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Original Article

Safety evaluation of 2-aza-8-oxohypoxanthine based on *in vitro* and human patch tests

Hisae Aoshima[1], Sayuri Hyodo[1], Rinta Ibuki[1], Jing Wu[2], Jae-Hoon Choi[2] and Hirokazu Kawagishi[2,3,4]

[1]Department of Research and Development, Vitamin C60 BioResearch Corp. Nihonbashidori-nichoume Bldg. 4F 2-2-6 Nihonbashi Chuo-ku, Tokyo 103-0027, Japan
[2]Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan
[3]Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan
[4]Graduate School of Integrated Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

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ABSTRACT — 2-Azahypoxanthine (AHX) and imidazole-4-carboxamide (ICA) are fairy-ring causing compounds from a mushroom-forming fungus, *Lepista sordida*, and 2-aza-8-oxohypoxanthine (AOH) is a metabolite of AHX in plants. However, the safety of AOH had not yet been elucidated. In this study, we focused on AOH and performed safety evaluations of the compound using *in vitro* and human patch tests for cosmetic applications. In the Ames test, AOH was not mutagenic to any of the test bacterial strains (> 5000 μg/plate). *In vitro* skin irritation and skin sensitization studies using reconstructed human epidermis and peptides that contained lysine and cysteine showed that AOH was not a skin irritant (cell viability > 50%) and did not exhibit skin sensitization. This compound also did not exhibit cytotoxicity under ultraviolet- or sham-irradiation in the alternative phototoxicity test using BALB/c 3T3 cells (mean photo effect < 0.1) and no skin reaction was observed in the patch test on human skin. Thus, we concluded that AOH is safe as a cosmetic ingredient. This is the first study in which safety evaluation tests were performed on AOH.

Key words: Ames test, Direct reactivity assay, Fairy ring, Phototoxicity, Skin irritation, Skin sensitization

INTRODUCTION

Turfgrass occasionally grows in the shape of a ring, with thicker growth than the surrounding grass, and thereafter, mushrooms form in the same circle (Fig. 1). This occurs on lawns in parks and golf courses. This phenomenon is called a “fairy ring” and, in Western legend, it is said that fairies make these circles and dance inside them (Ramsbottom, 1926; Shantz and Piemeisel, 1917; Smith, 1957). In 1675, the first scientific paper on fairy rings was published and, since that paper was introduced to Nature in 1884, the cause of the phenomenon remained a mystery for a long time (Evershed, 1884).

We previously cultured the hyphae of one of fairy-ring forming fungi, *Lepista sordida*, and discovered a plant growth stimulator, 2-azahypoxanthine (AHX) from the culture broth. We also found imidazole-4-carboxamide (ICA) from the broth, which suppresses the growth of turfgrass (Choi *et al*., 2010a, 2010b). Furthermore, it has been revealed that AHX is converted into 2-aza-8-oxo-

Correspondence: Hisae Aoshima (E-mail: hisae.aoshima@vc60.com)
Hirokazu Kawagishi (E-mail: kawagishi.hirokazu@shizuoka.ac.jp)

Fig. 1. Fairy ring.

hypoxanthine (AOH) when absorbed into plants (Fig. 2) (Choi *et al*., 2014). We call AHX, ICA, and AOH fairy chemicals (FCs), after the title of the article in Nature that covered our study (Mitchinson, 2014). Subsequent studies have demonstrated that FCs endogenously exist in edible parts of the three major cereals, rice, wheat, and corn (Choi *et al*., 2014); thus, people have eaten FCs for a long time. Furthermore, FCs increased the yield of rice, wheat, and other crops in field and greenhouse conditions (Asai *et al*., 2015; Tobina *et al*., 2014). Choi *et al*., 2010a). These results indicate the possibility that FCs are a new family of plant hormones (Kawagishi, 2018, 2019).

