# New Whitening agent: Kojyl-APPA

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Abstract

Exposure of the human skin to UV-light can cause sun-tanning, photoaging and even photo-carcinogenesis. Melanin is important in protecting the skin against UV damage, but excessive or uneven melanin production can lead to the formation of freckles and aged spot. Control of hyperpigmentation is becoming even more important as aged population continues to grow. These needs led us to develop effective and safe depigmenting-agent, kojyl 3-aminopropyl phosphate (kojyl-APPA), called Whitegen.

The development of whitegen was based on the fact that phosphate group of 3-aminopropyl phosphate can make kojic acid more compatible to the skin membrane and more stable. Instability of kojic acid has been a problem in cosmetic use. The insertion of phosphoester group has been recognized as a powerful tool to improve such physical properties as solubility and stability, because the phosphodiester residue is well charaterized as a non-toxic moiety, having a high affinity for cell membranes.

Kojyl-APPA showed no tyrosinase inhibition effect compared to kojic acid *in vitro*, but showed tyrosinase inhibition effect *in situ*. It means that kojyl-APPA is converted to kojic acid enzymatically in cells. Kojyl-APPA showed the inhibitory activity on melanin synthesis in mouse melanoma and normal humal melnaocytes and also showed long-lasting stability in comparison with its original form (kojic acid). Kojyl-APPA showed depigmenting effects when applied to UVB-induced hyperpigmentated region of guinea pig skin.

Based on these results, kojyl 3-aminopropyl phosphate can be used as a safe and effective ingredient for the brightness and cleanness of skin

# I. INTRODUCTION

Melanogenesis is the process of production of melanin by melanocytes within the skin and hair follicles (1,2). Melanocytes have specialized lysosome-like organelles, termed melanosomes, which contain several enzymes that mediate the production of melanin. Many factors are known as melanogenic inducers such as ultraviolet irradiation, inflammation (3) and other signaling molecules including alpha-melanocyte stimulating hormone (4) or endothelin-1 (5).

Tyrosinase, which catalyzes the hydroxylation of tyrosine to dopa and the oxidation of dopa to dopaquinone, is well-known as a key enzyme responsible for the production of melanin. Kojic acid is known to be a effective tyrosinease inhibitor (6), but the stability of this compound in solution limit the use of this compound.

Kojyl 3-aminopropyl phosphate (Kojyl-APPA) is a new compound which is made esterification of kojic acid and 3-APPA (Figure 1). Instability of kojic acid has been a problem in cosmetic use. The insertion of phosphoester group has been recognized as a powerful tool to improve such physical properties as solubility and stability, because the phosphodiester residue is well charaterized as a non-toxic moiety that has a high affinity for cell membranes and prevent degradation of kojic acid.

