 (56), 203 (43), 102 (34), 77 (16). HRMS: *m/z* calcd for $C_{20}H_{18}N_2O_5$ [M$^+$]: 366.1215; found: 366.1207.

(9) **Compound 16**
A soln of **15** (66.8 mg, 0.182 mmol) and MsOH (3.5 mL, 0.055 mmol) in *o*-dichlorobenzene (2.0 mL) was stirred at 150 °C for 2 h. The reaction mixture was poured into a sat. aq soln of $NaHCO_3$ and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane–EtOAc = 1:2) to afford **16** (55.6 mg, 91%). Yellow crystals; mp 190–191 °C (hexane–EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.89 (d, *J* = 4.5 Hz, 1 H), 8.50 (s, 1 H), 8.03 (s, 1 H), 7.60 (d, *J* = 4.5 Hz, 1 H), 7.45 (ddd, *J* = 7.9, 7.1, 1.3 Hz, 1 H), 7.33 (dd, *J* = 7.9, 1.3 Hz, 1 H), 7.27 (ddd, *J* = 8.4, 7.1, 1.3 Hz, 1 H), 7.13 (dd, *J* = 8.4, 1.3 Hz, 1 H), 4.13 (s, 3 H), 3.98 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.8, 165.7, 162.9, 149.6, 142.6, 142.1, 136.8, 135.0, 132.1, 131.8, 130.7, 130.2, 129.0, 125.9, 121.6, 121.4, 116.3, 63.9, 52.7. IR (KBr): 3058, 2950, 1735, 1660, 1591, 1475, 1286, 1195, 1120 cm^{-1}. MS (70 eV): *m/z* (%) = 334 (100) [M$^+$], 317 (96), 305 (46), 277 (29), 246 (27), 218 (31), 203 (49), 102 (44).

HRMS: *m/z* calcd for $C_{19}H_{14}N_2O_4$ [M^+]: 334.0953; found: 334.0947.

(10) **Compound 17**

An aq soln of KOH (2 M, 1.0 mL) was added to a soln of **16** (89.3 mg, 0.267 mmol) in MeOH (0.4 mL), and the mixture was stirred at r.t. for 8.5 h. The reaction mixture was concentrated in vacuo to remove the solvent. The residue was dissolved in H_2O, and the pH was adjusted to 6 with an aq soln of 2 M HCl. The mixture was extracted with $CHCl_3$ and EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica gel ($CHCl_3$–MeOH = 2:1) to afford a carboxylic acid derivative (62.2 mg, 73%). Diphenyl phosphoryl azide (0.8 ml, 3.76 mmol) and Et_3N (0.8 ml, 5.74 mmol) were added to a soln of the above compound (192.5 mg, 0.601 mmol) in *t*-BuOH (3.0 mL) at r.t., and the mixture was refluxed for 27.5 h. The solvent was removed in vacuo, and the residue was purified by column chromatography on silica gel (hexane–EtOAc = 1:1) to afford **17** (103.7 mg, 44%). Pale yellow crystals; mp 108–109 °C (EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.84 (s, 1 H), 8.81 (d, *J* = 4.6 Hz, 1 H), 8.49 (s, 1 H), 7.51 (s, 1 H), 7.44 (d, *J* = 4.6 Hz, 1 H), 7.42 (ddd, *J* = 8.0, 7.2, 1.2 Hz, 1 H), 7.35 (dd, *J* = 7.6, 1.2 Hz, 1 H), 7.26 (ddd, *J* = 7.6, 7.2, 1.2 Hz, 1 H), 7.14 (dd, *J* = 8.0, 1.2 Hz, 1 H), 4.16 (s, 3 H), 1.56 (s, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 167.5, 156.0, 152.1, 148.9, 144.4, 141.4, 135.0, 133.6, 132.0, 130.8, 130.2, 125.7, 125.5, 121.3, 120.1, 119.0, 112.9, 81.3, 63.4, 28.3 (3 C). IR (KBr): 3433, 2978, 1732, 1657, 1591, 1523, 1479, 1353, 1336, 1234, 1155 cm^{-1}. MS (70 eV): *m/z* (%) = 391 (5 [M^+], 335 (11), 291 (27), 274 (13), 262 (27), 57 (100), 41 (52). HRMS: *m/z* calcd for $C_{22}H_{21}N_3O_4$ [M^+]: 391.1532; found: 391.1538.

(11) **Compound 1**

A soln of BBr_3 in CH_2Cl_2 (1 M, 170 μL, 0.170 mmol) was added to a soln of **17** (13.4 mg, 0.034 mmol) in CH_2Cl_2 (0.4 mL) at –78 °C, and the mixture was stirred at –78 °C for 2 h. The mixture was poured into a sat. aq soln of $NaHCO_3$ and extracted with $CHCl_3$. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was roughly purified with preparative thin-layer chromatography (hexane–EtOAc = 1:5) and the powder obtained was further purified by washing with $CHCl_3$ and MeOH to afford **1** (3.5 mg, 38%). Ocher powder; mp (dec.). ^{1}H NMR (600 MHz, DMSO-d_6): δ = 15.23 (s, 1 H), 10.42 (s, 1 H), 8.51 (d, *J* = 4.6 Hz, 1 H), 7.43 (dd, *J* = 1.5, 8.0 Hz, 1 H), 7.41 (dd, *J* = 1.5, 8.0 Hz, 1 H), 7.30 (d, *J* = 4.6 Hz, 1 H), 7.29 (dd, *J* = 1.5, 6.9 Hz, 1 H), 7.24 (dt, *J* = 1.5, 8.0 Hz, 1 H), 7.20 (s, 1 H), 5.82 (s, 2 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 176.1, 157.5, 147.3, 144.9, 141.4, 140.6, 137.1, 131.7, 130.2, 128.0, 125.6, 120.7, 120.3, 117.9, 111.8, 104.5. IR (KBr): 3469, 3298, 3193, 1637, 1593, 1521, 1469, 1409, 1353, 1313, 1286, 1259 cm^{-1}. MS (70 eV): *m/z* (%) = 277 (100) [M^+], 259 (63), 231 (40), 204 (32), 177 (18), 102 (11), 77 (7). HRMS: *m/z* calcd for $C_{16}H_{11}N_3O_2$ [M^+]: 277.0851; found: 277.0856.