Kinetin (N6-furfuryladenine) is a member of the plant growth hormone family, cytokinins, and is generally known for its growth-promoting and antiaging effects on plants (Fig. 2). Stanley B. Levy of Revlon Consumer Products Corporation has stated that kinetin is also useful against human skin aging (Baran and Maibach, 2017). Many studies have reported the antiaging effects of kinetin on human skin cells and in clinical trials (Berge *et al*., 2006, 2008; Chiu *et al*., 2007; McCullough and Weinstein, 2002; Wu *et al*., 2007). Therefore, kinetin is used as an ingredient for cosmetics and cosmeceuticals. Like kinetin, FCs contain a purine carbon skeleton (Fig. 2) and regulate plant growth. Therefore, we decided to study the potential of FCs as cosmetic ingredients. Preliminary experiment examining their effects on the skin have indicated that, of the three FCs, AOH has the greatest potential as a cosmetic ingredient (preparing for submission); thus, we focused on examining the safety of AOH.

In this study, the toxicity of AOH was investigated using the Ames, alternative skin irritation, skin sensitization, and phototoxicity tests *in vitro*. The safety of AOH to human skin cells was also evaluated using the skin patch test.

MATERIALS AND METHODS

Chemicals and reagents

AOH was synthesized from 5-aminoimidazole-4-carboxamide, as previously described (Choi *et al*., 2016; Ikeuchi *et al*., 2014).

Ames test: The test strains *Salmonella typhimurium* TA98, TA100, TA 1535, and TA1537 and *Escherichia coli* WP2*uvrA* were obtained from the National Institute of Health Sciences (April 12th, 2017) (Kanagawa, Japan). S9 mix and cofactor FA were used in BoZo Research Center Inc. (Tokyo, Japan). The media used in the Ames test were as follows: Nutrient broth No.2 (Oxoid Ltd., Cambridge, UK), Bacto agar (Becton Dickinson and Co., Franklin Lakes, NJ, USA), and minimal glucose agar plate (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan). Soft agar was prepared from d-biotin, l-histidine hydrochloride monohydrate, and l-tryptophan (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2), sodium azide (SAZ), 2-aminoanthracene (2-AA), 2-methoxy-6-chloro-9-[3-(2-

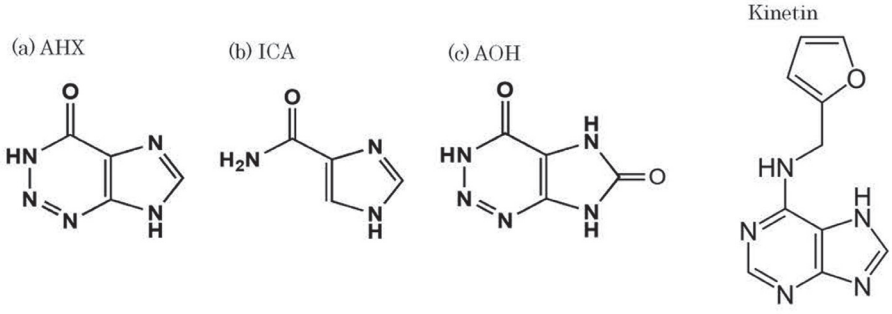

Fig. 2. Structure of (a) AHX, (b) ICA, (c) AOH, and kinetin.

chloroethyl)aminopropylamino]acridine·2HCl (ICR-191), and benzo[a]pyrene (B[a]P) from Fujifilm Wako were used as positive controls in the Ames test. Dimethyl sulfoxide (DMSO), sodium phosphate buffer (pH 7.4), and sodium chloride were purchased from Fujifilm Wako.

Skin irritation test: SkinEthic™ reconstructed human epidermis (RHE) was obtained from Episkin (Lyon, France). Phosphate buffered saline (PBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), and sodium dodecyl sulfate (SDS) were purchased from Fujifilm Wako. For the culture and maintenance of RHE, the medium attached to the kit was used.