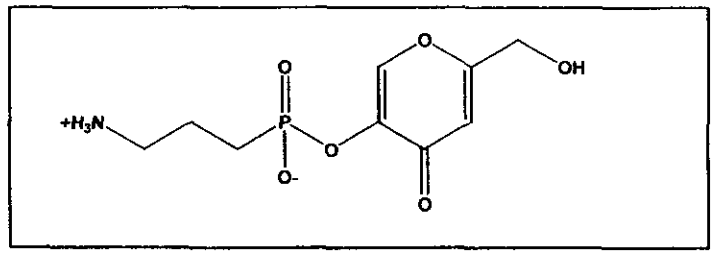


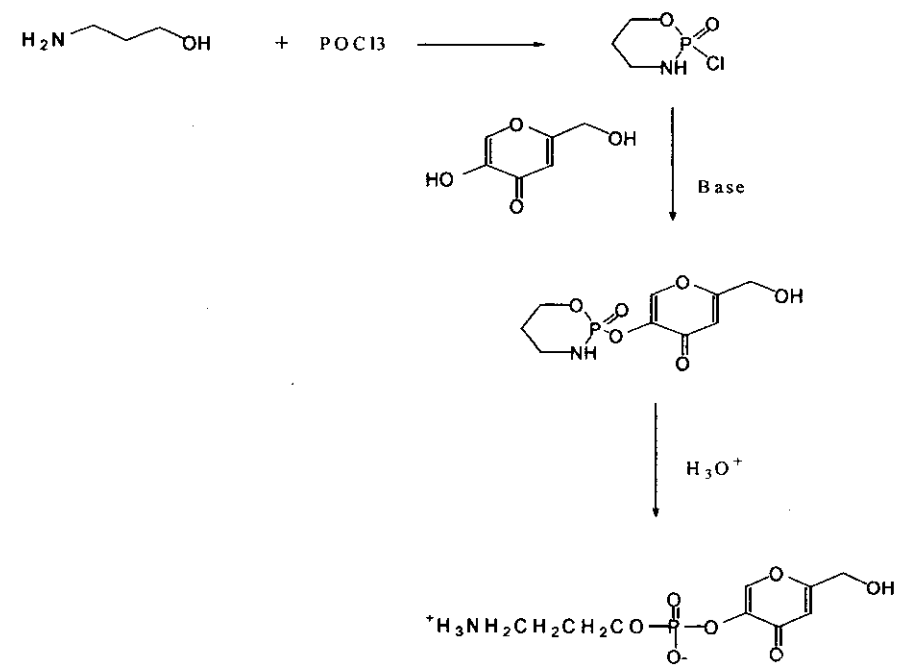

Fig.1. Sturcture of 5-[(3-aminopropyl)phosphinooxy]-2-(hydroxy-methyl)-4H-pyran-4-one

## 2. MATERIALS and METHODS

*Synthesis*

The syntheses of kojyl 3-aminopropyl phosphate are based on the reaction of 2,6-oxazaphosphoryl chloride with kojic acid in the presence of the organic base such as pyridine or triethylamine. As an illustration for synthesis, the route to phosphates is shown at scheme.

**SCHEME**




*The stability of kojyl-APPA on pH variation was measured by HPLC*

Each 1g of kojyl 3-aminopropyl phosphate and kojic acid were dissolved with 50ml water. While maintaining in 50 ℃ thermostatic bath for three weeks, the preservation of each compound was observed. Then, residual ratio of each compound was analyzed by using HPLC. Here, amine column, eluate solution of 50 % aqueous $CH_3CN$ solution including 50 mM $(NH_4)H_2PO_4$, flow rate of 0.8

ml/min and detection of 254 nm was used as HPLC analysis condition of kojyl 3-aminopropyl phosphate and kojic acid for control.

*Tyrosinase assay*

To determine the mushroom tyrosinase activity, we modified the method described by A. Vanni et al. (7). For the assay, we used 1.5 ml of a reaction mixture: 0.5 ml each of 0.1 M potassium phosphate buffer (pH 6.8) and 1.5 mM L-tyrosine solution, and 0.5 ml of mixture consisting of 100 units of mushroom tyrosinase to which solution of the test material has been added.

Tyrosinase derived from normal human melanocytes was also studied as follows. Normal human melanocytes were inoculated into 12 ml medium (MGM2) in a 75 $cm^2$ culture flask. After being cultured at 37 C° under 5 % $CO_2$ for four days, the cells were collected with a cell scraper and washed thoroughly using PBS. This cell pellet was added to 1 ml of 80 mM phosphate solution buffered at pH 6.8, containing 1 % triton X-100 and sonicated. The liquid was centrifuged at 12,000 X g for 10 minutes to obtain supernatant as a crude tyrosinase enzyme. The activity of tyrosinase was measured using [$^3$H]tyrosine by Pomerantz method.

*De novo melanin synthesis in mouse melanoma cells*

The Mel-Ab cell line is C57BL/6 mouse-derived spontaneously-immortalized melanocyte cell lines. This melanocytes are grown on plastic tissue culture flasks. Media for Mel-Ab cell is Dulbecco's Minimal Essential Media (DMEM) supplemented with 10 % fetal bovine serum (Gibco Life Tech.), 100 nm 12-O-tetradecanoylphorbol-13-acetate (Sigma), 1 nM cholera toxin (Sigma), 0.001 % streptomycin (Gibco Life Tech.) and 10,000 U/L penicillin.