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Molecular cloning of apoptosis-inducing Pierisin-like proteins, from two species of white butterfly, *Pieris melete* and *Aporia crataegi*☆

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ABSTRACT

Pierisin-1, present in cabbage butterfly, *Pieris rapae*, induces apoptosis against various kinds of cancer cell lines. Another cabbage butterfly, *Pieris brassicae*, also has an apoptosis-inducing protein, Pierisin-2. These proteins exhibit DNA ADP-ribosylating activity. Pierisin-like proteins are found to be distributed in subtribes Pierina, Aporiina and Appiadina. In this study, we performed the cDNA cloning of Pierisin-like proteins designated Pierisin-3 from gray-veined white, *Pieris melete*, and Pierisin-4 from black-veined white, *Aporia crataegi*. The nucleotide sequences of Pierisin-3 and -4 encode an 850 and an 858 amino acid protein, respectively. The partial peptide sequences of Pierisin-3 and -4 purified from pupae were identical to the deduced amino acid sequence of ORF. The deduced amino acid sequence revealed that Pierisin-3 is 93% similar to Pierisin-1 and Pierisin-4 is 64%. Pierisin-3 and -4 synthesized *in vitro* with the rabbit reticulocyte lysate exhibited apoptosis-inducing activity against human cervical carcinoma HeLa and human gastric carcinoma TMK-1 cells. Site-directed mutagenesis at a glutamic acid residue comprising the NAD-binding site resulted in a significant decrease in cytotoxicity of both proteins. Moreover, the proteins incubated with calf thymus DNA and β-NAD resulted in the formation of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine, as in the case of Pierisin-1 and -2. These findings could provide useful information for understanding the importance of apoptosis-inducing ability and molecular evolution of Pierisin-like proteins in family Pieridae.

1. Introduction

Pierisin-1, an apoptosis-inducing protein, has been identified from the cabbage butterfly, *Pieris rapae* (Koyama et al., 1996; Watanabe et al., 1998). Pierisin-1 has potent cytotoxicity against various human cancer cell lines, with 50% inhibitory concentration values ranging from 0.043–150 ng/ml (Kono et al., 1999). The amino acid sequence of this protein deduced from the cDNA contains 850 amino acids with a calculated molecular weight of 98,000. Amino acid alignment indicates that Pierisin-1 is 32% similar to the mosquitocidal toxin (MTX), a kind of ADP-ribosyltransferase from *Bacillus sphaericus* SSII-1 (Watanabe et al., 1999; Carpusca et al., 2006).

ADP-ribosylating toxins, such as pertussis and cholera toxins, target α-subunits of G-proteins (Zhang et al., 1995; Locht and Keith, 1986) while diphtheria toxin ADP-ribosylates a diphthamide residue on elongation factor 2 (Bell and Eisenberg, 1996). MTX ADP-ribosylates proteins with molecular masses of 42 and 38 kDa in lysates of mosquito *Culex quinquefasciatus* G7 cells (Thanabalu et al., 1993) and *Escherichia coli* elongation factor Tu (Schirmer et al., 2002). ADP-ribosyltransferases also exist in mammals. For example, mammalian ectoenzyme ART2-catalyzed ADP-ribosylation of cell membrane proteins induces formation of cytolytic membrane pores by activating the P2X7 purinoceptor (Koch-Nolte et al., 2008). On the other hand, Pierisin-1 modifies N^2 amino groups of guanine residues in DNA to yield N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine (Takamura-Enya et al., 2001). The 27-kDa N-terminal region of Pierisin-1 has DNA ADP-ribosylating activity, and the 71-kDa C-terminal region binds to glycosphingolipid receptors such as globotriaosylceramide (Gb3) and globotetraosylceramide (Gb4) on the surfaces of mammalian cells and incorporates Pierisin-1 into cells (Kanazawa et al., 2001; Matsushima-Hibiya et al., 2003). The mRNA of pierisin-1 is highly expressed in the fifth instar larvae, and the protein is accumulated in the fat bodies during the fifth instar larvae to early pupae (Watanabe et al., 2004). Therefore, Pierisin-1 may act to remove the unnecessary cells of larval tissues in the pupation. Moreover, Pierisin-1 may have the ability to protect from natural enemies, such as parasitic wasps, by exhibiting potent cytotoxicity in *P. rapae*.

The other cabbage butterfly, *Pieris brassicae*, also contains an apoptosis-inducing protein named Pierisin-2. cDNA of Pierisin-2 encodes 850 amino acids. The amino acid sequence deduced from the cDNA shows that Pierisin-2 is 91% similar to Pierisin-1. Pierisin-2 exhibits cytotoxicity similar to Pierisin-1 (Matsushima-Hibiya et al., 2000). Pierisin-2 catalyzes

Abbreviations: Gb3, globotriaosylceramide; Gb4, globotetraosylceramide.

☆ Database: The nucleotide sequences of pierisin-3 from *Pieris melete* and pierisin-4 from *Aporia crataegi* have been submitted to the DDBJ/EMBL/GenBank database under the accession numbers AB477051 (pierisin-3) and AB477052 (pierisin-4).

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ADP-ribosylation of dG in DNA to give the same reaction product as demonstrated for Pierisin-1 (Takamura-Enya et al., 2004).

Recently, we reported the distribution of Pierisin-like proteins in the subfamily Pierinae, family Pieridae (Matsumoto et al., 2008). Protein extracts from 13 species of butterflies in this subfamily exhibited DNA ADP-ribosylating activity and cytotoxicity against the human cervical carcinoma HeLa and gastric carcinoma TMK-1 cells. All of these extracts contained substances recognized by anti-Pierisin-1 antibodies. Moreover, sequences containing NAD-binding sites, conserved in ADP-ribosyltransferases, were amplified from genomic DNA from 13 species of butterflies by PCR. However, three species of butterflies in the subfamily Pierinae and four species of butterflies in the subfamily Coliadinae, the family Pieridae showed neither cytotoxicity nor ADP-ribosylating activity, and did not contain substances recognized by anti-Pierisin-1 antibodies. Sequences containing NAD-binding sites were not amplified from the genomic DNA from these seven species (Matsumoto et al., 2008). Thus, Pierisin-like proteins are distributed in butterflies not only of the subtribe Pierina, including the genus *Pieris*, but also of the subtribes Aporiina and Appiadina. Interestingly, the subfamily Pierinae is divided into two types by the morphology of the pupal stage (Braby et al., 2006), and this classification of pupal morphology corresponds with the distribution of Pierisin-like protein, except for that in *Appias lyncida* (Matsumoto et al., 2008).