Skin sensitization: Direct reactivity assay (DPRA): Peptides that contained lysine (Ac-RFAAKAA-COOH) and cysteine (Ac-RFAACAA-COOH) (both with a purity 90-95%) were obtained from Scrum Inc. (Tokyo, Japan). Sodium dihydrogen phosphate, sodium hydrogen phosphate, ammonium acetate, cinnamaldehyde (positive control), high-performance liquid chromatography (HPLC)-grade acetonitrile, and trifluoroacetic acid (TFA) were purchased from Fujifilm Wako.

Phototoxicity test: The media and reagents used in the phototoxicity test were as follows: Dulbecco modified Eagle medium (DMEM; Merck, Darmstadt, Germany); fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA); all ingredients in Hanks buffered salt solution (HBSS; Fujifilm Wako and Merck); chlorpromazine (CPZ; Merck); SDS, ethanol, acetic acid, penicillin, and streptomycin (Fujifilm Wako); and neutral red (NR; Merck).

Skin patch test: Aqueous solution of 6.5 mM (0.1%) AOH was used for all human tests. Scanpor Tape was purchased from Smart Practice Japan (Kanagawa, Japan).

Ames test

The Ames test was conducted according to the Organization for Economic Cooperation and Development (OECD) guideline for testing of chemicals (no. 471, 1997). Mutagenicity of AOH was assessed using a bacterial reverse mutation assay with *S. typhimurium* TA98, TA100, TA1535, and TA1537 and *E. coli* WP2*uvrA* (Green and Muriel, 1976; Maron and Ames, 1983). The test was performed using the preincubation method in the presence or absence of S9 mix. The optimal dose of AOH was determined by performing the test with the following dose: 1.22, 4.88, 19.5, 78.1, 313, 1250, and 5000 μg/plate. The highest dose was determined on the basis of the required dose mentioned in the guidelines. DMSO was used as a negative control. The doses of the positive controls, AF-2, SAZ, 2-AA, ICR-191, and B[a]P, were fixed at specific values that are known to induce positive responses. For each treatment, 0.1 mL of the test substance solution, negative control, or positive control was added to a sterilized test tube. Assays were performed by mixing 0.5 mL of a 0.1 mol/L sodium phosphate buffer (pH 7.4) or S9 mix with 0.1 mL of the bacterial suspension. The mixture was pre-incubated with gentle shaking (90 times/min) for 20 min at 37°C. After preincubation, 2 mL of molten top agar was added to this mixture, which was then poured onto the minimal glucose agar plate. After the overlaid agar solidified, the plates were incubated for 50 hr at 37°C. Revertant colonies in each plate were counted with an automatic colony analyzer CA-11D systems (System Science Co. Ltd., Tokyo, Japan). The test substances were judged as mutagenic when they produced a dose-dependent and reproducible increase in the number of revertant colonies that was equal to or greater than twice the number of colonies in the negative control, regardless of the presence or absence of S9 mix. The test substance was considered non-mutagenic when a reproducible increase in the number of revertant colonies was less than twice the number of the negative control.

In vitro skin irritation test

The skin irritation test was performed on the RHE model as per the OECD guideline for testing of chemicals (no. 439, 2010). The experiment was conducted according to the "SkinEthic Skin Irritation Test-42bis Test Method for the Prediction of Acute Skin Irritation of Chemicals: 42 Minutes Application + 42 Hours Post-Incubation, Standard Operation Procedure (SOP)". SkinEthic™-RHE was transferred to the maintenance medium after the RHE was cultured in the growth medium for 24 hr at 37°C with 5% CO_2. Sterile purified water (10 μL) was dropped on top of the RHE, then AOH (16 mg) was added to the RHE and this was incubated for 42 min at 37°C with 5% CO_2. PBS (16 μL) and 5% SDS (16 μL) were used as a negative control and positive control, respectively. After incubation, the treated RHEs were washed with PBS and further incubated in growth medium for 42 hr at 37°C with 5% CO_2. After incubation, the treated RHEs were transferred to the maintenance medium that contained 1 mg/mL MTT solution and this was incubated for 3 hr at 37°C. The formazan was extracted by isopropanol and absorbance was measured at 570 nm. The percentage viability was calculated from absorbance values at 570 nm in treated and control groups.