Confluent Mel-Ab cultures are removed from plastic using 0.25 % trypsin/EDTA.

Cells are placed into Falcon 24 well plastic culture plates at a density of $10^5$ cells/well and incubated for 24 hours in media prior to treatment with the designated compound. After 24 hours, the media are replaced with 990 μl of fresh medium. Then 10 μl of the test compounds (dissolved at vehicle which is composed of 50 % propylene glycol, 30 % ethanol and 20 % water) are added to duplicate wells. Control wells are treated with the same vehicle alone. The feeding and agent treatment is repeated daily for three days with no additional treatment on the fourth day. Following four days of treatment, the remaining adherent cells are assayed. One well of the duplicate wells is used for melanin content determination and the other is for cell viability observation. The melanin content of melanocytes after treatment is determined by the addition of 1 ml/well of 1 N NaOH to cells. The crude cell extracts are assayed using a spectrophotometer at 400 nm. Cell viability is measured by dyeing live cells with crystal violet.

*De novo melanin synthesis in Normal human melanocytes*

Noe-melanin synthesis was measured by the rate of incorporation of L-[3-$^{14}$C]3,4-dihydroxyphenylalanine into newly synthesized melanins (8) during the last 48-96 h of melanocyte treatment as described previously (9). This assay measures the complete reaction sequence of melanin biosynthesis and reflects the melanogenic activity of tyrosinase, tyrosinase-related proteins I and II, and inhibitory factors involved in this process.

*In Situ tyrosinase assays*

The early rate-limiting step of the biosynthetic pathway of melanin (hydroxylation of tyrosinase) was estimated during the last day of treatment from the amount of $^3H_2O$ released into the medium during the conversion of L-

[*ring*-3′,5′-$^{3}$H]tyrosine to dihydroxyphenylalanine according to an adaptation (10) of the methods of Pomerantz (11) and Oikawa *et al.* (12) as described previously (13). Cells were seeded into 48 well culture plate at 2 X $10^{5}$ cells per well and allowed to attach overnight. The medium was then exchanged for growth medium supplemented with compounds under investigation. 24 h before the termination of the experiment, medium was supplemented with 2 μCi[$^{3}$H]tyrosine per ml. At the end of the experiment the radiolabeled medium was assayed for the presence of $^{3}H_2O$.

*Test for UVB-induced hyperpigmentation in brown guinea pig*

UVB-induced hyperpigmentation is elicited on the backs of brownish guinea pigs weighing about 500 g each. Guinea pigs are anesthetized with pentobarbital (30 mg/kg) and 12 separate areas (1 cm diametral circle) on the black of each animal are exposed to UVB radiation (Waldmann UV 800, Herbert Waldmann GmbH&E, Philis TL/12 lamp emitting 280-305nm). The total energy dose of UVB is 500 mJ/$cm^{2}$ per exposure. Group of 4 animals are used in experiment. The animals are exposed to UVB radiation one time a week for 3 consecutive weeks. Candidates for whitening agent are then topically applied to the hyperpigmented areas (5ul/circle) two times a day for 8 weeks from next day of the last tanning. Visual scoring is used to determine the degree of depigmentation one time a week for 8 weeks.

*Safety of Kojyl-APPA*

To insure safety of the compounds, acute oral toxicity, acute dermal toxicity, primary skin irritation, acute eye irritation, skin sensitization, human patch and repeat insult human patch tests were performed.

## 3. RESULTS and DISCUSSION

*Stability*

kojyl 3-aminopropyl phosphate, which is more stable than kojic acid itself at 50 °C over the pH 2 ~ 8 for 3 weeks. As the pH rises above 6, kojyl 3-aminopropyl phosphate was decomposed. The results are shown in Figure 2.