In this study, we report the cDNA cloning of apoptosis-inducing proteins named Pierisin-3 from the gray-veined white, *Pieris melete*, and named Pierisin-4 from the black-veined white, *Aporia crataegi*. These two species belong to subtribes Pierina and Aporiina respectively, and these extracts had been shown to have Pierisin-like activity (Matsumoto et al., 2008). The deduced amino acid sequence from the cDNA indicated that Pierisin-3 and -4 have a close amino acid similarity to Pierisin-1 and -2. Both *in vitro* expressed proteins exhibited cytotoxicity and DNA ADP-ribosylating activity similar to Pierisin-1 and -2. The evolutionary conservation for amino acid sequences of Pierisin-3 and -4 to compare with Pierisin-1 and -2 is discussed.

2. Materials and methods

2.1. Insects and RNA isolation

The fifth instar larvae of *P. melete* were collected in the Tochigi Prefecture, Japan, and were reared on natural host plants, cabbage, *Brassica oleracea*, at room temperature until pupation in our laboratory and stored at −80 °C until use. The fifth instar larvae of *A. crataegi* were purchased from the Eikoh Science Corp. (Osaka, Japan), and were reared on natural host plants, Yoshino cherry, *Prunus yedoensis*, at room temperature until pupation in our laboratory and stored at −80 °C until use. Some fifth instar larvae of *P. melete* and *A. crataegi* were frozen at day 1 and stored at −80 °C for the preparation of total RNA. Total RNA was prepared from the whole body of a day 1 fifth instar larvae by using Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The 5′ RACE and 3′ RACE were performed by using GeneRacer™ Kit (Invitrogen) according to the manufacturer's instructions and 1 μg of total RNA as starting material. The 5′- and 3′- phosphate groups of RNA were dephosphorylated, the 5′-cap structures of full length mRNA were replaced by GeneRacer™ RNA Oligo by oligo-capping method (Maruyama and Sugano, 1994). Then, first-strand cDNA synthesis was carried out with SuperScript® III Reverse Transcriptase using GeneRacer™ Oligo dT Primer.

2.2. cDNA cloning of Pierisin-3 and -4

For cDNA cloning of Pierisin-3 and Pierisin-4 from *P. melete* and *A. crataegi*, respectively, the sequences containing NAD-binding sites, which are conserved in ADP-ribosyltransferase were cloned. Then, the full length of cDNAs was obtained by PCR, as described below.

The sequences containing NAD-binding sites were amplified using degenerate primers PierisinDP_NF1 and PierisinDP_NR1 deduced from the Pierisin-1 and -2 amino acid sequences (DDBJ accession numbers AB030305 and AB037676) (Watanabe et al., 1999; Matsushima-Hibiya et al., 2000). The PCR was performed for 2 min at 98 °C followed by 35 cycles of 10 s at 98 °C, 20 s at 58 °C, 1 min at 72 °C, and a final extension for 7 min at 72 °C by using Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) in iCycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The purified PCR products were cloned into pCR®-Blunt II-TOPO® vector (Invitrogen), and were then sequenced in both directions, using ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Amersham Place, England).

In Pierisin-3, Pm_F5 primer and GeneRacer™ 3′ Nested Primer were used for 3′ RACE and Pm_R2 primer and GeneRacer™ 5′ Nested Primer were used for 5′ RACE. The PCR was performed for 2 min at 98 °C followed by 35 cycles of 30 s at 98 °C, 30 s at 58 °C, 1–4 min at 72 °C, and a final extension for 7 min at 72 °C. The PCR products were excised from the agarose gel and purified by using Wizard® SV Gel and the PCR Clean-Up System, and then the PCR fragments were cloned into the pCR®-Blunt II-TOPO® vector or StrataClone™ PCR Cloning Vector pSC-B (STRATAGENE, La Jolla, CA, USA) and sequenced. The full length of cDNA was obtained by PCR using forward primer PmF_F and reverse primer PmF_R. The amplified PCR product was purified by Wizard® SV Gel and PCR Clean-Up System and subjected to the PCR direct sequencing to determine the full length cDNA sequence.