In vitro skin sensitization test

DPRA was performed according to the OECD guideline for testing of chemicals (no. 442C, 2015). AOH (31.13 mg for cysteine, 28.38 mg for lysine) was mixed

with 2 mL of ultrapure water. Stock solutions (0.667 mM) of peptides that contained cysteine and lysine were prepared in phosphate (100 mM sodium dihydrogen phosphate and 100 mM sodium hydrogen phosphate; pH 7.5) and ammonium acetate (100 mM; pH adjusted to 10.2 by dropwise addition of ammonium hydride) buffers, respectively, prior to use. Test chemical (50 μL), 200 μL of acetonitrile, and 750 μL of stock solution of peptide that contained cysteine or test chemical (250 μL) and 750 μL of stock solution of peptide that contained lysine were incubated in the dark for 24 hr at 25°C. Samples and calibration standards of each peptide (0.0167-0.534 mM), prepared in the appropriate buffer that contained acetonitrile (20%, v/v), were analyzed in triplicate for each peptide using HPLC. The peptide deletion (%) promoted by each test chemical was obtained according to the following equation: depletion (%) = [1 – (peptide peak area of test chemical / peptide peak area of control)] × 100. Negative depletion was considered as zero. The mean peptide depletion value was used to classify each chemical as negative or positive and determine its reactivity class as defined by the OECD TG 422C (Table 1).

The HPLC system (Shimadzu Corp., Kyoto, Japan) was equipped with an autosampler (model SIL-20ACHT), UV-visible detector (model SPD-20AV), and quaternary pump (model LC-20AD). The injection volume for all samples was 3 μL on to the L-column2 ODS (2.1 mm I.D. × 100 mm, Chemicals Evaluation and Research Institute, Tokyo, Japan). The autosampler and column temperatures were 25 and 30°C, respectively. The mobile phase consisted of 0.1% (v/v) TFA in ultrapure water (A) and 0.085% (v/v) TFA in acetonitrile (B), both at 0.35 mL/min. For the separation of peptides, a gradient of 10 to 25% (B) over 10 min was followed by an increase to 90% over 3.5 min and a decrease to 10% (B) over 20 min. Before running, the entire system was equilibrated at 30°C with phases A and B (1:1) for 2 hr. In addition, the column was re-equilibrated under the initial conditions for 7 min between injections. The chromatograms were extracted at 220 nm. Quantitation was carried out using LabSolutions (Shimadzu Corp.).

Table 1. Cysteine/ lysine prediction model for DPRA.

Mean of cysteine and lysine depletion (%)	Reactivity class	Prediction model [a)]
0 < depletion ≤ 6.38	No or minimal	Negative
6.38 < depletion ≤ 22.62	Low	Positive
22.62 < depletion ≤ 42.47	Moderate	Positive
42.47 < depletion ≤ 100	High	Positive

a) Prediction model based on OECD TG 442C (2015).

Alternative phototoxicity test

An *in vitro* 3T3 NR uptake toxicity test was performed by modifying the OECD guideline for testing of chemicals (no. 432, 2004). The phototoxicity of each test substance was estimated based on the differences in their cytotoxic concentrations for BALB/c 3T3 cells that had or had not been subjected to ultraviolet A (UVA) irradiation. BALB/c 3T3 cells were seeded in two 96-well microplates that contained DMEM supplemented with 10% FBS, 100 IU penicillin, and 100 μg/mL streptomycin. The cells were cultured for 24 hr. After cultivation for 1 hr in HBSS supplemented with various concentrations of test substances, the cells were irradiated with 5 J/cm^2 using an illuminant (SOL500, Hönle UV Technology, Munich, Germany) and an illuminometer (UV-Meter HighEnd, Hönle UV Technology). UVA or sham-irradiated CPZ and SDS were used as the positive and negative control, respectively. After 24 hr, the concentrations of the substances that exhibited cytotoxicity were measured using the NR assay. The cells were cultivated for 3 hr with NR (final concentration, 50 μg/mL) in DMEM. NR incorporated into living cells was quantified based on the difference in the absorbance of cell lysates solubilized in 50% ethanol in 1% acetic acid at 540 nm. Cell viability was expressed as a percentage of the control (absorbance of sample-untreated × 100 / absorbance of UVA-irradiated or sham-irradiated cell).