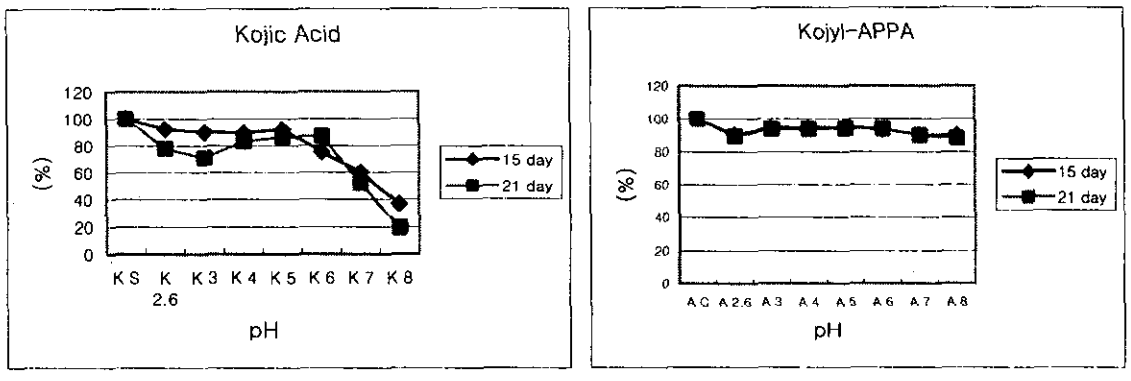



Figure 2. Stability of kojyl 3-aminopropyl phosphate and kojic acid.

*Effects on Tyrosinase activity*

Inhibitory effects of kojyl-APPA on tyrosinase activity were checked in vitro. As expected, kojyl-APPA did not inhibit mushroom and melanocyte derived tyrosinase directly.

Mushroom Tyrosinase

Kojyl 3-aminopropyl phosphate didn't have mushroom tyrosinase inhibition acitivity. But kojic acid has strong tyrosinase inhibition activity in vitro (Figure 3).

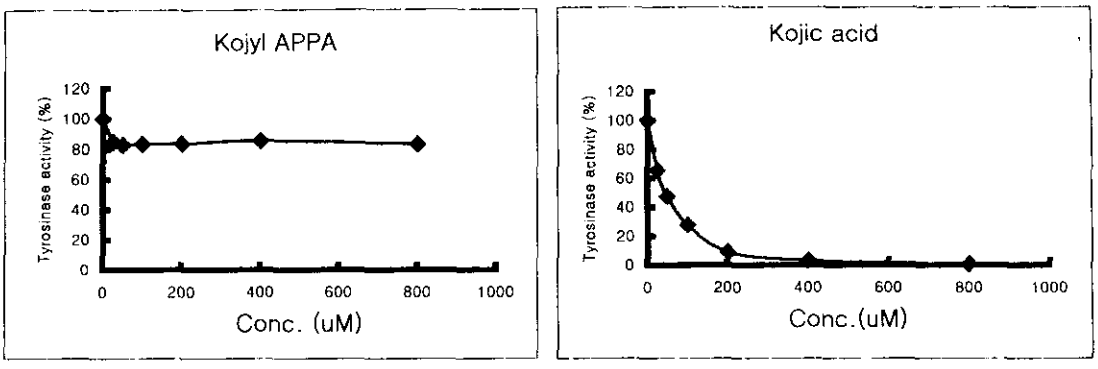



Figure 3. Mushroom tyrosinase activity.

Normal human melanocyte ext.

In *in vitro* tyrosinase assays using human melanocyte, kojyl 3-aminopropyl phosphate showed no inhibition activities, but kojic acid showed tyrosinase inhibition activities in a dose dependent manner (Figure 4).

