The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Original Article

Safety evaluation of exomaltotetraohydrolase from *Pseudomonas stutzeri*

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(Received July 27, 2024; Accepted August 24, 2024)

ABSTRACT — Exomaltotetraohydrolase (G4ase) catalyzes the hydrolysis of (1->4)-α-D-glucosidic linkages in amylaceous polysaccharides from the non-reducing ends removing successive maltotetraose residues. A safety assessment was conducted for G4ase produced by the non-genetically modified strain of *Pseudomonas stutzeri*, MO-19. Two standardized acute oral toxicity studies using female rats were performed on G4ase having a TOS not determined for the first study and 7.13% TOS for the second. The 50% lethal dose (LD_{50}) of G4ase was determined to be more than 2000 mg/kg, corresponding to more than 143 mg-TOS/kg. A 2-week oral repeated toxicity study in rats at 1000 mg/kg/day (highest dose; TOS not determined) demonstrated no treatment related toxicity and was used to identify the appropriate dose for a 90-day study. Results of a standardized 90-day oral repeated toxicity study (gavage) of G4ase (7.13%-TOS) using rats demonstrated that the No Observed Adverse Effect Level (NOAEL) of G4ase was 1000 mg/kg/day (the highest dose), corresponding to 71.3 mg-TOS/kg. Four standardized genotoxicity studies of bacterial reverse mutation, chromosomal aberration, and *in vivo* and *in vitro* micronucleus tests were performed on G4ase (5.19, 5.19, 7.13 and 6.65%-TOS, respectively). It was concluded that G4ase did not induce gene mutation in *Salmonella typhimurium* and *Escherichia coli*, did not induce chromosomal aberrations in cultured mammalian cells, and did not induce micronucleated erythrocytes in rat bone marrow cells or human spleen cell line lymphoblasts. Taken together these data indicate that G4ase from *P. stutzeri* strain MO-19 is safe for use as a processing aid in manufacturing food for human consumption.

Key words: Exomaltotetraohydrolase, Glucan 1,4-α-maltotetraohydrolase, G4ase, *Pseudomonas stutzeri*

INTRODUCTION

Exomaltotetraohydrolase (G4ase) is a catalyst hydrolyzing (1->4)-α-D-glucosidic linkages in amylaceous polysaccharides, such as starch, amylopectin, amylose, glycogen, maltoheptaose and maltohexaose, to remove successive maltotetraose residues (G4) from non-reducing chain ends and is assigned the International Union of Biochemistry and Molecular Biology (IUBMB) number and name of EC 3.2.1.60 and glucan 1,4-α-maltotetraohydrolase, respectively (Kim *et al*., 1995).

G4ase was first identified in the culture broth of *Pseudomonas stutzeri* by Robyt and Ackerman as an amylase forming G4 (Robyt and Ackerman, 1971). Fujita *et al.* first reported the nucleotide sequence of the G4ase gene (*amyP*) for G4ase from *P. stutzeri* strain MO-19 (Fujita *et al.*, 1989). The nucleotide sequence contained an open reading frame coding for a secreted precursor (547 amino acid residues) of G4ase, which had a signal peptide

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of 21 amino acid residues at its N-terminus. G4ase was reported to be an extracellular G4-forming amylase produced in several forms (Schmidt and John, 1979; Sakano *et al.*, 1983). Nakada *et al*. reported that *P. stutzeri* strain MO-19 produced two active forms of extracellular G4ase (57 and 46 kDa) using SDS-PAGE. The 46-kDa form is derived from proteolysis of the C-terminal region of the 57-kDa form by an endogenous protease (Nakada *et al*., 1990). Morishita and co-workers reported the crystal structure of G4ase from *P. stutzeri* strain MO-19 had 429 amino acid residues and a molecular mass of 47,200 (Morishita *et al*., 1997).

G4ase is currently used as a processing aid (biocatalyst) to produce food/food ingredients, such as G4-rich starch syrup and in bakery dough. After hydrolysis, the G4ase no longer performs technological functions in food due to inactivation and/or purification steps during the production of final foods.

G4ase is listed in List of Existing Food Additives under the name of "Exomaltotetraohydrolase" (MOH, 1996) in accordance with Article 14 of Food Sanitation Act in Japan (Act No. 233, 1947) and G4ase derived from *Streptomyces thermoviolaceus*, *Streptomyces violaceoruber* and *P. stutzeri* is monographed in Japan's Specifications and Standards for Food Additives (MHLW, 2018). In the US, the Food and Drug Administration (FDA) has issued a "no questions letter" to Generally Recognized as Safe (GRAS) Notice GRN No. 277 for a G4ase preparation from *B. licheniformis* expressing a modified G4ase gene from *P. stutzeri* (US FDA, 2009). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated the safety of G4ase from *P. stutzeri* expressed in *B. licheniformis* and established an Acceptable Daily Intake (ADI) of "not specified" (JECFA, 1995). G4ase from *P. stutzeri* or other microorganisms (including genetically modified microorganisms) is also accepted in several countries including France, Australia/New Zealand, and Korea.

The American Type Culture Collection (ATCC) and DSMZ-German Collection of Microorganisms and Cell Culture GmbH (DSMZ) categorize strains of *P. stutzeri* as Biosafety Level (BSL) 1 and Risk Group 1, respectively. These substances are materials not known to consistently cause disease in immunocompetent adult humans and that present minimal potential hazard to laboratory personnel and the environment, and agents that are not associated with disease in healthy adult humans, respectively.

The strain MO-19 was obtained by traditional mutation and selection techniques to provide higher G4ase activity and lower side activities such as lipase from the parent strain isolated from soil in Okayama, Japan. It is not genetically modified. To ascertain the safety of the use of G4ase from *P. stutzeri* strain MO-19 this manuscript describes key toxicity assessment studies on G4ase. These consist of standardized oral toxicity tests (acute, 2-week and 90-day repeated) in rats and genotoxicity tests, including bacterial reverse mutation, chromosomal aberration and *in vitro* and *in vivo* micronucleus tests.

MATERIALS AND METHODS

Test substance

The test substances used for all G4ase studies were prepared by the identical fermentation process of a pure culture of the non-pathogenic, non-toxigenic, non-genetically modified *Pseudomonas stutzeri* strain MO-19. Purification was performed by removal of microbial cells, concentration and sterilization by filtration at Nagase Viita Co., Ltd. (Okayama, Japan). G4ase was stored frozen until used for each test. It was confirmed to be stable after up to 3 cycles of freeze (-20°C) and thaw. The total organic solids (TOS) of G4ase (6561 U/g; Lot No. 60423) used for the second acute oral toxicity study, 90-day repeated-oral toxicity study and the *in vivo* micronucleus test in rats was 7.13%. The bacterial reverse mutation and chromosomal aberration tests were performed using Lot No. 60614 with 6865 U/g of enzyme activity and 5.19% TOS, and Lot No. 30429 with 6091 U/g of enzyme activity and 6.65% TOS was used for the *in vitro* micronucleus test. The enzyme activity of Lot No. 60311 used for the first acute oral toxicity and 2-week repeated-oral toxicity studies was 3570 U/g (TOS not determined). In each test, it was also confirmed that G4ase was stable during the experimental period at the 3rd party testing laboratory.

Animal maintenance

Crl:CD(SD) strain rats used for the oral toxicity (acute, 2-week, and 90-day repeated) and *in vivo* micronucleus tests were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan), and quarantined and acclimated for 7 days until testing. Animals were housed in an animal room at the controlled temperature of 20-26°C, humidity of 35-70%, ≥ 12 times/hour ventilation, and 12-hr lighting cycle during the study. Animals were housed individually in wire-mesh cages hung in an automatic water flushing breeding rack and allowed access to a standard diet (CRF-1: Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. The condition of each animal was observed daily, and the body weight was measured at receipt and at the end of the quarantine and acclimation period. It was confirmed that none of the animals showed any signs of disease or abnormal body

weight change before testing.

Safety studies in rats

All the animal studies (acute, 2-week and 90-day repeated oral toxicity tests, and *in vivo* micronucleus test) were conducted at the Public Interest Incorporated Foundation, Biosafety Research Center (Shizuoka, Japan; BSRC) in compliance with the Act on Welfare and Management of Animals in Japan (Act No. 105 of October 1, 1973; recent revision: Act No. 46 of May 30, 2014) and Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Ministry of the Environment Notification No. 88, April 28, 2006; recent revision: Ministry of the Environment Notification No. 84, August 30, 2013). The animal studies were reviewed and approved before initiation by the Institutional Animal Care and Use Committee of BSRC and were performed in accordance with the ethics criteria contained in the BSRC Guidance's for Animal Testing (June 2, 2014).

Oral toxicity studies

Acute oral toxicity tests

Acute oral gavage toxicity studies were performed twice on G4ase in rats using the Organisation of Economic Co-operation and Development (OECD) Guideline for the Testing of Chemicals No. 420 (17th December 2001).

A first study was conducted under non-Good Laboratory Practice (non-GLP) using 10 female 8-week-old Crl:CD(SD) strain rats from March 22, 2016, to May12, 2016. Because the test substance is used as a food additive in Japan it is required to be of low acute toxicity to rats. Therefore, doses of 1000 and 2000 mg/kg were selected which were the maximum dose set in the OECD Guidelines for acute toxicity test and repeated toxicity test, respectively. The body weight range of the animals was 187 to 203 g at dosing. G4ase solutions were prepared at 200 and 400 mg/mL for the 1000 and 2000 mg/kg groups, respectively, by dissolution/dilution with water for injection (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan; OPFI) and was administered at 1.0 mL/100 g-body weight by gavage to animals fasted overnight. The day of dosing was defined as Day 0 and the period of Days 0-14 was defined as the observation period. On Day 0, all animals were observed for general conditions once within 30 min and once each at 1, 2, 3 and 4 hr after dosing. During the observation period (Days 1-14), the general conditions of the animals were observed once daily. All animals were weighed on Days 0 (before dosing), 7 and 14. On Day 14 all surviving animals were macroscopically examined for any pathological changes on their external surface and orifices, and organs and tissues in the abdominal, thoracic, pelvic and cranial cavities. Statistical analysis was not used.