The photoirritation factor (PIF) was estimated from the 50% toxic concentration (IC_{50}) of UVA- and sham-irradiated cells. PIF ≥ 5 indicates photoirritation, 2 < PIF < 5 indicates likely photoirritation, and PIF ≤ 2 indicates no photoirritation. The mean photo effect (MPE) was calculated based on the comparison of the complete concentration-response curves and was defined as the weighted average across a representative set of photo effect values. MPE ≥ 0.15 indicates photoirritation, 0.1 < MPE < 0.15 indicates likely photoirritation, and MPE ≤ 0.1 indicates no photoirritation.

Skin patch test

The study was approved by the human ethics committee of DRC Co., Ltd. (number N20190308-1, Approval date: 8 Mar 2019) and written informed consent was obtained from subjects prior to enrollment. Additionally, this test was performed in accordance with the Declaration of Helsinki.

The patch test was performed on the skin of 9 men and 19 women from Japan, aged 20-57 y. The test sample (0.1% AOH aqueous solution) was placed on a Finn Chamber on Scanpor Tape and applied to each subject's upper back for 24 hr in an occlusive condition. Skin con-

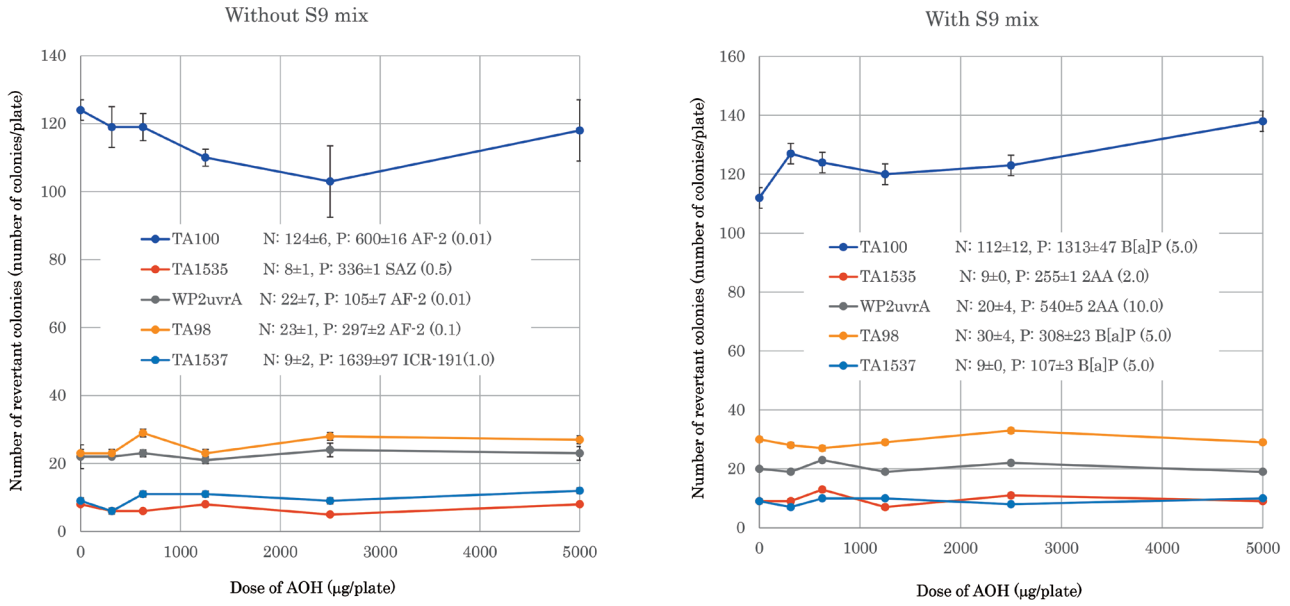

Fig. 3. Ames test of AOH. (A) without S9 mix, (B) with S9mix. N: Negative control, DMSO; positive control: mean ± S.D. of various compounds (μg/mL), n = 3.

ditions were evaluated 1 and 24 hr after the removal of the test sample. Skin reactions were evaluated according to the International Contact Dermatitis Research Group standards and the guidelines of the Japanese Dermatological Association (2009).

RESULTS AND DISCUSSION

Ames test

The mutagenicity of AOH was examined using bacterial reverse mutation assays with various bacteria (Fig. 3). AOH was not soluble in water and DMSO at 50 mg/mL. The homogeneity of the suspension in DMSO was better than that in water. Thus, DMSO was used to suspend the compound and was selected as the negative control for the