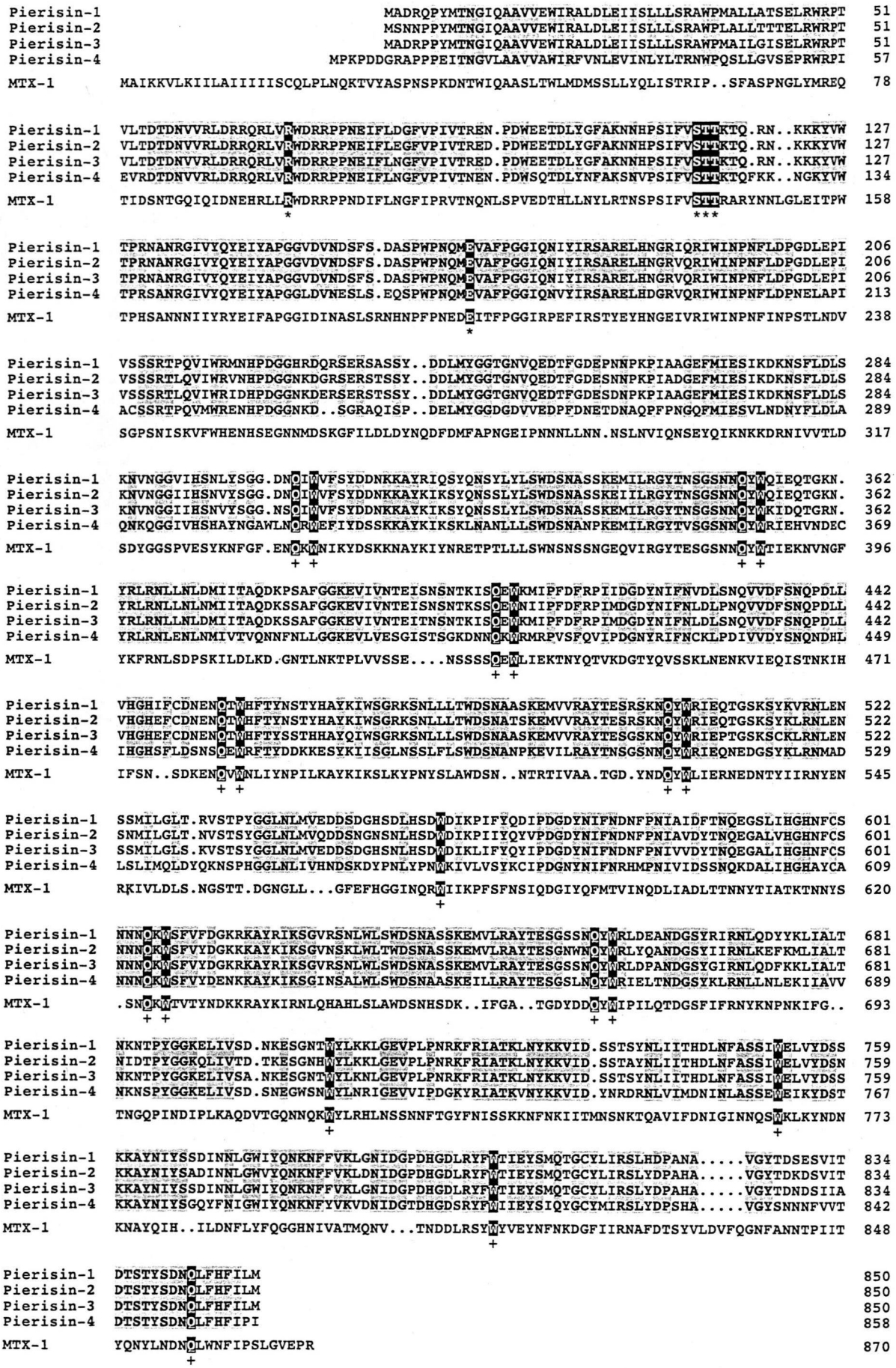
In the case of Pierisin-4, Ac_F1 primer and GeneRacer™ 3′ Primer were used for 3′ RACE and Ac_R3 primer and GeneRacer™ 5′ Nested Primer for 5′ RACE were used. Moreover, the full length of cDNA was obtained by the PCR using forward primer AcF_F and reverse primer AcF_R. The amplified PCR product was purified and sequenced in both directions as in Pierisin-3.

2.3. Construction of clones of the coding region of the Pierisin-3 and -4 genes

The coding region of the *Pierisin-3* gene, with 442 bp of 3′-flanking region, was amplified, and *SalI* and *EcoRI* restriction sites were introduced into the 5′ and 3′ ends, using the primer pair Pm_Sal and Pm_Eco. The amplified fragment was digested with *SalI* and *EcoRI* and inserted into the *SalI-EcoRI* site of pMAL-p2x (New England Biolabs, Ipswich, MA, USA) in the opposite direction to the *tac* promoter. In the same way, the coding region of the *Pierisin-4* gene, with 355 bp of 3′-flanking regions, was amplified, and *NheI* and *SacI* restriction sites were introduced into the 5′ and 3′ ends, using the primer pair Ac_Nhe and Ac_Sac. These clones were confirmed by sequencing the insert in both directions.

2.4. Purification and amino acid sequence determination of Pierisin-3 and -4

Because the chromatographic characteristics of *Pierisin-3* from *P. melete* were expected to be similar to *Pierisin-1*, purification of *Pierisin-3* from pupae of *P. melete* was carried out using the same method for *Pierisin-1* (Watanabe et al., 1998, 1999). The crude extracts from 112 pupae were added with ammonium sulfate to give 35% saturation and then centrifuged. The precipitates were dialyzed and applied to an anion-exchange DEAE-cellulose DE52 column (2.5×7 cm; Whatman, Kent, England). The fractions containing the cytotoxicity were separated by SDS-PAGE. About 98-kDa bands of these fractions were excised and digested in gel with trypsin. The digested peptides were separated by reverse-phase-HPLC with a Symmetry® C18 3.5 μm column (1.0×150 mm; Waters, Milford, MA, USA). Amino acid sequences of the isolated peptides were determined by a Procise 494 cLC Protein Sequencing System (Applied Biosystems).