A second study was conducted under OECD GLP from January 20, 2017, to March 31, 2017. The study included 5 female 8-week-old rats with 1 animal for the sighting study and 4 animals for the main study. The body weight range of the animals was 196-204 g at dosing. The dose level of the sighting study was selected at 2000 mg/kg, the upper limit level described in the guideline. From the results of the sighting study, 2000 mg/kg was selected as the dose level for the main study. The subsequent procedure was the same as the first study.

2-week repeated oral dose toxicity test

A 2-week repeated oral dose toxicity study was conducted under OECD GLP from April 25, 2016, to July 1, 2016, on G4ase using rats as a dose finding study for a subsequent 90-day repeated-oral dose toxicity study with reference to OECD Guideline 408 (21st September 1998).

Thirty (30) male and 30 female 5-week-old Crl:CD(SD) strain rats were purchased, 25 male and 25 female rats were randomized into 5 groups of 5 male and 5 female animals based on their body weights before testing, which ranged from 166.2-176.6 g for males and from 128.8-148.3 g for females. The weight variation of the animals was within ± 20% of the mean weight of the males/females. The dose levels were 30, 100, 300 and 1000 mg/kg/day by dissolution and dilution with water for injection (OPFI, Japan). The doses of 0.5 mL per 100 g-body weight were administered by gavage once a day for 2 weeks. Water for injection was used as the negative control (0 mg/kg/day). The rationale for the highest dose was selected because of the acute oral toxicity study in which no abnormal results were reported.

The day of dosing was defined as Day 1. Clinical signs of the animals were observed twice a day (before and after dosing) from Days 1 to 14, and on Day 15. Animals were weighed before the start of dosing on Day 1, and then on Days 4, 8, 11 and 14. After an overnight fast on Day 14, blood for a standard hematology panel was drawn from all animals via the abdominal aorta on Day 15. Animals were then euthanized by exsanguination under isoflurane anesthesia, and necropsied for gross findings, and organs weighed (brain, thymus, heart, lungs (including bronchi), liver, kidneys, adrenal glands, spleen, epididymides, testes, uterus, and ovaries) and organ weights per body weight were assessed. The external surface and orifices were observed, followed by observation of the organs and tissues in the abdominal, thoracic, pelvic and cranial cavities. If an abnormality was observed,

the organ or tissue was fixed in 10% neutral buffered formalin and stored.

The quantitative data (body weight, feed consumption, hematological examination, clinical chemistry, tissue/organ weights and ratio of organ/tissue weights per body weight) were analyzed by the Bartlett's test for equality of variance. When the Bartlett's test showed homoscedasticity, the data were analyzed by Dunnett's multiple comparison test to assess the statistical significance of differences between the control group and each test substance-treated group. When the Bartlett's test showed heteroscedasticity, the data were analyzed by the Steel's test to assess the statistical significance of differences between the control group and each test substance-treated group. The significance level of the Bartlett's test was two-sided 5%, and the significance levels of the other tests were two-sided 5% and 1%, respectively. No statistical tests were used for clinical signs and findings at necropsy.

90-day repeated dose oral toxicity test

To evaluate the potential of repeated oral toxicity of G4ase in rats, a study was conducted from May 31, 2016, to September 8, 2016, under OECD GLP according to OECD Guideline No. 408 (21st September 1998). Forty-five (45) male and 45 female rats were purchased and for the study, 40 male and 40 female rats were randomized into 4 groups of 10 male and 10 female animals based on their body weights before testing, which ranged from 184-217 g for males and from 155-176 g for females. The weight variation of the animals was within ± 20% of the mean weight of the males/females. Based on the results of the preliminary 2-week repeated-oral toxicity study results, 1000 mg/kg/day, the maximum dose in the guideline, was set as the highest dose, and 300 and 100 mg/kg/day were selected for the lower dose levels using a common ratio of approximately 3. G4ase solutions were prepared with water for injection (OPFI, Japan) which was used as negative control (0 mg/kg/day) and were administered by gavage to the animals once a day for 90 days. Water for injection was used as the negative control (0 mg/kg/day).

The general conditions of the animals were observed twice a day (before and after dosing) during the administration period. On the day of the scheduled necropsy, an examination was performed before the animals were sacrificed. The Functional Observation Battery (FOB) was conducted once during the quarantine period, and once a week thereafter to examine detailed behavioral, physiological, and neuronal functions. All animals were subjected to observations including home in cage activity, responses on removal from cage, and behavior in an open field. Tests for sensorimotor function, grip strength and locomotor activity were conducted during the 13th week of the administration period. The animals were weighed weekly from Days 1 (before grouping) to 90, and body weight gain on Days 1-90 was calculated. The animals to be necropsied were weighed on the necropsy day (Day 91). The mean daily feed consumption was calculated by subtracting the amounts of feed supplied from the remaining feed on the next measurement. Hematological and blood chemical examinations were conducted on Days 91 and 92. After overnight fasting, blood samples were collected from the abdominal aorta of the animals under isoflurane anesthesia, and plasma and serum samples were prepared for hematological and blood chemical examination. Fresh urine (within 3 hr after urination) and pooled urine (for 24 hr) were collected on Days 87 and 88 under fed and water-supplied conditions for urinalysis. The residue of the pooled urine was stained using the New Sternheimer method, and examined microscopically for erythrocytes, casts, leukocytes, fat globules, squamous cells, mucous threads, transitional epithelial cells, crystals, and renal tubular epithelial cells. Ophthalmological examinations were conducted on animals in the control and 1000 mg/kg/day groups on Days -7 and -4 during the quarantine period and Day 85 during the administration period. Observations included appearance and light reflex, and examination of the anterior part of the eyeballs, optic media, and fundus oculi. The organs of all animals were weighed, and the organ to body weight ratio was calculated.

The external surface and orifices were macroscopically observed, followed by observation of the organs and tissues in the abdominal, thoracic, pelvic, and cranial cavities. Specimens for histopathological examination were prepared from skin, mammary gland, lymph nodes (mesenteric and cervical), pancreas, spleen, kidneys, salivary glands (sublingual and mandibular), sternum, femur, bone marrow (sternum and femur), thymus, trachea, lungs, heart, thyroid glands, parathyroid glands, tongue, esophagus, stomach, duodenum, jejunum, ileum (including Peyer's patch), cecum, colon, rectum, liver, adrenal glands, urinary bladder, seminal vesicle (including coagulating glands), prostate (including urethra), testes, epididymides, ovaries, oviducts, uterus, vagina, eyes (including optic nerve), harderian glands, brain, pituitary gland, spinal cord (cervical, thoracic and lumbar), skeletal muscle (femoral region), sciatic nerve, Zymbal's glands and aorta. The tissues were fixed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Only specimens from the control and 1000 mg/kg/day groups were microscopically examined.

The quantitative data were initially analyzed by the Bartlett's test for equality of variance. When the Bartlett's test showed homoscedasticity, the data were analyzed by Dunnett's multiple comparison test to assess the statistical significance of differences between the control group and each G4ase-treated group. When the Bartlett's test showed heteroscedasticity, the data were analyzed by the Steel's test to assess the statistical significance of differences between the control group and each G4ase-treated group. FOB data were analyzed by Steel's test. The significance level of the Bartlett's test was two-sided 5%. The significance levels of the other tests were two-sided 5% and 1%, respectively. No statistical analysis was performed for clinical signs, ophthalmological findings, gross and histopathological findings.

Genotoxicity studies

Bacterial reverse mutation test

To assess the potential of G4ase to induce gene mutations, a bacterial reverse mutation test (Ames test) was conducted on *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and on *Escherichia coli* WP2*uvrA* by the pre-incubation method in both the absence (-S9 assay) and presence (+S9 assay) of the metabolic activation system (S9 mix). This study was conducted from January 26, 2017, to February 17, 2017, in accordance with OECD Guideline No. 471 (21st July 1997) under OECD GLP.

The G4ase doses tested ranged from 8.19 to 5000 µg/plate for the dose-finding study and from 313 to 5000 µg/plate for the main study as specified in the guideline, with or without S9 mix. Water for injection (OPFI, Japan) which was used as the solvent to prepare G4ase solutions was used as the negative control. 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2; Wako Pure Chemical Industries, Ltd., Osaka, Japan; WPCI), sodium azide (NaN_3; WPCI, Japan), 9-ammoacridine hydrochloride (9-AA; Sigma-Aldrich Japan K.K., Tokyo, Japan; SAJ) and 2-aminoanthracene (2-AA; WPCI, Japan) were used as the positive controls.

For the dose-finding study, 5 dose levels of G4ase (range 0.0819-8.00 mg/mL) were prepared by series dilution. One hundred (100) µL of the negative control solution, the G4ase solution or the positive control solutions were transferred into a test tube. In addition to 500 µL of 0.1 mol/L sodium-phosphate buffer (pH 7.4) with or without the S9 mix, 100 µL of each bacterial suspension were added to the test tube, and the mixtures were pre-incubated with shaking for 20 min at 37°C. After pre-incubation, 2 mL of top agar was added to the test tubes. The contents were mixed, and then poured and spread onto plates. The plates were incubated for 48 hr at 37°C. After incubation, growth conditions of the bacterial background on the plates were observed under a stereoscopic microscope to check for bacterial growth inhibition by G4ase. Revertant colonies were counted with an automated colony analyzer, correcting for the area and count loss.

Based on the results of the dose-finding study, 5 dose levels of the test substance were selected for the main study, namely, 313-5000 µg/plate (maximum 260 µg-TOS/plate) as described above. For the main study, the same procedure was used as for the dose-finding study.

When the mean number of revertant colonies on a plate treated with G4ase increased by two-fold or more as compared with that in the negative control group, and the increase was reproducible or dose-dependent, the result was judged to be positive. No statistical analysis was performed for the evaluation.

Chromosomal aberration test

Under OECD GLP, a chromosomal aberration test was performed with G4ase using Chinese hamster lung fibroblast cell lines (CHL/IU) from July 4, 2016, to August 23, 2016, in compliance with OECD Guideline No. 473 (26 September 2014).