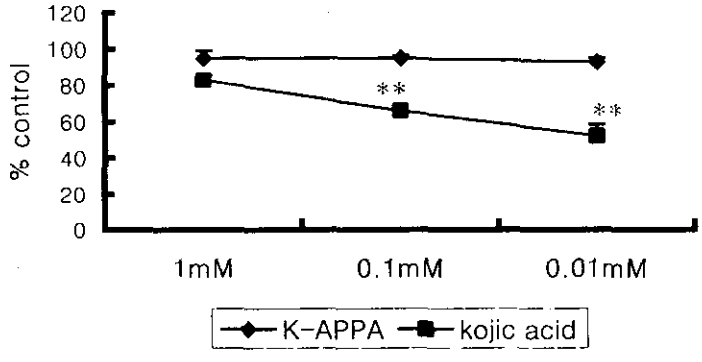



Figure 4. *In vitro* tyrosine hydroxylase using human tyrosinase. ** $P < 0.01$.

*Effects on Melanin synthesis in Mel-Ab cells*

Inhibition of melanin synthesis in Mel-Ab cell treated with different concentrations of Kojyl APPA or Kojic acid was measured (Fig. 5). Kojyl APPA and Kojic acid similarly decreased the melanin content of Mel-Ab cells to 75 % of control. But concentration dependence was not observed.

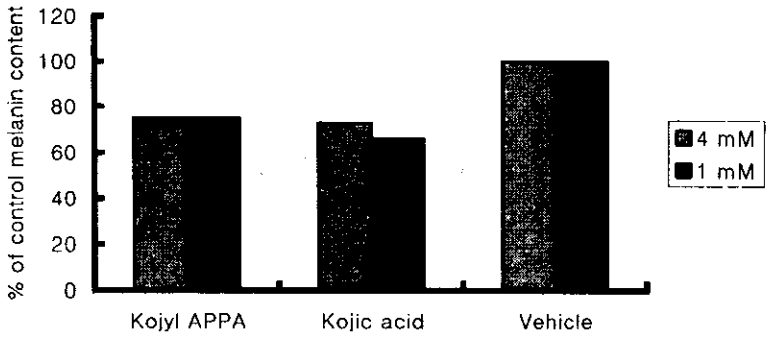
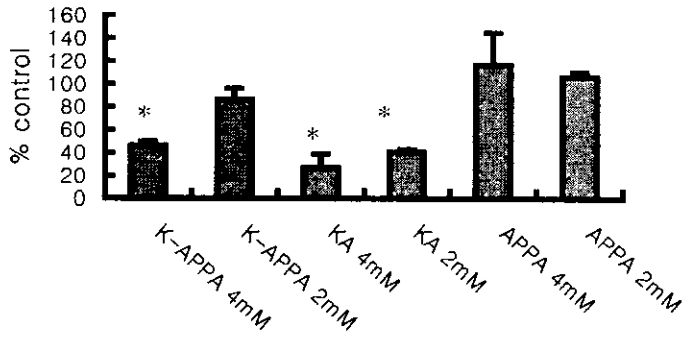


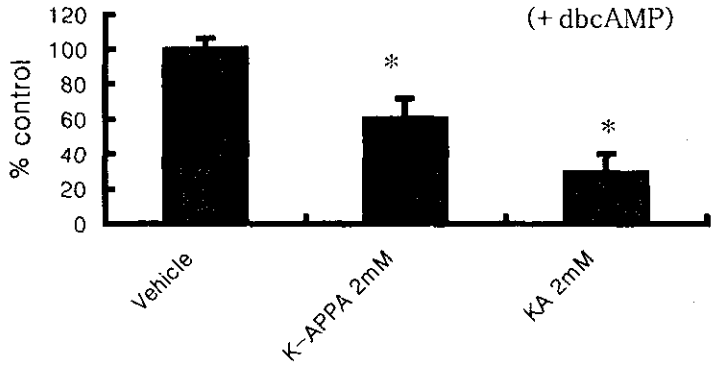

Figure 5. Inhibition of melanin synthesis in Mel-Ab cell treated with different concentrations of Kojyl APPA or Kojic acid was measured.

*Effects on Melanin synthesis in normal human melanocytes*

Inhibition of neo-melanin synthesis in normal human melanocyte treated with different concentrations of Kojyl-APPA or Kojic acid was measured. Kojyl APPA decreased the melanin content of melanocytes to 40 % of control at 4 X $10^{-3}$ M (Fig. 4. A). Kojyl-APPA also decreased neomelanin synthesis to 40 % of control at 2 X $10^{-3}$ M in c-AMP activated melanocytes (Figure 4. B).