Pierisin-1 MADRQPYMTNGIQAAVVEWIRALDLEIISLLLSRAWPMALLATSELRWRPT 51
Pierisin-2 MSNNPPYMTNGIQAAVVEWIRALDLEIISLLLSRAWPLALLTTTELRWRPT 51
Pierisin-3 MADRPPYMTNGIQAAVVEWIRALDLEIISLLLSRAWPMAILGISELRWRPT 51
Pierisin-4 MPKPDDGRAPPPEITNGVLAAVVAWIRFVNLEVINLYLTRNWPQSLLGVSEPRWRPI 57
MTX-1 MAIKKVLKIILAIIIIISCQLPLNQKTVYASPNSPKDNTWIQAASLTWLMDMSSLLYQLISTRIP..SFASPNGLYMREQ 78
Pierisin-1 VLTDTDNVVRLDRRQRLVRWDRRPPNEIFLDGFVPIVTREN.PDWEETDLYGFAKNNHPSIFVSTTKTQ.RN..KKKYVW 127
Pierisin-2 VLTDTDNVVRLDRRQRLVRWDRRPPNEIFLEGFVPIVTRED.PDWEETDLYGFAKNNHPSIFVSTTKTQ.RN..KKKYVW 127
Pierisin-3 VLTDTDNVVRLDRRQRLVRWDRRPPNEIFLNGFVPIVTRED.PDWEETDLYGFAKNNHPSIFVSTTKTQ.RN..KKKYVW 127
Pierisin-4 EVRDTDNVVRLDRRQRLVRWDRRPPNEIFLNGFVPIVTNEN.PDWSQTDLYNFAKSNVPSIFVSTTKTQFKK..NGKYVW 134
MTX-1 TIDSNTGQIQIDNEHRLLRWDRRPPNDIFLNGFIPRVTNQNLSPVEDTHLLNYLRTNSPSIFVSTTRARYNNLGLEITPW 158
Pierisin-1 TPRNANRGIVYQYEIYAPGGVDVNDSFS.DASPWPNQMEVAFPGGIQNIYIRSARELHNGRIQRIWINPNFLDPGDLEPI 206
Pierisin-2 TPRNANRGIVYQYEIYAPGGVDVNDSFS.DASPWPNQMEVAFPGGIQNIYIRSARELHNGRVQRIWINPNFLDPGDLEPI 206
Pierisin-3 TPRNANRGIVYQYEIYAPGGVDVNDSFS.DASPWPNQMEVAFPGGIQNIYIRSARELHNGRVQRIWINPNFLDPGDLEPI 206
Pierisin-4 TPRSANRGIVYQYEIYAPGGLDVNESLS.EQSPWPNQMEVAFPGGIQNVYIRSARELHDGRVQRIWINPNFLDPNELAPI 213
MTX-1 TPHSANNNIIYRYEIFAPGGIDINASLSRNHNPFPNEDEITFPGGIRPEFIRSTYEYHNGEIVRIWINPNFINPSTLNDV 238
Pierisin-1 VSSSRTPQVIWRMNHPDGGHRDQRSERSASSY..DDLMYGGTGNVQEDTFGDEPNNPKPIAAGEFMIESIKDKNSFLDLS 284
Pierisin-2 VSSSRTLQVIWRVNHPDGGNKDGRSERSTSSY..DDLMYGGTGNVQEDTFGDESNNPKPIADGEFMIESIKDKNSFLDLS 284
Pierisin-3 VSSSRTLQVIWRIDHPDGGNKDERSERSTSSY..DDLMYGGTGNVQEDTFGDESDNPKPIAAGEFMIESIKDKNSFLDLS 284
Pierisin-4 ACSSRTPQVMWRENHPDGGNKD..SGRAQISP..DELMYGGDGDVVEDPFDNETDNAQPFPNGQFMIESVLNDNYFLDLA 289
MTX-1 SGPSNISKVFWHENHSEGNNMDSKGFILDLDYNQDFDMFAPNGEIPNNNLLNN.NSLNVIQNSEYQIKNKKDRNIVVTLD 317
Pierisin-1 KNVNGGVIHSNLYSGG.DNQIWVFSYDDNKKAYRIQSYQNSYLYLSWDSNASSKEMILRGYTNSGSNNQYWQIEQTGKN. 362
Pierisin-2 KNVNGGIIHSNVYSGG.DNQIWVFSYDDNKKAYKIKSYQNSSLYLSWDSNASSKEIILRGYTNSGSNNQYWQIEQTGKN. 362
Pierisin-3 KNVNGGIIHSNVYSGG.NSQIWVFSYDDNKKAYKIKSYQNSSLYLSWDSNASSKEMILRGYTNSGSNNQYWKIDQTGRN. 362
Pierisin-4 QNKQGGIVHSHAYNGAWLNQRWEFIYDSSKKAYKIKSKLNANLLLSWDSNANPKEMILRGYTVSGSNNQYWRIEHVNDEC 369
MTX-1 SDYGGSPVESYKNFGF.ENQKWNIKYDSKKNAYKIYNRETPTLLLSWNSNSSNGEQVIRGYTESGSNNQYWTIEKNVNGF 396
Pierisin-1 YRLRNLLNLDMIITAQDKPSAFGGKEVIVNTEISNSNTKISQEWKMIPFDFRPIIDGDYNIFNVDLSNQVVDFSNQPDLL 442
Pierisin-2 YRLRNLLNLNMIITAQDKSSAFGGKEVIVNTEISNSNTKSSQEWNIIPFDFRPIMDGDYNIFNLDLPNQVVDFSNQPDLL 442
Pierisin-3 YRLRNLLNLDMIITAQDKSSAFGGKEVIVNTEITNSNTKISQEWKMIPFDFRPIMDGDYNIFNLDLSNQVVDFSNQPDLL 442
Pierisin-4 YRLRNLENLNMIVTVQNNFNLLGGKEVLVESGISTSGKDNNQKWRMRPVSFQVIPDGNYRIFNCKLPDIVVDYSNQNDHL 449
MTX-1 YKFRNLSDPSKILDLKD.GNTLNKTPLVVSSE....NSSSSQEWLIEKTNYQTVKDGTYQVSSKLNENKVIEQISTNKIH 471
Pierisin-1 VHGHIFCDNENQTWHFTYNSTYHAYKIWSGRKSNLLLTWDSNAASKEMVVRAYTESRSKNQYWRIEQTGSKSYKVRNLEN 522
Pierisin-2 VHGHEFCDNENQTWHFTYNSTYHAYKIWSGRKSNLLLTWDSNATSKEMVVRAYTESRSKNQYWRIEQTGSKSYKLRNLEN 522
Pierisin-3 VHGHEFCDNENQTWHFTYSSTHHAYQIWSGRKSNLLLSWDSNAASKEMVVRAYTESGSKNQYWRIEPTGSKSCKLRNLEN 522
Pierisin-4 IHGHSFLDSNSQEWRFTYDDKKESYKIISGLNSSLFLSWDSNANPKEVILRAYTNSGSNNQYWRIEQNEDGSYKLRNMAD 529
MTX-1 IFSN..SDKENQVWNLIYNPILKAYKIKSLKYPNYSLAWDSN..NTRTIVAA.TGD.YNDQYWLIERNEDNTYIIRNYEN 545
Pierisin-1 SSMILGLT.RVSTPYGGLNLMVEDDSDGHSDLHSDWDIKPIFYQDIPDGDYNIFNDNFPNIAIDFTNQEGSLIHGHNFCS 601
Pierisin-2 SNMILGLT.NVSTSYGGLNLMVQDDSNGNSNLHSDWDIKPIIYQYVPDGDYNIFNDNFPNIAVDYTNQEGALVHGHNFCS 601
Pierisin-3 SSMILGLS.KVSTSYGGLNLMVEDDSDGHSNLHSDWDIKLIFYQYIPDGDYNIFNDNFPNIVVDYTNQEGALIHGHNFCS 601
Pierisin-4 LSLIMQLDYQKNSPHGGLNLIVHNDSKDYPNLYPNWKIVLVSYKCIPDGNYNIFNRHMPNIVIDSSNQKDALIHGHAYCA 609
MTX-1 RKIVLDLS.NGSTT.DGNGLL...GFEFHGGINQRWIIKPFSFNSIQDGIYQFMTVINQDLIADLTTNNYTIATKTNNYS 620
Pierisin-1 NNNQKWSFVFDGKRKAYRIKSGVRSNLWLSWDSNASSKEMVLRAYTESGSSNQYWRLDEANDGSYRIRNLQDYYKLIALT 681
Pierisin-2 NNNQKWSFVYDGKKKAYKIKSGVNSKLWLTWDSNASSKEMVLRAYTESGNWNQYWRLYQANDGSYIIRNLKEFKMLIALT 681
Pierisin-3 NNNQKWSFVYDGKRKAYRIKSGVRSNLWLSWDSNASSKEMVLRAYTESGSSNQYWRLDPANDGSYGIRNLQDFKKLIALT 681
Pierisin-4 NNNQKWSFVYDENKKAYKIKSGINSALWLSWDSNAASKEILLRAYTESGSLNQYWRIELTNDGSYKLRNLLNLEKIIAVV 689
MTX-1 .SNQKWTVTYNDKKRAYKIRNLQHAHLSLAWDSNHSDK..IFGA..TGDYDDQYWIPILQTDGSFIFRNYKNPNKIFG.. 693
Pierisin-1 NKNTPYGGKELIVSD.NKESGNTWYLKKLGEVPLPNRKFRIATKLNYKKVID.SSTSYNLIITHDLNFASSIWELVYDSS 759
Pierisin-2 NIDTPYGGKQLIVTD.TKESGNHWYLKKLGEVPLPNRKFRIATKLNYKKVID.SSTAYNLIITHDLNFASSIWELVYDSN 759
Pierisin-3 NKNTPYGGKELIVSA.NKESGNTWYLKNLGEVPLPNRKFRIATKLNYKKVID.SSTSYNLIITHDLNFASSIWELVYDSS 759
Pierisin-4 NKNSPYGGKELIVSD.SNEGWSNWYLNRIGEVVIPDGKYRIATKVNYKKVID.YNRDRNLVIMDNINLASSEWEIKYDST 767
MTX-1 TNGQPINDIPLKAQDVTGQNNQKWYLRHLNSSNNFTGYFNISSKKNFNKIITMNSNKTQAVIFDNIGINNQSWKLKYNDN 773
Pierisin-1 KKAYNIYSSDINNLGWIYQNKNFFVKLGNIDGPDHGDLRYFWTIEYSMQTGCYLIRSLHDPANA.....VGYTDSESVIT 834
Pierisin-2 KKAYNIYSADINNLGWVYQNKNFFVKLDNIDGPDHGDLRYFWTIEYSMQTGCYLIRSLYDPAHA.....VGYTDKDSVIT 834
Pierisin-3 KKAYNIYSSDINNLGWIYQNKNFFVKLGNIDGPDHGDLRYFWTIEYSMQTGCYLIRSLYDPAHA.....VGYTDNDSIIA 834
Pierisin-4 KKAYNIYSGQYFNIGWIYQNKNFYVKVDNIDGTDHGDSRYFWIIEYSIQYGCYMIRSLYDPSHA.....VGYSNNNFVVT 842
MTX-1 KNAYQIH..ILDNFLYFQGGHNIVATMQNV...TNDDLRSYWYVEYNFNKDGFIIRNAFDTSYVLDVFQGNFANNTPIIT 848
Pierisin-1 DTSTYSDNQLFHFILM 850
Pierisin-2 DTSTYSDNQLFHFILM 850
Pierisin-3 DTSTYSDNQLFHFILM 850
Pierisin-4 DTSTYSDNQLFHFIPI 858
MTX-1 YQNYLNDNQLWNFIPSLGVEPR 870