In the cell growth inhibition test (dose-finding study), 0.391-12.5 mg/mL solutions of the test substance were prepared by dissolution and diluted in water for injection (OPFI, Japan). The cells were exposed to the solutions at the maximum concentration of 5000 µg/mL for 6 hr with or without metabolic activation or for 24 hr without metabolic activation.

Based on the results of the dose-finding study no inhibition of 50% or more in cell growth, even at 5000 µg/mL was observed. Solutions of 12.5-50.0 mg/mL of G4ase were prepared using water for injection for the main test, and the cells were exposed to G4ase solutions of 1250, 2500 and 5000 µg/mL (maximum 260 µg-TOS/mL) for 6 hr with or without the S9 mix or for 24 hr without metabolic activation. Water for injection, the solvent to prepare the G4ase solutions, was used as the negative control. Mitomycin C (MMC; Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) and cyclophosphamide (CP; Shionogi & Co., Ltd., Osaka, Japan; SCL) were used as positive controls for -S9 and 24 hr assays and for the +S9 assay, respectively, as noted in the guideline. MMC was used at the concentrations of 0.1 and 0.05 µg/mL for -S9 and 24 hr assays, respectively, and CP was used at the concentrations of 12.5 µg/mL for +S9 assay.

In the cell growth inhibition test, the number of cells was counted using an automated cell counter. The Population

Doubling (PD) was calculated using the formula below.

PD = log [(Number of cells at the end of the culture) / (Number of cells at the start of treatment)] / log2.

The ratio of PD in each treatment group to the negative control group was calculated as the relative cell growth rate using the formula below.

Relative cell growth rate (%) = (PD in each treatment group) / (PD in the negative control group) × 100.

In the main test, precipitation and other changes were checked by unmagnified examination of samples at the start and end of the treatment with the test substance in each assay. The number of cells was counted according to the same method described above and the relative cell growth rate was calculated. Chromosome slides were prepared and 150 metaphases (chromosome number: 25 ± 2) per plate (300 metaphases/concentration) were examined microscopically (×600). The structural aberrations were classified into the following types: gap, chromatid break, chromosome break, chromatid exchange, chromosome exchange, and others. Gap was recorded in cases in which a cutting-like chromatid break was observed in an unstained region of the chromatid or chromosome, and the split (unstained) part was clearly smaller in width than the width of the chromatid and was not dislocated from the axis. In addition, the number of polyploid cells (chromosome numbers: 38 or more) was counted by observing 300 metaphases for each concentration. The evaluation was made on the total incidence of aberrant cells minus the number of cells with only gaps (-gap). The incidence of aberrant cells was analyzed by Fisher's exact test (one-sided significance level 2.5%). If significant differences were observed in the test substance group as compared to the negative control group, concentration dependency was analyzed by the Cochran-Armitage trend test (one-sided significance level 2.5%).

In vivo micronucleus test

An *in vivo* micronucleus test was conducted with G4ase using male rats from February 9, 2017, to March 22, 2017, in compliance with OECD Guideline No. 474 (29 July 2016) under OECD GLP. At the end of the quarantine and acclimation period 30 animals were assigned to groups by a random sampling method based on their body weights. Body weights of the animals ranged from 261 to 291 g and the weight range of the animals was within the mean weight ± 20%.

Based on the results of the acute and preliminary 2-week repeated-oral toxicity studies, 3 dose levels were selected, including the highest dose of 2000 mg/kg, which is the highest dose specified in the guideline, along with 2 lower doses of 1000 and 500 mg/kg. G4ase solutions were prepared with water for injection (OPFI, Japan) at concentrations of 50, 100 and 200 mg/mL (maximum 14.3 mg-TOS/mL). Water for injection and 1 mg/mL solution of CP (SCL, Japan) were used as the negative and positive controls, respectively. G4ase and negative control solutions were administered at 1.0 mL/100 g-body weight via gavage to animals once a day for 2 consecutive days, with a 24-hr interval between the doses. The positive control solution was administered by gavage at 1.0 mL/100 g-body weight. To ensure that data from at least 5 animals were available for each group, 6 animals were treated in each group. In the G4ase and negative control groups, the animals were observed for any abnormal clinical signs at 1, 24 and 25 hr after the first dosing and before preparation of the bone marrow sample (48 hr after the first dosing). The body weights of the animals were measured before the preparation of the bone marrow samples.

Rats were euthanized at 24 hr after the final dosing (at 24 hr after dosing of the positive control), the femur was removed, and the bone marrow cells were flushed out with calf serum inactivated at 56°C for 30 min (Life Technologies Japan Ltd., Tokyo, Japan; LTJ). The bone marrow cell suspension samples were prepared by the standard procedures from all animals using Dulbecco's phosphate-buffered saline (SAJ, Japan), 10% neutral buffered formalin solution (WPCI, Japan) and a cell strainer (Corning International K.K., Tokyo, Japan). From the bone marrow samples, 4000 immature erythrocytes (IE) per animal were analyzed using a fluorescent microscope (×800) equipped with a blue excitation filter and a barrier filter, and the number of micronucleated immature erythrocytes (MNIE) was counted. To investigate the influence of G4ase on bone marrow cell proliferation, the number of IE out of a total of 500 erythrocytes was also counted. The ratio of IE to analyzed erythrocytes and the frequency of MNIE in relation to the total number of IE were calculated as follows.

Ratio of IE (%) = (Number of IE) / (Number of erythrocytes analyzed) × 100

Frequency of MNIE (%) = (Number of MNIE) / (Number of IE analyzed) × 100

The data on the frequency of MNIE were analyzed by the Conditional Binomial test (Kastenbaum and Bow-

man method) to compare the findings between the negative control group and each of the other groups. If significant differences were observed in the test substance groups, dose dependency was analyzed by the Cochran-Armitage trend test. The data on the ratio of IE to the analyzed erythrocytes were subjected to Dunnett's multiple comparison test, to determine the existence of any significant differences between the negative control group and each G4ase-treated group.

In vitro micronucleus test (MNvit test)

An *in vitro* micronucleus test was conducted with G4ase on a lymphoblast cell line derived from the spleen of a human (TK6) from July 14, 2023, to September 12, 2023, under OECD GLP in compliance with OECD Guideline No. 487 (29 July 2016).

In the cell growth inhibition test (dose-finding study), the maximum concentration of G4ase was set at 5000 μg-TOS/mL in water for injection (OPFI, Japan) as prescribed in the guideline, and concentrations of 2500, 1250, 625, 313, 156, 78.1 and 39.1 μg-TOS/mL were selected. The water for injection was used as the solvent to prepare the test substance formulations. G4ase was added to the cell suspension and incubated at 37°C for 3 hr with or without S9 mix (+S9 assay and -S9 assay, respectively) and for 24 hr without S9 mix (24 hr assay). MMC (SAJ, Japan), CP (SCL, Japan) and Colchicine (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), were used as positive controls for the -S9 assay, +S9 assay and 24 hr assay, respectively. Water for injection was used as the negative control, and water for injection and/or physiological saline was used as solvent and/or diluent for the positive controls. Precipitation and changes in color of the medium were checked with the naked eye at the start and end of treatment with the test substance. Following the completion of the incubation, slides of the cells were prepared. For measurement of cytotoxicity, the cells on the slides were stained with acridine orange (LTJ, Japan) and 500 cells per culture were analyzed using a fluorescent microscope (×400) equipped with a blue excitation filter and a barrier filter, and the number of mono-, bi- and multinucleate cells were recorded. As an index of cytotoxicity, the Replication Index (RI) was calculated using the formula below.

RI = {[(Number of binucleate cells) + 2 × (Number of multinucleate cells)] / [Total number of cells (the treatment group)]} / {[(Number of binucleate cells) + 2 × (Number of multinucleate cells)] / [Total number of cells (the negative control group)] × 100

Cytostasis, which indicates inhibition of cell growth, was calculated using the following formula.

Cytostasis (%) = 100 - RI

In the main test (MNvit test), 1200-2400, 400-2400 and 50.0-300 μg-TOS/mL of G4ase were selected for the -S9 assay, +S9 assay and 24 hr assay, respectively, based on the results of the cell growth inhibition test. The procedures for the -S9 assay, +S9 assay and 24 hr assay were the same as the cell growth inhibition test. For micronucleus analysis, 1000 binucleate cells per culture (2000 binucleate cells per concentration) were analyzed using a fluorescent microscope (×400) equipped with a blue excitation filter and a barrier filter, and the number of micronucleated binucleate cells (MNBN) was recorded. Binucleate cells were analyzed only if the two nuclei were approximately equal in size and their nuclear boundaries were clear. The criteria for scoring micronuclei were as follows:

- The diameter should be less than 1/2 of the main nucleus.
- The micronuclei should be separate from or marginally overlap with the main nucleus as long as there is clear identification of the nuclear boundary.
- The micronuclei should have similar characteristics as the main nucleus.

The frequency of MNBN to analyzed binucleate cells was calculated using the formula below:

Frequency of MNBN (%) = (Number of MNBN) / (Number of binucleate cells analyzed) × 100

The frequency of MNBN was analyzed by Fisher's exact test (one-sided, upper significance level of 0.025) between the negative control group and each group. If significant differences were observed in the test substance group, the concentration dependence was analyzed by the Cochran-Armitage trend test (one-sided, upper significance level of 0.025).

RESULTS

Oral toxicity studies

Acute oral toxicity tests

In both the first and second tests, all animals administered G4ase (maximum 2000 mg/kg; 144.0 mg-TOS/kg for the second GLP study), or control survived, and no animals showed abnormal clinical signs or body weight gain during the 14-day observation period. Further, no abnormal gross findings in the skin, orifices or examined

organs and tissues were noted, except for 2 animals in the 2000 mg/kg group of the first study. A dilated pelvis renalis and single depressed focal lesion (10 × 2 mm) were observed in the right kidney of one animal each. However, the pathological abnormalities were solitary and not observed in the other kidney of the same animals. In addition, the abnormalities are frequently observed in the same strain rats of the same age. Therefore, it was judged that the observations were spontaneous changes.