A




B

Figure 6. Inhibition of neomelanin synthesis in normal human melanocytes. A, test materials were treated for 48 hours. B, test materials were treated for 48 hours with 100 uM dibutyladenosine 3'5-cyclicmonophosphate(dbcAMP). * $p < 0.05$.

*Effects on in situ tyrosinase activity*

*In situ* tyrosinase activity was measured in normal human melanocyte cultured for 12 and 24 hours. The inhibitory effects were evident at 24 hours after kojyl-APPA and kojic acid treatment. Tyrosinase activity was reduced to almost 30 % by kojyl-APPA treatment and 33 % by kojic acid (Figure 7).

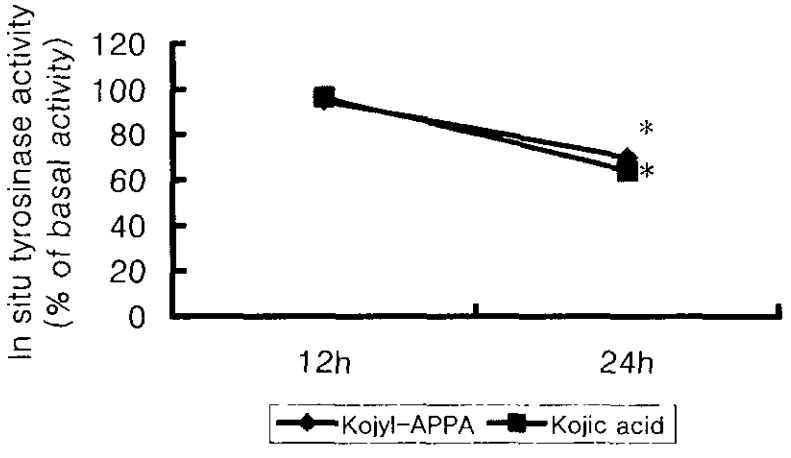



Figure 7. In situ tyrosine hydroxylase activity. Melanocyte were seeded to 48 well culture dishes at 2 X $10^5$ cells per well and were treated with 2 mM kojyl-APPA and kojic acid, where containing 2 μCi [$^3$H] tyrosine per ml. * $P < 0.05$

*Effects on UVB-induced Hyperpigmentation in Guinea-Pig*

Representative result of the lightening effect of Kojyl APPA after 8 weeks treatment on UVB-induced hyperpigmentation is shown in figure 8. There was depigmenting effects

of Kojyl-APPA after 8 weeks treatment compared with vehicle treatment without any side effects.