Pierisin-4 was isolated from the 19 pupae of *A. crataegi* using a method similar to that for *Pierisin-3*, except for the purification with Phenyl–Sepharose CL-4B hydrophobic interaction column (1.5×6 cm; GE Healthcare) after purification with DEAE-cellulose DE52 column.

2.5. In vitro transcription and translation of Pierisin-3 and -4

To express *Pierisin-3* and *-4* genes, *in vitro* transcription and translation were carried out as previously described (Watanabe et al., 1999). To obtain cDNA fragments containing a T7 promoter sequence at the 5′ end of the coding region, the primer pairs Pm_T7 and Pm_R3 for *Pierisin-3*, Ac_T7 and Ac_R2 for *Pierisin-4*, were used for the PCR. The amplified PCR products were transcribed using MEGAscript® T7 Kit (Ambion, Austin, TX, USA) and translated using rabbit reticulocyte lysate (Retic Lysate IVT™, Ambion). The translation efficiencies were confirmed by autoradiography of SDS-PAGE gels of [^{35}S]methionine incorporated products.

2.6. Site-directed mutagenesis

A DNA fragment containing a sequence alteration at the desired position was amplified from an intact cDNA subclone of Pierisin-3 and -4 with overlap PCR technique described previously (Matsushima-Hibiya et al., 2003; Nakano et al., 2006) To obtain overlapped 5′- and 3′- fragments of Pierisin-3, two separate PCR reactions were carried out using a 5′ primer Pm_T7 and 3′ primers PmD_R or PmQ_R for 5′-fragment, and 5′ primers PmD_F or PmQ_F and 3′ primer Pm_R3 for 3′-fragment. These mutations are that the glutamic acid residue at position 165 was replaced with aspartic acid or glutamine. The 5′ and 3′ fragments were mixed together and used as the template for a second round PCR to obtain full length mutated DNA fragments.

In the case of Pierisin-4, two separate PCR reactions were carried out using a 5′ primer Ac_T7 and 3′ primers AcD_R or AcQ_R for 5′-fragment, and 5′ primers AcD_F or AcQ_F and 3′ primer Ac_R2 for 3′-fragment. These mutations are that the glutamic acid residue at position 172 was replaced with aspartic acid or glutamine. The 5′ and 3′ fragments were mixed together and used as the template for a second round PCR to obtain full length mutated DNA fragments. The appropriate recombination was confirmed by DNA sequencing, and the resultant DNA was used as the template for the *in vitro* expression system described here.