2-week repeated-oral dose toxicity test in rats

A 2-week repeated-oral dose toxicity test was performed in rats as a dose-finding study for the subsequent 90-day repeated-oral dose toxicity test. No animals treated with even the highest dose (1000 mg/kg/day) of G4ase, or control for two weeks died or showed abnormal signs during the administration period. There were no significant differences between control or G4ase-treatment groups of either sex in body weight or feed consumption. In the 30 mg/kg/day group, activated partial thromboplastin time (APTT) in males was significantly longer ($p \leq 0.05$), and the differential leukocyte ratios of neutrophil count (NEUT) and lymphocyte count (LYMPH) in females were significantly higher and lower ($p \leq 0.05$), respectively, than in the control groups (Table 1). However, these changes were not considered to be treatment-related effects because there was no significant change in the 1000 mg/kg/day group and no dose-dependency. In the 100 mg/kg/day group, the relative weights of the brain and testes in the males were significantly lower ($p \leq 0.05$) than those in the control group. Again, these changes were considered incidental because there was no change in the 300 or 1000 mg/kg/day groups, therefore there was no dose relationship. There were no gross nec-

Table 1. Hematology in the 2-week repeated oral toxicity test.

Sex	Male					Female				
Dose (mg/kg/day)	0	30	100	300	1000	0	30	100	300	1000
	Mean (S.D.)									
HCT (%)	42.6 (1.6)	41.8 (1.3)	41.9 (1.5)	41.9 (2.4)	41.1 (1.5)	42.1 (1.1)	41.4 (0.6)	42.3 (1.2)	41.3 (2.2)	41.1 (0.8)
HGB (× 10^6/mm^3)	14.6 (0.6)	14.2 (0.5)	14.3 (0.4)	14.3 (0.7)	14.0 (0.7)	14.7 (0.3)^N	14.5 (0.2)	14.9 (0.3)	14.4 (0.9)	14.4 (0.3)
RBC (g/dL)	6.93 (0.16)^N	6.61 (0.13)	6.81 (0.26)	6.64 (0.48)	6.63 (0.15)	7.22 (0.44)	7.09 (0.14)	7.18 (0.25)	6.94 (0.42)	7.00 (0.33)
MCV (μm^3)	61.4 (1.5)	63.3 (2.3)	61.6 (2.7)	63.2 (1.7)	62.0 (2.4)	58.4 (2.4)	58.4 (1.5)	58.9 (1.1)	59.5 (1.2)	58.8 (1.9)
MCH (pg)	21.1 (0.8)	21.6 (0.8)	20.9 (0.8)	21.5 (0.7)	21.1 (1.0)	20.3 (0.8)	20.5 (0.5)	20.7 (0.4)	20.9 (0.5)	20.6 (0.8)
MCHC (%)	34.3 (0.6)	34.1 (0.2)	34.0 (0.4)	34.1 (0.4)	34.1 (0.4)	34.8 (0.3)	35.1 (0.2)	35.2 (0.4)	35.1 (0.5)	35.1 (0.3)
Reticulocyte (%)	4.1 (0.5)	4.6 (1.0)	4.8 (0.3)	4.8 (0.5)	4.5 (1.0)	2.8 (1.0)	2.4 (0.5)	2.8 (0.7)	2.3 (0.3)	3.0 (0.5)
Reticulocyte (× 10^9/L)	284.1 (36.6)	304.0 (62.6)	327.6 (20.0)	315.8 (23.0)	292.6 (59.8)	196.1 (68.5)	167.0 (33.4)	198.4 (40.8)	162.5 (18.2)	206.6 (27.6)
PLT (× 10^3/mm^3)	1127 (104)	1142 (124)	1217 (80)	1165 (78)	1195 (113)	1291 (91)	1297 (68)	1135 (161)	1213 (84)	1294 (92)
WBC (× 10^3/mm^3)	7.09 (1.13)	8.43 (1.46)	6.95 (0.95)	8.90 (1.58)	7.45 (2.25)	4.85 (0.75)	4.17 (1.92)	6.04 (1.37)	4.39 (1.71)	5.68 (1.56)
Differential leukocyte ratios (%)										
NEUT	16.9 (3.5)	21.5 (6.9)	15.6 (5.4)	16.7 (3.7)	12.7 (2.9)	12.7 (3.2)	18.0 (1.5)*	16.1 (3.2)	13.2 (2.7)	12.1 (2.7)
LYMPH	78.6 (3.8)	74.1 (7.4)	80.6 (5.6)	79.9 (3.4)	82.9 (3.5)	82.9 (4.1)	77.6 (2.0)*	79.9 (3.8)	83.1 (2.5)	84.4 (2.7)
MONO	3.0 (0.7)	2.7 (1.0)	2.5 (0.5)	2.2 (0.4)	2.7 (0.9)	2.3 (1.3)	2.5 (0.8)	1.8 (0.5)	1.6 (0.6)	1.8 (0.6)
EOSN	1.0 (0.2)	0.8 (0.4)	0.9 (0.2)	0.7 (0.2)	1.0 (0.3)	1.4 (0.3)	1.3 (0.3)	1.7 (0.5)	1.4 (0.6)	1.1 (0.3)
BASO	0.1 (0.0)^N	0.1 (0.1)	0.1 (0.0)	0.1 (0.0)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.2 (0.1)	0.1 (0.1)	0.1 (0.0)
LUC	0.5 (0.1)	0.6 (0.3)	0.3 (0.1)	0.4 (0.2)	0.6 (0.3)	0.6 (0.2)	0.5 (0.2)	0.4 (0.2)	0.5 (0.2)	0.4 (0.1)
NEUT (× 10^3/mm^3)	1.20 (0.28)	1.82 (0.65)	1.12 (0.50)	1.45 (0.25)	0.95 (0.41)	0.63 (0.20)	0.76 (0.39)	0.95 (0.18)	0.58 (0.26)	0.67 (0.18)
LYMPH (× 10^3/mm^3)	5.57 (0.95)	6.24 (1.24)	5.56 (0.44)	7.14 (1.45)	6.19 (1.88)	4.00 (0.48)	3.23 (1.44)	4.86 (1.21)	3.65 (1.45)	4.80 (1.36)
MONO (× 10^3/mm^3)	0.21 (0.07)	0.23 (0.10)	0.17 (0.05)	0.20 (0.05)	0.20 (0.06)	0.12 (0.08)	0.10 (0.05)	0.11 (0.03)	0.07 (0.04)	0.11 (0.05)
EOSN (× 10^3/mm^3)	0.07 (0.01)^N	0.07 (0.03)	0.06 (0.01)	0.06 (0.01)	0.07 (0.01)	0.07 (0.03)	0.06 (0.04)	0.10 (0.03)	0.06 (0.03)	0.06 (0.02)
BASO (× 10^3/mm^3)	0.01 (0.00)	0.01 (0.01)	0.01 (0.00)	0.01 (0.00)	0.01 (0.01)	0.00 (0.01)	0.00 (0.00)	0.01 (0.01)	0.00 (0.01)	0.01 (0.00)
LUC (× 10^3/mm^3)	0.04 (0.01)	0.05 (0.03)	0.02 (0.01)	0.04 (0.02)	0.04 (0.02)	0.03 (0.01)	0.02 (0.01)	0.02 (0.02)	0.02 (0.01)	0.02 (0.01)
PT (sec.)	12.6 (1.1)	13.7 (1.9)	12.8 (2.6)	11.6 (0.8)	13.8 (2.3)	8.8 (0.3)	8.6 (0.2)	8.6 (0.3)	8.9 (0.3)	8.7 (0.3)
APTT (sec.)	23.4 (1.3)	26.2 (1.3)*	23.7 (2.4)	21.9 (0.4)	25.9 (2.0)	16.7 (2.0)	16.5 (1.8)	16.6 (1.2)	17.8 (1.8)	18.5 (0.7)

*$p \leq 0.05$ (Dunnett's multiple test, significant difference from control (0 mg/kg/day) group),
^{N}Nonparametric analysis.
Abbreviations: HCT, Hematocrit; HGB, hemoglobin; RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, Platelet count; WBC, White blood cell count; NEUT, neutrophil count; LYMPH, lymphocyte count; MONO, Monocyte count; EOSN, Eosinophil count; BASO, basophil count; LUC, large unstained cell count; PT, Prothrombin time; APTT, activated partial thromboplastin time.

Table 2. Gross necropsy findings in the 2-week repeated oral toxicity test.

Sex		Male					Female				
Dose level (mg/kg/day)		0	30	100	300	1000	0	30	100	300	1000
Organ	Findings										
RESPIRATORY SYSTEM											
Lungs	Brown patch	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	1/5
URINARY SYSTEM											
Kidneys	Cyst	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5
	Depression, focal	1/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
REPRODUCTIVE SYSTEM											
Testes	Small	0/5	1/5	0/5	0/5	0/5	-	-	-	-	-
Epididymides	Small	0/5	1/5	0/5	0/5	0/5	-	-	-	-	-
NERVOUS SYSTEM											
Brain	Defect	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
	Dilated ventricle	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5

-: Not applicable

ropsy findings related to G4ase-treatment in the 1000 mg/kg/day group of either sex (Table 2). Because all lesions observed were sporadic and focal, and often historically observed in this strain, these were concluded to be spontaneously occurring.