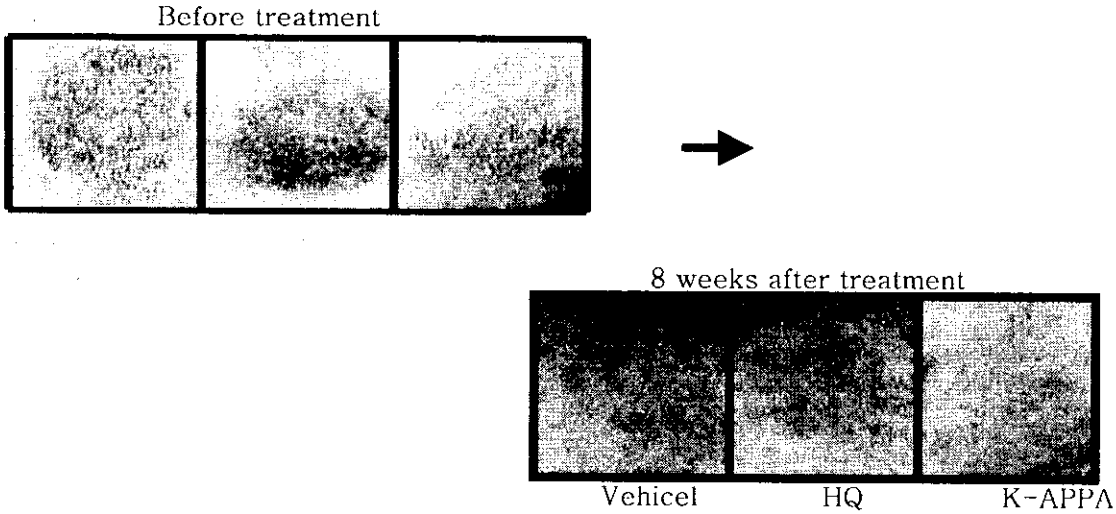



Fig. 8. The depigmenting effect of Kojyl-APPA on UVB-induced hyperpigmentation in Guinea Pig skin.

*Safety*

**To estimate the safety of the synthesized compounds, various safety tests were carried out. All of tested compounds were safe in these tests**

1-1. Acute oral toxicity test in mice: 1 ml/kg of the sample was administrated to total 10 mice (male and female were separately 5 mice). As a result, no death of mice was observed. And the difference in the body weight after administration was also insignificant.

1-2. Acute dermal toxicity test in mice and rabbits: 0.2 ml/kg of the sample was administrated percutaneously once to a total of 10 mice (5 male and 5 female). After 2 weeks of observation, no mice administered with the sample exhibited abnormal symptoms or changes in body weight. When the same experiment was performed on rabbits, No abnormal symptom and change of body weight were observed in rabbit administrated with the sample.

1-3. Primary skin irritation test: 0.1 ml of test sample was applied to back site (2.5 cm X 2.5 cm) of each of 12 rabbits depilated 24 hours before. As a result, no skin irritation was observed.

1-4. Eye irritation test: The sample was diluted with saline to produce 2 % of test sample, and 0.1 ml of the diluted solution was applied to the eye of each of 9 rabbits. As a result, no eye irritation on cornea, iris and conjunctiva was observed.

1-5. Skin sensitization test: Test was carried out for 6 (3 males and 3 females) Guinea pigs according to Maggnusson and Kligman's procedure. As a

result, no skin abnormality such as erythema, edema or freckles was observed.

1-6. Human patch test: Human patch test was carried out for 30 healthy women aged 20~28 according to CTFA Guideline(The Cosmetic Toiletry and Fragrance Association, INC., Washington, D. C. 20036, 1991). As a result, primary irritation response was not observed.

1-7. Repeat Insult Human Patch Test: Human patch test was carried out for subjects according to CTFA Guideline. As a result, no repeat irritation or no sensitive irritation was observed.

As mentioned in the results of the above safety experiments, kojil-3-aminopropylphosphate derivatives according to the present invention are materials safe for skin application.

## 4. CONCLUSIONS

We have put unprecedented concentration on the development of a novel compound by conjugating kojic acid with other compound, thus making kojic acid stabile and efficiently skin-absorbable simultaneously. we synthesized and tested kojyl 3-aminopropyl phosphate, which was easily prepared by the reaction of kojic acid with 2,6-oxazaphosphoryl chloride, to prevent the degradation of kojic acid.

This compounds showed higher stability and remarkably reduced skin irritating potential compared to kojic acid. Also, in the epidermis, these were enzymatically cleaved into its component molecules which exhibited their own biological efficacy.

Kojyl-APPA showed no tyrosinase inhibition effect compared to kojic acid *in vitro*, but showed tyrosinase inhibition effect *in situ*. It means that kojyl-APPA is converted to kojic acid enzymatically in cells. Kojyl-APPA showed the inhibitory activity on melanin synthesis in mouse melanoma and normal humal melnaocytes. It also showed long-lasting stability in comparison with its original form (kojic acid). Kojyl-APPA showed depigmentating effects when applied to UVB-induced hyperpigmentated region of guinea pig skin.

Based on these testing, kojyl 3-aminopropyl phosphate is safe and effective ingredient for the brightness and cleanness of skin

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