2.7. Analysis of cytotoxicity and DNA ADP-ribosylating activity of in vitro expressed proteins

The cytotoxicity of the *in vitro* expressed proteins against HeLa cells and TMK-1 cells was examined by using WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium (Dojindo Laboratory, Kumamoto, Japan)] cell proliferation assay as described previously (Watanabe et al., 1998; Kono et al., 1999). Apoptotic cells were stained with TdT-mediated dUTP-biotin nick-end labeling (TUNEL) assay using *in situ* Apoptosis Detection Kit (Takara Bio, Otsu, Japan) and Hoechst 33342 after the formalin fixation, and analyzed with a fluorescence microscope. DNA ADP-ribosylating activity was determined by HPLC analysis as previously described (Matsumoto et al., 2008; Nakano et al., 2006).

To confirm the structure of the ADP-ribosylated DNA, the reaction of the *in vitro* expressed proteins, DNA and β-NAD was performed and the detection of the reaction products was carried out as described previously

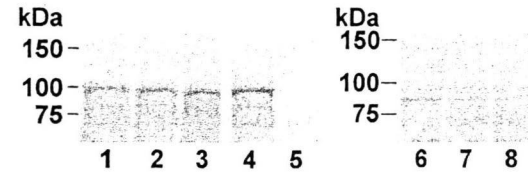


Fig. 2. Detection of *in vitro* expressed [^{35}S]methionine-labeled Pierisin-3 and -4, and their mutated proteins by SDS-PAGE. Five-microliters each of *in vitro* expressed lysates were loaded; lane 1, Pierisin-3 wild type; lane 2, Pierisin-3 E165D; lane 3, Pierisin-3 E165Q; lane 4, Pierisin-1 wild type; lane 5, lysate without template RNA; lane 6, Pierisin-4 wild type; lane 7, Pierisin-4 E172D; lane 8, Pierisin-4 E172Q.

(Nakano et al., 2006). Fifty microliters of the *in vitro* expressed proteins were incubated with 0.1 mg of calf thymus DNA, 2 μmol β-NAD in 1 ml of reaction buffer for 2 h at 37 °C. The DNA was recovered, digested by treatment with micrococcal nuclease and phosphodiesterase II, and treated with bacterial alkaline phosphatase. The reaction products were analyzed by HPLC with a LC-10A system (Shimadzu, Kyoto, Japan) armed with a SPD 10Avp photodiode array detector (Shimadzu) and a Develosil RPAQUEOUS column (4.6×250 mm; Nomura Chemical, Seto, Japan). These products were also analyzed by electrospray ionization-mass spectrometry using ZQ 2000 instrument (Micromass, Manchester, England) armed with an HP 1000 HPLC system (Hewlett-Packard, Palo Alto, USA).

3. Results

3.1. Identification of Pierisin-3 in P. melete

To obtain partial cDNA fragments of Pierisin-3, degenerate primers were designed from the conserved catalytic site motif of ADP-ribosylating protein. Total RNA was extracted from the fifth instar larva of *P. melete*. About 300-bp PCR products were cloned and sequenced to design the primer for 5′ and 3′ RACE. In addition, the PCR amplified Pierisin-3 coding sequence was inserted into the pMAL-p2x vector. The basal-level expression of the *Pierisin-3* gene would be highly toxic to *E. coli*, as expected from our studies of Pierisin-1 and -2, and we inserted the PCR product in the vector in an opposite direction to the *tac* promoter. The positive clones were sequenced and confirmed that the two of three clones had no nonsynonymous substitution. One of these two clones had less synonymous substitutions (G1516A, T2137C, T2176A). Thus, we concluded that the clone is an intact clone at the amino acid level, and it was employed for the Pierisin-3 expression *in vitro*.

The complete cDNA sequence of Pierisin-3 consists of 3347 bp associated with a putative initiator codon ATG at position 95–97, a stop codon at position 2645–2647 and a polyadenylational signal at position 3328–3333 close to the poly A sequence (Suppl. Fig. 1A). The ORF encodes 850 amino acids with a calculated molecular weight of 97,598. From the deduced amino acid sequence, Pierisin-3 is 93% similar to Pierisin-1 and 91% to Pierisin-2 (Fig. 1). Furthermore, the partial peptide sequences of Pierisin-3 purified from pupae in the same process as Pierisin-1 were identical to the deduced amino acid sequence of ORF. The identified three internal amino acid sequences, Glu91-Gly102, Gly135-Ala144 and Ser816-Gly825, are shown in Suppl. Fig. 1A (bold type with underlines). The essential regions for ADP-ribosyltransferase activity (Masignani et al., 2000; Domenighini

Fig. 1. Alignment of the deduced amino acid sequences of Pierisin-3 and -4. These sequences were aligned together with that of Pierisin-1 (DDBJ accession number AB030305), Pierisin-2 (DDBJ accession number AB037676) and MTX (DDBJ accession number M60446). Identical amino acid residues in Pierisins are highlighted in gray. The conserved arginine (Region I), Ser–Thr–Thr motif (Region II), and glutamic acid residues (Region III) for ADP-ribosylating activity and the QXW motifs for receptor binding are boxed in black with asterisks (*) or plus signs (+). Sequence alignment between Pierisins and MTX was decided referring to Watanabe et al. (1999) and Carpusca et al. (2006).