90-day repeated-oral toxicity test

During the test period, no animals in the control or G4ase-treated groups (maximum 1000 mg/kg/day: 71.3 mg-TOS/kg/day) of either sex died or showed abnormal signs. No noteworthy FOB-related abnormalities in response to removal from cage, condition on hand-held observation, behavior in the open-field or sensorimotor reactivity were observed at any examination time. Furthermore, there were no significant differences in any of the quantitative data between the control group and each G4ase-treated group. There were no significant differences in feed consumption and body weights of the animals of either sex during the administration period and in overall body weight gains from Days 1-90 between the control group and the G4ase-treatment groups. As shown in Table 3, hematological analyses provide the following statistically significant differences ($p \leq 0.05$). The basophil count (BASO) in the males of the 1000 mg/kg/day group was significantly lower, and APTT in the males, and hemoglobin (HGB) concentration in the females in the 300 mg/kg/day group were significantly higher than the control group. These changes were not considered to be treatment-related because the BASO value difference was very slight and within the normal range for this strain of rat, whereas the APPT and HGB values were not dose dependent. The results of the blood chemical analyses demonstrated that the γ-globulin ratio and concentration of males in the 100 mg/kg/day group were significantly higher ($p \leq 0.05$) than the control group. In females, the γ-globulin concentration in the 100 and 1000 mg/kg/day groups, and the β-globulin ratio, γ-globulin ratio and the γ-glutamyl transpeptidase (γ-GTP) level in the 100 mg/kg/day group were significantly higher than the control group. The total protein level in the female 300 mg/kg/day group and the albumin concentration in the female 100 mg/kg/day group were significantly lower than the control group (Table 4). However, these changes in males and females were not considered to be treatment-related effects because there were no clinical or histopathological findings in the 1000 mg/kg/day group or a dose relationship. The results of the urinalysis are shown in Table 5. It demonstrated 1 male with occult blood (3+) and erythrocytes (3+) in the 100 mg/kg/day group, which were judged incidental to treatment because the same findings were noted in the control group. There were no changes in the other variables of either sex in each test group. No ophthalmological findings related to G4ase-treatment in the 1000 mg/kg/day group, as compared to the control group, in either sex was observed. There were no significant changes attributable to the dosing of G4ase on organ weight in either sex. The relative weights of the heart and liver in females of the 300 mg/kg/day group were significantly lower than those in the control group as shown in Table 6. These changes were considered incidental because there was no dose relationship, no gross necropsy findings related to G4ase-treatment in either sex, and all lesions observed were determined to be sporadic, focal, often observed in this strain and spontaneously occurring. There were no gross necropsy findings assessed to G4ase-treatment in either sex because all lesions observed were sporadic, focal and often observed in this strain animal (Table 7). No histopathological findings were noted related to G4ase-treatment when the control and 1000 mg/kg/day groups of either sex were compared. Any findings

Table 3. Hematology in the 90-day repeated oral toxicity test.

Sex	Male				Female			
Dose (mg/kg/day)	0	100	300	1000	0	100	300	1000
	Mean (S.D.)							
HCT (%)	44.4 (2.8)	44.0 (1.3)	44.2 (1.7)	45.2 (1.3)	42.3 (1.1)	43.1 (1.5)	43.5 (1.2)	42.4 (1.6)
HGB ($\times 10^6$/mm^3)	15.5 (1.1)^N	15.2 (0.5)	15.4 (0.7)	15.7 (0.4)	14.9 (0.4)	15.2 (0.5)	15.4 (0.4)*	14.9 (0.6)
RBC (g/dL)	8.60 (0.54)^N	8.55 (0.28)	8.59 (0.30)	8.74 (0.20)	7.97 (0.24)	8.02 (0.38)	8.18 (0.26)	8.10 (0.30)
MCV (μm^3)	51.7 (1.4)	51.5 (1.4)	51.5 (1.9)	51.7 (1.0)	53.1 (1.6)	53.8 (2.2)	53.2 (1.1)	52.4 (1.4)
MCH (pg)	18.0 (0.5)	17.8 (0.5)	17.9 (0.8)	18.0 (0.5)	18.7 (0.5)	19.0 (0.7)	18.9 (0.4)	18.5 (0.5)
MCHC (%)	34.8 (0.3)^N	34.5 (0.3)	34.8 (0.4)	34.9 (0.7)	35.2 (0.4)	35.2 (0.6)	35.5 (0.3)	35.3 (0.3)
Reticulocyte (%)	2.1 (0.9)^N	2.0 (0.3)	1.8 (0.3)	1.8 (0.4)	2.0 (0.5)	1.7 (0.4)	1.6 (0.3)	1.6 (0.3)
Reticulocyte ($\times 10^9$/L)	176.5 (56.8)^N	167.7 (22.7)	148.5 (20.2)	155.7 (31.5)	156.6 (41.3)	132.7 (28.9)	132.5 (18.1)	131.0 (25.8)
PLT ($\times 10^3$/mm^3)	1006 (159)	982 (86)	983 (68)	1003 (123)	1062 (97)^N	960 (330)	1052 (79)	1152 (323)
WBC ($\times 10^3$/mm^3)	8.94 (3.81)^N	9.63 (2.15)	7.10 (1.27)	7.10 (2.30)	3.83 (1.17)	3.81 (1.27)	3.75 (1.64)	4.03 (1.34)
Differential leukocyte ratios (%)								
NEUT	18.9 (12.5)^N	18.6 (4.8)	16.6 (5.3)	19.2 (5.2)	18.0 (6.3)	17.0 (6.0)	13.7 (3.0)	15.0 (6.5)
LYMPH	76.3 (11.9)^N	76.9 (5.3)	77.9 (5.8)	76.0 (5.4)	75.8 (6.2)	77.6 (6.8)	81.5 (3.5)	79.2 (6.5)
MONO	2.7 (1.1)	2.4 (0.8)	3.1 (1.0)	2.6 (1.0)	3.0 (0.8)	3.0 (1.2)	2.5 (1.1)	2.9 (0.7)
EOSN	1.5 (0.5)	1.6 (0.6)	1.9 (0.6)	1.7 (0.7)	2.7 (1.3)	1.8 (0.8)	1.9 (0.7)	2.4 (0.7)
BASO	0.1 (0.0)^N	0.1 (0.0)	0.1 (0.1)	0.1 (0.0)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.1 (0.0)
LUC	(0.4) 0.4	0.4 (0.2)	0.5 (0.3)	0.4 (0.2)	0.5 (0.2)	0.5 (0.3)	0.4 (0.2)	0.4 (0.2)
NEUT ($\times 10^3$/mm^3)	2.06 (2.83)^N	1.76 (0.47)	1.19 (0.49)	1.31 (0.36)	0.65 (0.21)^N	0.61 (0.16)	0.56 (0.41)	0.55 (0.15)
LYMPH ($\times 10^3$/mm^3)	6.48 (1.48)	7.45 (1.90)	5.51 (0.95)	5.45 (2.03)	2.94 (1.07)	3.00 (1.19)	3.01 (1.15)	3.25 (1.24)
MONO ($\times 10^3$/mm^3)	0.22 (0.07)	0.22 (0.06)	0.23 (0.10)	0.19 (0.08)	0.12 (0.06)	0.11 (0.05)	0.10 (0.07)	0.12 (0.06)
EOSN ($\times 10^3$/mm^3)	0.13 (0.03)	0.15 (0.06)	0.14 (0.06)	0.12 (0.05)	0.09 (0.03)	0.07 (0.04)	0.07 (0.03)	0.09 (0.03)
BASO ($\times 10^3$/mm^3)	0.01 (0.00)^N	0.01 (0.01)	0.01 (0.01)	0.00 (0.01)$^\#$	0.01 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
LUC ($\times 10^3$/mm^3)	0.04 (0.03)	0.04 (0.02)	0.03 (0.02)	0.03 (0.03)	0.02 (0.02)	0.02 (0.01)	0.02 (0.01)	0.02 (0.01)
PT (sec.)	11.8 (1.6)	13.0 (2.4)	13.1 (2.1)	13.0 (3.0)	8.5 (0.2)	8.6 (0.3)	8.6 (0.2)	8.6 (0.2)
APTT (sec.)	26.1 (2.4)	27.6 (2.2)	28.4 (1.7)*	27.5 (1.6)	18.8 (1.2)^N	18.2 (1.9)	17.6 (2.9)	18.5 (1.4)
Fibrinogen (mg/dL)	294 (41)	275 (29)	302 (47)	286 (26)	207 (12)^N	224 (36)	220 (20)	212 (36)

$\#p \leq 0.05$ (Steel's test, significant difference from control (0 mg/kg/day) group),
$*p \leq 0.05$ (Dunnett's multiple test, significant difference from control (0 mg/kg/day) group),
^{N}Nonparametric analysis.
Abbreviations: HCT, Hematocrit; HGB, hemoglobin; RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, Platelet count; WBC, White blood cell count; NEUT, neutrophil count; LYMPH, lymphocyte count; MONO, Monocyte count; EOSN, Eosinophil count; BASO, basophil count; LUC, large unstained cell count; PT, Prothrombin time; APTT, activated partial thromboplastin time.

were comparable to the control group and/or the lesions were sporadic, focal, and common to this strain of rats. Since these observations were considered spontaneous, only the 1000 mg/kg/day treatment group was histopathologically examined.

Bacterial reverse mutation test

The results of the main study are shown in Table 8. In both the dose-finding and main studies, the number of revertant colonies did not increase by twofold or more in the G4ase-treated groups (maximum 5000 μg/plate; 260 μg-TOS/plate), as compared with the negative control group, in any of the strains in either the -S9 or the +S9 assays. No growth inhibition of any test strain was observed. All positive and negative controls resulted in revertant colony data demonstrating that the study was valid.

Chromosomal aberration test

The cell growth inhibition test showed no inhibition of 50% or more in the -S9, +S9 or 24 hr assays, and no precipitation was observed at any concentration (maximum 5000 μg/mL; 260 μg-TOS/plate) at either the start or the end of the treatments. Therefore, 5000 μg/mL was set as the maximum concentration for the subsequent chromosomal aberration test.

The results of the -S9, +S9 and 24 hr assays of the main test are shown in Table 9. The incidences of cells with structural chromosomal aberrations and the incidences of polyploid cells in the groups treated with G4ase at any concentration were not statistically different

Table 4. Blood chemistry in the 90-day repeated oral toxicity test.