test. AF-2, SAZ, 2-AA, ICR-191, and B[a]P were used as positive controls because these substances are widely used in reverse mutation assays using bacteria and are recommended in the guideline on genotoxicity using bacteria. The positive controls, in the presence or absence of S9 mix, showed positive responses by the respective test strains, as evidenced by the number of revertant colonies being greater than 2-fold that of the respective negative control value. Consequently, the validity of the test was confirmed. The number of revertant colonies in AOH-treated groups was less than twice that in the corresponding negative control-treated groups for all test strains, both in the presence and absence of S9 mix. Microbial toxicity was not observed in any of the test strains regardless of the presence or absence of S9 mix. The results showed that AOH was not mutagenic under the conditions used in this study.

In vitro skin irritation test

The skin irritation of AOH was examined using the MTT assay with RHE (Fig. 4). The cell viability of the negative control (PBS) was 100.0 ± 3.5%, whereas the viability of positive control (5% SDS) was 1.2 ± 0.1%. The cell viability was 100.6 ± 1.2% in the presence of AOH. This result indicates that AOH is a non-irritant (> 50% non-irritant) (Fig. 4).

In vitro skin sensitization tests

The skin sensitization of AOH was examined using DPRA. Table 2 shows the mean values of cysteine and lysine depletion by AOH and cinnamaldehyde according to the DPRA prediction model. The peptide depletion of cysteine and lysine by cinnamaldehyde was 73.2% and 65.4%, respectively. This result indicated that the test system was established. The peptide depletion of cysteine and lysine by AOH was 6.8% and 0.8%, respectively. Therefore, the reactivity of AOH was none or very low, and the skin sensitization of AOH was negative under the test condition.

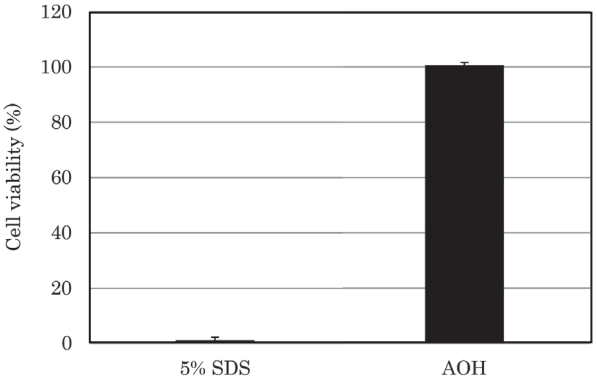

Fig. 4. *In vitro* skin irritation test.