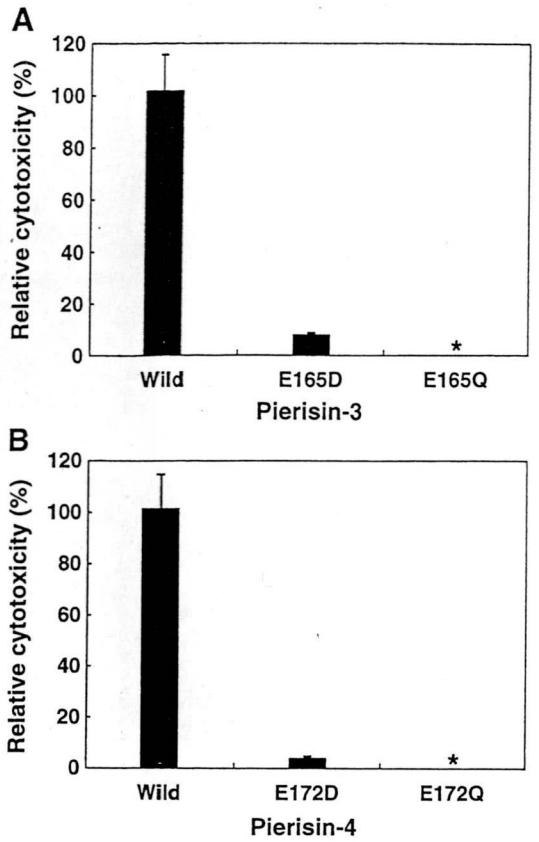


Fig. 3. Cytotoxicities of wild type and mutated Pierisin-3 or -4 against HeLa cells. Cells were incubated with a final concentration of 1% of rabbit reticulocyte lysate containing *in vitro* expressed Pierisin-3 (A) and Pierisin-4 (B) for 48 h at 37 °C and subjected to WST-1 cell proliferation assay. The cytotoxicity of mutated proteins relative to the wild type proteins is indicated. Asterisks show cytotoxicity not detected in this assay. Data are means of three independent experiments. Error bars represent standard deviations.

and Rappuoli, 1996), and ricin B chain-like QXW motif that binds glycosphingolipid receptors, such as Gb3 and Gb4, are conserved, as shown in Fig. 1 (Matsushima-Hibiya et al., 2003).

3.2. Identification of Pierisin-4 in A. crataegi

cDNA sequence and subcloning of Pierisin-4 were determined using the same method as that for the Pierisin-3. Three positive clones were sequenced and confirmed that all the clones had no synonymous nor nonsynonymous substitution. Thus, we concluded that these clones are intact clones, and one of the clones was employed for the Pierisin-4 expression *in vitro*. Pierisin-4 consists of 3319 bp associated with a putative initiator codon at position 77–79, a stop codon at position 2651–2653 and a polyadenylational signal at position 3293–3298 close to the poly A sequence l (Suppl. Fig. 1B). The ORF encodes 858 amino acids with a calculated molecular weight of 99,204. Amino acid alignment showed that Pierisin-4 is 64% similar to Pierisin-1, -2 and -3 (Fig. 1). Deduced amino acid sequence entirely encodes the partial peptide sequences of purified Pierisin-4 from pupae of *A. crataegi*. The essential regions for ADP-ribosyltransferase activity (Masignani et al., 2000; Domenighini and Rappuoli, 1996), and ricin B chain-like QXW motif are also conserved, as shown in Fig. 1 (Domenighini and Rappuoli, 1996).

3.3. Cytotoxicity and apoptosis-inducing activity of in vitro expressed Pierisin-3 and -4

Pierisin-3 and -4 were expressed by rabbit reticulocyte lysate, since it is impossible to express Pierisin-3 and -4 in *E. coli*, probably due to the high toxicity of the Pierisins. The translation efficiency and molecular mass of the *in vitro* expressed Pierisin-3 and their mutated products were confirmed by SDS-PAGE of [^{35}S]methionine-labeled proteins, and those of the *in vitro* expressed Pierisin-4 and their mutated products were also confirmed in the similar way (Fig. 2). To examine whether Pierisin-3 and -4 have cytotoxicity and apoptosis-inducing activity against mammalian cells, HeLa and TMK-1 cells were treated with *in vitro* expressed Pierisin-3 and -4. Both expressed proteins showed cytotoxicity (Fig. 3), and similar cytotoxicity of both expressed proteins was observed in TMK-1 cells (data not shown). Furthermore, the cytotoxicity levels of the *in vitro* expressed Pierisin-3 and -4 were almost equivalent to the *in vitro* expressed Pierisin-1. Both expressed proteins induced chromatin condensation and nuclear fragmentation indicating apoptotic cell death, in HeLa cells, as observed by fluorescence microscopy (Fig. 4). Glutamic acid residues, Glu165 in Pierisin-3 and Glu172 in Pierisin-4 (Fig. 1, boxed in black),

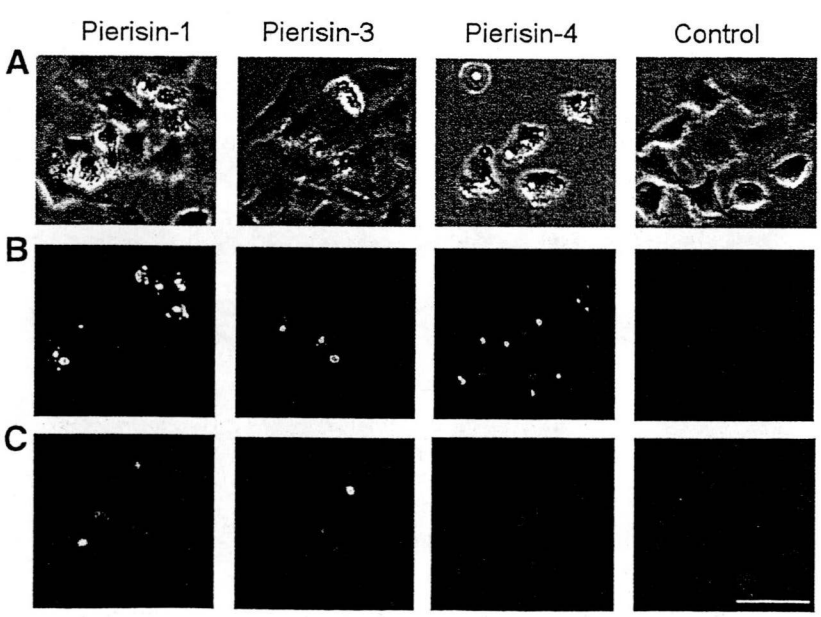


Fig. 4. Morphological analysis of HeLa cells treated with the *in vitro* expressed Pierisin-3 and -4 proteins. The cells were treated for 24 h with 1% of *in vitro* expressed proteins or 1 ng/ml purified Pierisin-1. (A) Phase-contrast micrographs for morphological changes analysis. (B) Fluorescence micrograph of TUNEL assays for detection of DNA fragmentation undergoing apoptotic cells. The TUNEL-positive cells were labeled by FITC. (C) Fluorescence micrograph of Hoechst 33342-stained cells. Bar = 50 μm.