Sex	Male				Female			
Dose (mg/kg/day)	0	100	300	1000	0	100	300	1000
	Mean (S.D.)							
Glucose (mg/dL)	164 (25)	165 (16)	167 (21)	173 (20)	145 (13)	143 (22)	142 (23)	155 (14)
Triglyceride (mg/dL)	75 (27)	68 (20)	68 (24)	71 (26)	45 (49)^{N}	36 (14)	34 (25)	52 (57)
Total cholesterol (mg/dL)	67 (12)	68 (12)	66 (10)	60 (18)	76 (19)	65 (10)	66 (14)	70 (17)
BUN (mg/dL)	13.5 (2.0)	14.6 (2.5)	14.0 (2.5)	14.4 (1.5)	15.0 (2.8)	13.6 (1.6)	14.4 (1.9)	15.0 (3.6)
Creatinine (mg/dL)	0.31 (0.04)	0.31 (0.05)	0.31 (0.06)	0.32 (0.05)	0.36 (0.09)^{N}	0.34 (0.03)	0.36 (0.04)	0.36 (0.04)
Total bilirubin (mg/dL)	0.06 (0.01)	0.06 (0.01)	0.06 (0.01)	0.07 (0.01)	0.08 (0.02)	0.09 (0.02)	0.08 (0.01)	0.08 (0.01)
AST (U/L)	67 (14)	65 (7)	68 (6)	64 (9)	71 (13)	77 (12)	77 (12)	73 (18)
ALT (U/L)	28 (7)	25 (5)	25 (5)	27 (5)	22 (5)	25 (5)	24 (9)	24 (11)
ALP (U/L)	291 (47)	283 (48)	281 (27)	315 (61)	108 (29)	128 (28)	136 (28)	124 (27)
γ-GTP (U/L)	0.7 (0.2)	0.7 (0.2)	0.6 (0.2)	0.7 (0.2)	0.8 (0.2)	1.2 (0.3)**	0.9 (0.2)	0.8 (0.3)
Calcium (mg/dL)	9.97 (0.34)	9.96 (0.32)	9.89 (0.31)	9.95 (0.22)	10.16 (0.23)	10.00 (0.24)	9.96 (0.30)	10.17 (0.33)
I.phosphorus (mg/dL)	5.97 (0.47)	5.93 (0.51)	6.14 (0.37)	6.12 (0.70)	4.97 (0.80)	4.65 (0.63)	5.04 (0.57)	4.56 (0.90)
Sodium (mmol/L)	144.1 (0.9)	143.7 (0.7)	143.8 (0.5)	144.2 (0.9)	142.4 (1.4)	142.8 (1.2)	143.1 (0.7)	143.0 (0.9)
Potassium (mmol/L)	4.55 (0.23)	4.69 (0.29)	4.77 (0.18)	4.73 (0.19)	4.43 (0.15)^{N}	4.43 (0.21)	4.44 (0.17)	4.50 (0.43)
Chloride (mmol/L)	106.3 (1.5)	106.8 (1.2)	106.4 (0.9)	106.0 (1.4)	106.6 (1.8)	107.5 (1.8)	107.8 (1.8)	106.7 (1.8)
Albumin (%)	50.7 (2.8)	49.6 (2.2)	50.3 (2.9)	51.3 (1.5)	58.6 (1.7)	56.5 (2.7)	57.8 (2.1)	58.1 (3.4)
α_1-Globulin (%)	21.7 (3.6)	22.3 (2.5)	21.1 (2.6)	21.4 (1.9)	17.2 (1.4)	16.1 (2.5)	15.9 (2.6)	15.9 (3.5)
α_2-Globulin (%)	7.9 (1.7)	7.7 (0.9)	8.2 (1.3)	8.2 (1.4)	6.6 (0.8)	6.6 (1.1)	6.9 (0.6)	6.5 (1.2)
β-Globulin (%)	15.3 (2.2)^{N}	14.9 (1.0)	15.2 (1.1)	14.6 (0.7)	12.7 (1.2)	14.1 (0.5)**	13.3 (1.1)	13.3 (1.0)
γ-Globulin (%)	4.4 (1.1)	5.6 (0.8)*	5.2 (1.2)	4.5 (0.7)	5.0 (1.0)	6.7 (1.2)**	6.2 (1.1)	6.2 (1.3)
A/G (%)	1.04 (0.12)	0.99 (0.08)	1.02 (0.12)	1.06 (0.06)	1.42 (0.10)	1.30 (0.14)	1.37 (0.12)	1.40 (0.21)
Total protein (g/dL)	6.00 (0.34)	6.05 (0.37)	5.98 (0.21)	5.95 (0.23)	6.75 (0.52)^{N}	6.43 (0.20)	6.32 (0.24)$^{\#}$	6.74 (0.22)
Albumin (g/dL)	3.04 (0.26)	3.00 (0.16)	3.01 (0.20)	3.05 (0.14)	3.96 (0.39)	3.63 (0.19)*	3.65 (0.20)	3.92 (0.32)
α_1-Globulin (g/dL)	1.30 (0.27)	1.35 (0.20)	1.26 (0.16)	1.27 (0.15)	1.16 (0.12)	1.04 (0.18)	1.01 (0.17)	1.07 (0.22)
α_2-Globulin (g/dL)	0.47 (0.08)	0.47 (0.07)	0.49 (0.08)	0.49 (0.07)	0.44 (0.07)	0.42 (0.07)	0.44 (0.05)	0.44 (0.09)
β-Globulin (g/dL)	0.92 (0.12)	0.90 (0.08)	0.91 (0.06)	0.87 (0.05)	0.85 (0.06)^{N}	0.91 (0.02)	0.84 (0.06)	0.90 (0.06)
γ-Globulin (g/dL)	0.26 (0.07)	0.34 (0.05)*	0.31 (0.07)	0.27 (0.04)	0.33 (0.07)	0.43(0.07)**	0.39 (0.07)	0.41 (0.08)*

$^{\#}p \leq 0.05$ (Steel's test, significant difference from control (0 mg/kg/day) group),
$^{*}p \leq 0.05$, $^{**}p \leq 0.01$ (Dunnett's multiple test, significant difference from control (0 mg/kg/day) group),
^{N}Nonparametric analysis.
Abbreviations: BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyl transpeptidase; I.phosphorus, inorganic phosphorus.

($p \leq 0.025$) as compared to the incidence in the negative control group. No inhibition of cell growth and no precipitation was observed at any concentration. The incidences of cells with chromosomal aberrations in both the negative and positive control groups were within the acceptable range as compared with historical data. These results confirmed the validity of this study.

In vivo micronucleus test

The test results are shown in Table 10. The frequencies of MNIE in the G4ase-treated groups were 0.14, 0.13 and 0.11% in the 500, 1000 and 2000 mg/kg groups (maximum 143 mg-TOS/kg), respectively. No significant increase was noted in any of the G4ase-treated groups as compared with the negative control. The ratios of IE to the analyzed erythrocytes were 68.6, 67.6 and 71.4% in the 500, 1000 and 2000 mg/kg groups, respectively, and no significant decrease was noted in any of the G4ase-treated groups as compared with the negative control. Neither apparent suppression of body weight gain nor abnormal clinical signs were observed in any of the G4ase-treated groups. Data from the negative and positive controls demonstrated the validity of the study method.

In vitro micronucleus test

Results of the cell growth inhibition test demonstrated ≥ 50% inhibition of cell growth at 2500 μg-TOS/mL or more in the -S9 and +S9 assays, and at 313 μg-TOS/mL or more in the 24 hr assay. No precipitation of G4ase was observed in any of the assay at the start of the treatment, but precipitation was observed at 5000 μg-TOS/mL

Table 5. Urinalysis in the 90-day repeated oral toxicity test.

Sex		Male				Female			
Dose (mg/kg/day)		0	100	300	1000	0	100	300	1000
		Mean (S.D.)							
Volume (mL)		14.1 (5.6)	14.1 (4.4)	16.1 (7.2)	14.6 (5.4)	15.5 (9.4)	16.6 (5.4)	12.9 (9.3)	14.4 (8.3)
Osmotic pressure (mOsm/kg)		1396 (367)	1564 (579)	1393 (571)	1249 (470)	1367 (619)	1034 (293)	1397 (529)	1262 (534)
Sodium (mmol/L)		62.1 (27.8)	86.3 (46.9)	72.5 (43.0)	60.8 (41.8)	95.1 (43.1)	67.0 (20.9)	87.5 (37.6)	80.1 (41.5)
Potassium (mmol/L)		168.0 (46.2)	188.6 (69.7)	173.2 (89.5)	147.8 (71.4)	176.0 (83.5)	129.8 (35.0)	174.2 (76.5)	154.4 (68.2)
Chloride (mmol/L)		84.6 (32.1)	114.5 (60.4)	100.1 (62.8)	79.5 (54.9)	131.0 (72.5)[N]	86.9 (25.9)	118.0 (58.6)	106.3 (57.0)
Sodium (mmol/day)		0.84 (0.36)	1.06 (0.38)	0.96 (0.36)	0.76 (0.31)	1.17 (0.36)	1.04 (0.32)	0.89 (0.21)	0.92 (0.23)
Potassium (mmol/day)		2.22 (0.62)	2.42 (0.48)	2.30 (0.38)	1.89 (0.34)	2.17 (0.62)	2.02 (0.53)	1.78 (0.44)	1.80 (0.39)
Chloride (mmol/day)		1.13 (0.45)	1.42 (0.44)	1.30 (0.50)	0.99 (0.41)	1.55 (0.42)	1.37 (0.48)	1.20 (0.34)	1.21 (0.35)
		Number of animals							
Color	1	0	0	0	0	0	0	0	0
	2	10	9	9	10	10	10	10	10
	3	0	1	1	0	0	0	0	0
	4	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0
	11	0	0	0	0	0	0	0	0
pH	5	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0
	6.5	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	1	0
	7.5	0	0	0	1	1	2	1	0
	8	0	0	1	0	3	2	3	3
	8.5	5	6	5	6	5	6	2	6
	≥9	5	4	4	3	1	0	3	1
Occult blood	-	7	5	6	6	8	9	7	9
	+/-	2	2	3	3	2	1	3	0
	1+	0	2	1	1	0	0	0	1
	2+	0	0	0	0	0	0	0	0
	3+	1	1	0	0	0	0	0	0
Ketone bodies	-	1	1	1	6	8	10	7	9
	+/-	5	4	5	0	1	0	3	1
	1+	4	5	4	4	1	0	0	0
	2+	0	0	0	0	0	0	0	0
	3+	0	0	0	0	0	0	0	0
	4+	0	0	0	0	0	0	0	0
Glucose (g/dL)	-	10	10	10	10	10	10	10	10
	0.1	0	0	0	0	0	0	0	0
	0.25	0	0	0	0	0	0	0	0
	0.5	0	0	0	0	0	0	0	0
	≥1.0	0	0	0	0	0	0	0	0
Protein (mg/dL)	-	0	0	1	2	6	8	6	7
	+/-	3	0	2	4	2	2	2	1
	30	3	8	4	2	0	0	1	1
	100	4	2	3	2	2	0	1	1
	≥300	0	0	0	0	0	0	0	0
Bilirubin	-	10	10	10	10	10	10	10	10
	1+	0	0	0	0	0	0	0	0
	2+	0	0	0	0	0	0	0	0
	3+	0	0	0	0	0	0	0	0
Urobilinogen (E.U./dL)	0.1	8	8	8	10	8	10	7	8
	1.0	2	2	2	0	2	0	3	2
	2.0	0	0	0	0	0	0	0	0
	4.0	0	0	0	0	0	0	0	0
	8.0	0	0	0	0	0	0	0	0
	≥12	0	0	0	0	0	0	0	0
Casts	-	10	10	10	10	10	10	10	10
	+	0	0	0	0	0	0	0	0
Fat globules	-	10	10	10	10	10	10	10	10
	+	0	0	0	0	0	0	0	0
Mucous threads	-	10	10	10	10	10	10	10	10
	+	0	0	0	0	0	0	0	0
Crystals	-	0	0	2	0	0	0	0	0
	+	10	10	8	10	10	10	10	10