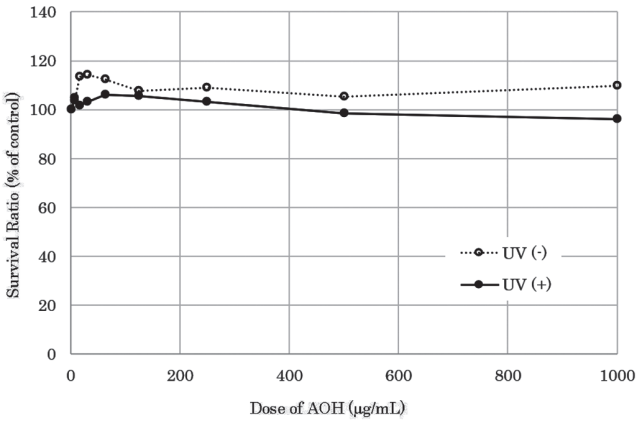

Fig. 5. Survival ratio of cells in the presence of AOH under UV (-) or UV (+) conditions.

Table 2. Mean cysteine and lysine depletion (*in vitro* skin sensitization test: DPRA).

Chemical Name	Cysteine Depletion (%)	Lysine Depletion (%)	Mean of cysteine and lysine depletion (%)	Reactivity class	DPRA prediction
AOH	6.8	0.8	3.8	No or minimal reactivity	Negative
Positive control (Cinnamaldehyde)	73.2	65.4	69.3	High reactivity	Positive

Table 3. Alternative phototoxicity test.

Sample	IC_{50} (mg/mL)		PIFMean	MPEMean	Judgment
	UV (-)	UV (+)			
AOH	-	-	-	0.068	Negative
CPZ	51.155	0.902	56.769	0.471	Positive
SDS	18.494	20.066	0.923	-0.020	Negative

Alternative phototoxicity test

The alternative phototoxicity of AOH was examined using the cell viability assay with BALB/c 3T3 cells. The PIF values of all tested substances are shown in Fig. 5. IC_{50}, PIF, and MPE values of the test samples are shown in Table 3. The IC_{50} of CPZ was 0.902 μg/mL under UVA-irradiation and 51.155 μg/mL under sham-irradiation. Thus, this test system was considered reliable. AOH, even at the maximum concentration of 1000 μg/mL, did not exhibit any cytotoxicity to the UV- or sham-irritated cells. This result indicated that AOH was not phototoxic under the conditions employed in this study.

Skin patch test

No serious skin reaction was observed 1 and 24 hr after the removal of the test sample in all human subjects (skin irritation index = 3.7, data not shown). The skin irritation index of AOH was < 5. These results allowed us to conclude that AOH was a safe product.

In conclusion, this is the first study that investigated the safety of AOH for external use on skin. We demonstrated that AOH did not induce any mutagenicity, skin irritation, skin sensitization, phototoxicity, or patch test reactions (Table 4). Therefore, we concluded that AOH can be safely used for external use on skin and as a cosmetic ingredient.

ACKNOWLEDGMENTS

We thank Nikoderm Research Inc. (Osaka, Japan) for performing the skin irritation, skin sensitization, and alternative phototoxicity tests *in vitro* and the phototoxic-

Table 4. Comparative results of the various safety tests for AOH.

Safety test	Guideline	Result
Ames test	OECD TG471	No mutagenicity
in vitro skin irritation	OECD TG439	No irritation
in vitro skin sensitization	OECD TG442C	No sensitization
in vitro phototoxicity	OECD TG432	No toxicity
Human skin patch test (0.1% AOH aqueous solution)	-	Negative (Skin irritation index: 3.7)

ity and photosensitization studies on human skin. We are also grateful to DRC Co., Ltd and BoZo Research Center Inc. for conducting the human patch test and Ames test, respectively. This work was partially supported by a Grant-in-Aid for Scientific Research on Innovative Areas "Frontier Research on Chemical Communications" (JP17H06402) from MEXT.

Conflict of interest---- This study was funded by Vitamin C60 BioResearch Corporation. Hisae Aoshima, Sayuri Hyodo, and Rinta Ibuki are employees of Vitamin C60 BioResearch Corporation.

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