Table 6. Organ to body weight ratio (relative organ weight) in the 90-day repeated-oral toxicity test.

Sex	Male				Female			
Dose (mg/kg/day)	0	100	300	1000	0	100	300	1000
	Mean (S.D.)							
Body weight (g)	519 (75)	513 (59)	516 (60)	483 (43)	275 (16)^{N}	268 (30)	275 (28)	286 (45)
Brain (%)	0.445 (0.064)	0.447 (0.042)	0.437 (0.041)	0.464 (0.041)	0.756 (0.063)	0.770 (0.097)	0.742 (0.074)	0.746 (0.105)
Heart (%)	0.295 (0.024)	0.290 (0.023)	0.296 (0.027)	0.290 (0.025)	0.338 (0.014)	0.326 (0.023)	0.315 (0.016)*	0.328 (0.025)
Lungs (%)	0.303 (0.033)	0.295 (0.021)	0.302 (0.033)	0.307 (0.020)	0.398 (0.024)	0.415 (0.047)	0.410 (0.035)	0.403 (0.039)
Liver (%)	2.519 (0.125)	2.609 (0.218)	2.541 (0.203)	2.383 (0.142)	2.506 (0.159)	2.415 (0.129)	2.269 (0.095)**	2.359 (0.168)
Kidneys (%)	0.618 (0.054)	0.614 (0.042)	0.628 (0.072)	0.611 (0.056)	0.691 (0.083)	0.663 (0.054)	0.640 (0.058)	0.643 (0.050)
Spleen (%)	0.149 (0.027)	0.154 (0.019)	0.146 (0.023)	0.142 (0.016)	0.165 (0.021)^{N}	0.185 (0.041)	0.161 (0.016)	0.168 (0.026)
Adrenal glands (%)	0.012 (0.001)	0.012 (0.001)	0.012 (0.002)	0.013 (0.002)	0.022 (0.003)	0.025 (0.005)	0.023 (0.005)	0.024 (0.002)
Testes (%)	0.674 (0.089)^{N}	0.671 (0.059)	0.602 (0.170)	0.672 (0.080)	-	-	-	-
Ovaries (%)	-	-	-	-	0.028 (0.005)	0.029 (0.006)	0.028 (0.006)	0.026 (0.006)
Thyroid glands (%)	0.005 (0.001)	0.006 (0.001)	0.005 (0.001)	0.006 (0.001)	0.007 (0.001)	0.007 (0.001)	0.007 (0.002)	0.007 (0.001)
Pituitary (%)	0.003 (0.000)	0.003 (0.000)	0.003 (0.000)	0.003 (0.000)	0.007 (0.001)	0.007 (0.001)	0.006 (0.001)	0.007 (0.001)
Thymus (%)	0.056 (0.011)^{N}	0.062 (0.019)	0.059 (0.023)	0.052 (0.007)	0.082 (0.017)	0.082 (0.022)	0.090 (0.018)	0.086 (0.014)
Prostate (%)	0.328 (0.045)	0.296 (0.044)	0.292 (0.037)	0.311 (0.043)	-	-	-	-
Uterus (%)	-	-	-	-	0.214 (0.066)	0.216 (0.063)	0.280 (0.132)	0.247 (0.114)
Epididymides (%)	0.258 (0.030)^{N}	0.256 (0.029)	0.241 (0.057)	0.257 (0.019)	-	-	-	-
Salivary glands (%)	0.142 (0.019)	0.134 (0.017)	0.137 (0.018)	0.144 (0.016)	0.171 (0.016)	0.167 (0.013)	0.165 (0.023)	0.160 (0.023)
Seminal vesicle (%)	0.316 (0.056)	0.311 (0.032)	0.301 (0.047)	0.317 (0.039)	-	-	-	-

Significant difference from the control; *$p \leq 0.05$, **$p \leq 0.01$ (Dunnett's multiple test),
^{N}Nonparametric analysis.

in the 24 hr assay at the end of the treatment. The color of the medium changed to brown at 2500 µg-TOS/mL or more in all assays at the start of the treatment, and at 2500 µg-TOS/mL or more in the -S9 and +S9 assay, and at 5000 µg-TOS/mL in the 24 hr assay at end of the treatment, without markedly changing the pH.

The main test (MNvit test) showed the percentages of cytostasis as 6.7, 14.7, 18.9, 18.3, 22.6, 31.7 and 51.2 at 1200, 1400, 1600, 1800, 2000, 2200 and 2400 µg-TOS/mL, respectively, in the -S9 assay, 0.7, 11.1, 19.0, 45.0, 55.2 and 76.6 at 400, 800, 1200, 1600, 2000 and 2400 µg-TOS/mL, respectively, in the +S9 assay, and 1.3, 8.2, 18.0, 30.5, 41.7 and 52.2 at 50.0, 100, 150, 200, 250 and 300 µg-TOS/mL, respectively, in the 24 hr assay. The results of the frequencies of MNBN are shown in Table 11. All the frequencies of MNBN in the groups treated with G4ase in any of the assays were not statistically significantly different as compared to the frequency in each negative control group. No precipitation of the test substance was observed in any of the assay at the start and end of the treatment. At the start and end of the treatment, the color of the medium changed to brown at 2000 µg-TOS/mL or more in the -S9 and +S9 assays with no marked changes in pH.

DISCUSSION

This manuscript provides the results of safety studies of G4ase from *P. stutzeri* strain MO-19 obtained by chemical mutagenesis (conventional mutation technique) from the wild-type strain isolated from a soil sample collected in Okayama, Japan.

All disclosed strains of *P. stutzeri* deposited in ATCC and the DSMZ are classified as BSL 1 in accordance with Biosafety in Microbiological and Biomedical Laboratories (BMBL), U.S. Department of Health and Human Services, and risk group 1 according to German Technical Rules for Biological Agents (TRBA), respectively (ATCC; DSMZ). These biosafety classifications mean that *P. stutzeri* is unlikely to cause human disease in immunocompetent adult humans.

Information from the BRENDA database (Chang *et al*., 2021) describes G4ase as naturally occurring in *P. stutzeri*, as well as in addition to *Bacillus* spp. (Kim *et al*., 1995), *Niallia circulans* (Takasaki *et al*., 1991), *Pelomonas saccharophilia* (Zhang *et al*., 2020), and *Pseudomonas* spp. (Fogarty *et al*., 1994; Kobayashi *et al*., 1998).

Allergenic homology searches on the amino acid sequence of G4ase from *P. stutzeri* strain MO-19 (Nakada

Table 7. Gross necropsy findings in the 90-day repeated oral toxicity test.

Sex		Male				Female			
Dose level (mg/kg/day)		0	100	300	1000	0	100	300	1000
Organ	Findings								
HEMATOPOIETIC SYSTEM									
Lymph nodes	Reddish	0/10	0/10	0/10	0/10	0/10	1/10	0/10	0/10
Thymus	Reddish	0/10	0/10	1/10	0/10	0/10	0/10	0/10	0/10
RESPIRATORY SYSTEM									
Lungs	Brown	0/10	0/10	1/10	0/10	0/10	0/10	0/10	0/10
	Brown patch	0/10	0/10	1/10	0/10	0/10	0/10	0/10	0/10
DIGESTIVE SYSTEM									
Stomach	Black patch	0/10	0/10	0/10	0/10	2/10	0/10	0/10	0/10
	White patch	1/10	1/10	0/10	0/10	1/10	0/10	0/10	0/10
Small intestine	Diverticulum	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	White patch	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Liver	Hepatodiaphragmatic nodule	0/10	0/10	0/10	0/10	0/10	1/10	0/10	0/10
	Small, caudate lobe	0/10	0/10	0/10	0/10	0/10	0/10	0/10	1/10
Abdominal cavity	Mass	0/10	0/10	0/10	0/10	1/10	0/10	0/10	0/10
URINARY SYSTEM									
Kidneys	Cyst	0/10	0/10	0/10	0/10	0/10	1/10	0/10	0/10
	Depression, focal	3/10	1/10	2/10	2/10	3/10	1/10	1/10	0/10
	Dilated pelvis	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
REPRODUCTIVE SYSTEM									
Testes	Small	0/10	0/10	1/10	0/10	-	-	-	-
Epididymides	Small	0/10	0/10	1/10	0/10	-	-	-	-
Ovaries	Cyst	-	-	-	-	0/10	0/10	1/10	0/10
Uterus	Cyst	-	-	-	-	1/10	0/10	0/10	0/10
	Dilated lumen	-	-	-	-	0/10	1/10	3/10	2/10
ENDOCRINE SYSTEM									
Thyroid	Glands nodule	0/10	0/10	1/10	0/10	0/10	0/10	0/10	0/10
NERVOUS SYSTEM									
Brain	Deformed	0/10	0/10	0/10	0/10	0/10	0/10	1/10	0/10

-: Not applicable

Table 8. Results of the bacterial reverse mutation test.

Dose (μg/plate)	Number of revertant colonies per plate				
	S. typhimurium				*E. coli*
	TA98	TA100	TA1535	TA1537	WP2*uvrA*
[-S9 Assay]			Mean (S.D.)		
0	31 (5)	111 (6)	11 (1)	9 (2)	27 (5)
313	30 (6)	106 (11)	8 (4)	8 (1)	32 (9)
625	26 (2)	113 (10)	10 (2)	6 (1)	28 (3)
1250	24 (2)	119 (11)	12 (5)	6 (1)	25 (4)
2500	29 (5)	114 (10)	13 (4)	11 (2)	28 (6)
5000	28 (5)	126 (11)	12 (4)	15 (1)	28 (3)
Positive control substance [μg/plate]	AF-2 [0.1]*	AF-2 [0.01]*	NaN_3 [0.5]*	9-AA [80]*	AF-2 [0.01]*
Revertant colonies per plate	623 (37)	684 (46)	532 (7)	167 (5)	96 (7)
[+S9 Assay]			Mean (S.D.)		
0	39 (5)	124 (19)	10 (2)	17 (1)	26 (4)
313	28 (8)	121 (5)	9 (2)	12 (1)	22 (5)
625	34 (8)	122 (4)	13 (3)	12 (1)	25 (4)
1250	32 (11)	121 (10)	11 (4)	16 (1)	35 (2)
2500	33 (2)	129 (16)	7 (4)	16 (2)	31 (5)
5000	40 (4)	134 (11)	15 (3)	21 (3)	34 (7)
Positive control substance [μg/plate]	2-AA [0.5]*	2-AA [1]*	2-AA [2]*	2-AA [2]*	2-AA [10]*
Revertant colonies per plate	395 (16)	1315 (131)	450 (9)	156 (9)	798 (77)

*AF-2, 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide; NaN_3, Sodium azide; 9-AA, 9-ammoacridine hydrochloride; 2-AA, 2-Aminoanthracene.

Table 9. Results of the chromosomal aberration test.

Dose (μg/mL)	Relative cell growth (%)	Number of cells with structural aberrations						Number of cells with aberrations -gap (%)	Number of polyploid cells (%)
		gap	ctb	cte	csb	cse	oth		
[-S9 Assay]									
0	100.0	2	2	0	0	0	0	2 (0.7)	0 (0.0)
1250	94.8	0	1	2	0	0	0	3 (1.0)	0 (0.0)
2500	118.7	1	1	2	0	0	0	3 (1.0)	0 (0.0)
5000	113.1	2	2	0	0	0	0	2 (0.7)	1 (0.3)
0.1 (MMC*)	90.7	8	93	134	0	0	0	178 (59.3)**	1 (0.3)
[+S9 Assay]									
0	100.0	0	0	0	0	0	0	0 (0.0)	0 (0.0)
1250	99.2	0	1	1	0	0	0	2 (0.7)	1 (0.3)
2500	109.3	0	0	1	0	0	0	1 (0.3)	0 (0.0)
5000	110. 8	0	0	1	0	0	0	1 (0.3)	0 (0.0)
12.5 (CP*)	66.9	3	6 39	122	0	0	0	141 (47.0)**	0 (0.0)
[24 hr Assay]									
0	100.0	0	1	2	0	0	0	3 (1.0)	1 (0.3)
1250	117 .5	0	0	1	0	0	0	1 (0.3)	1 (0.3)
2500	125.8	0	0	1	0	0	0	1 (0.3)	1 (0.3)
5000	117.8	0	0	1	0	0	0	1 (0.3)	0 (0.0)
0.05 (MMC)	107.7	3	47	78	0	0	0	111 (37.0)**	1 (0.3)

*MMC, Mitomycin C; CP, Cyclophosphamide; Positive controls.
Abbreviations: csb, chromosome break; cse, chromosome exchange; ctb, chromatid break; cte, chromatid exchange; oth, others than gap, chromatid break, chromatid exchange and chromosome exchange.
**Significant difference ($p \leq 0.025$) from negative control (0 μg/mL) (Fisher's exact test).

Table 10. Results of the *in vivo* micronucleus test.

Dose (mg/kg)	0	500	1000	2000	CP*
			Mean (S.D.)		
Frequency of MNIE (%)	0.14 (0.07)	0.14 (0.05)	0.13 (0.06)	0.11 (0.04)	1.96 (0.29)**[KB]
			(Min-Max)		
Range of MNIE/4000IE	2-9	3-8	3-9	3-6	66-97
			Mean (S.D.)		
Ratio of IE (%)	66.1 (5.6)	68.6 (4.1)	67.6 (9.4)	71.4 (2.7)	60.4 (3.5)

*CP, Cyclophosphamide (10 mL/kg; Positive control).
**Significant difference ($p \leq 0.025$) from negative control (0 mg/kg).
[KB]Kastenbaurn and Bowman method.

et al., 1990) for full-length, 80 amino acid alignments by FASTA and 8 amino acid exact matches using "The Food Allergy Research and Resource Program (FARRP) AllergenOnline.org database" (version 21) (Goodman *et al*., 2016) were performed on the amino acid sequence of G4ase from *P. stutzeri* strain MO-19 reported by Nakada *et al*. (Nakada *et al*., 1990). Results indicated that G4ase from *P. stutzeri* MO-19 is not likely to be homologous, is highly unlikely to be cross-reactive, and has an extremely low possibility to induce an allergenic reaction.

The data presented herein demonstrates that G4ase from *P. stutzeri* strain MO-19 showed no mutagenicity and no genotoxicity in the bacterial reverse mutation, chromosomal aberration, and *in vitro* and *in vivo* micronucleus tests, even at the highest concentrations used. High concentrations of G4ase were given by gavage to rats in standardized acute, 2-week, and 90-day repeated oral toxicity studies. All the animals given G4ase from *P. stutzeri* strain MO-19 survived. The single oral 50% lethal dose (LD_{50}) of G4ase from *P. stutzeri* strain MO-19 was greater than the highest doses tested, namely >2000 mg/kg (>143 mg-TOS/kg from the second GLP study). In the 2000 mg/kg dose group of five animals in the first study one had a dilated renal pelvis and a second a focal depression, while the second study had no such findings. Examination of gross necropsy findings in the 2-week and

Table 11. Results of the *in vitro* micronucleus test.

Dose (μg-TOS/mL)	MNBN (%)
-S9 assay	
0 (negative control)	0.95
1200	0.75
2000	1.15
2400	1.00
MMC* (positive control)	2.25**
+S9 assay	
0 (negative control)	0.65
400	0.95
1200	0.95
2000	0.85
CP* (positive control)	2.15**
24 hr assay	
0 (negative control)	0.65
100	0.75
200	0.65
300	0.80
COL* (positive control)	2.45**

*MMC, Mitomycin C; CP, Cyclophosphamide; COL, Colchicine
**Significant difference from negative control (Fisher's exact test): $p \leq 0.025$
Abbreviations: MNBN, Micronucleated binucleate cells

90-day repeated oral toxicity study also showed male and female animals with these formations, which concurred with the original determination that they are common to this strain and age of animals and are not dose dependent. Further, histopathological inspections suggested no treatment caused effect on the kidneys. The No Observed Adverse Effect Level (NOAEL) from the 90-day repeated oral toxicity study was at the highest doses tested, 1000 mg/kg/day (71.3 mg-TOS/kg/day), in male and female rats, indicating the enzyme to be non-toxic. These findings are consistent with the safe use of "Maltotetraohydrolase enzyme preparation from *Bacillus licheniformis* expressing a modified maltotetraohydrolase gene from *Pseudomonas stutzeri*", which is determined GRAS (U.S. FDA, 2009) and for which an ADI "not specified" was established (JECFA, 1995).

Supporting the claim of safe use of G4ase as a processing aid in food manufacturing is the fact that G4ase has been allowed for use in Japan for over 35 years. G4ase is found in the List of Existing Food Additives and monographed in Japan's Specifications and Standards for Food Additives under the name of "Exomaltotetraohydrolase" (MOH, 1996; MHLW, 2018). G4ase is commercially manufactured from *P. stutzeri* strain MO-19 and used to manufacture food ingredients such as maltotetraose (G4)-rich starch syrup and other similar products in Japan. Recently, G4ase is also used in dough to produce finished commercial bakery products. To the authors' knowledge no untoward effects have been reported by individuals involved in the manufacture and handling of G4ase, individuals using G4ase in production of food ingredients, individuals using G4ase in commercial products, or consumers that have ingested various final foods containing inactive G4ase, such as bakery and foods containing G4-rich starch syrup manufactured using G4ase.

In conclusion, under conditions of the 90-day repeated oral toxicity study using G4ase from *P. stutzeri* strain MO-19 on female and male rats the NOAEL was 1000 mg/kg/day (71.3 mg-TOS/kg/day). Using an uncertainty factor of 100 for intake of adult humans (70 kg), this allows for a safe consumption of this G4ase of 700 mg/person/day (49.9 mg-TOS/person/day). These data from the genotoxicity, and oral LD_{50} and NOAEL-generating toxicity studies on G4ase at the highest doses tested, and long history of safe use in Japan demonstrates that G4ase from *P. stutzeri* strain MO-19 is safe when used as a processing aid in food production in accordance with current Good Manufacturing Practices.

ACKNOWLEDGMENTS

Nagase Viita Co., Ltd. acknowledges the Public Interest Incorporated Foundation, Biosafety Research Center, Japan, 582-2 Shioshinden, Iwata, Shizuoka 437-1213, Japan, for conducting all studies reported in this manuscript as an independent third party.

Conflict of interest---- Shuji Matsumoto is an employee of Nagase Viita Co., Ltd., and Alan B. Richards is a consultant for Nagase Viita Co., Ltd.

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