Development of 5-[(3-Aminopropyl)phosphinooxy]-2-(hydroxymethyl)-4*H*-pyran-4-one as a Novel Whitening Agent

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A stable derivative of kojic acid, 5-[(3-aminopropyl)phosphinooxy]-2-(hydroxymethyl)-4*H*-pyran-4-one (Kojyl-APPA), was synthesized in good yield. The effects of Kojyl-APPA on tyrosinase activity and melanin synthesis were investigated. Kojyl-APPA showed tyrosinase inhibition effect (30%) *in situ*, but not *in vitro*. Kojyl-APPA inhibited tyrosinase activity significantly at 24 h after treatment in normal human melanocytes. It means that Kojyl-APPA is not a direct inhibitor of tyrosinase itself, but it is converted to a potential inhibitor kojic acid enzymatically in cells. In addition, Kojyl-APPA decreased melanin content to 75% of control in melanoma cells and decreased neomelanin synthesis to 43% of control in normal human melanocytes. Its permeation through skin increased by about 8 times as compared with kojic acid.

Key words Kojyl-APPA; kojic acid; tyrosinase inhibition; melanin synthesis

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. Although melanin has important role in protecting the skin against UV damage, its excessive or uneven melanin production can lead to the formation of freckles and aged spot. As melanogenic inducers, many factors such as ultraviolet irradiation, inflammation[3] and other signaling molecules including alpha-melanocyte stimulating hormone[4] or endothelin-1[5] are known. Among them, tyrosinase, which catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA quinone, is well-known as a key enzyme responsible for the production of melanin. Therefore, inhibition of tyrosinase is a effective means to reduce the production of melanin.

Kojic acid **1** (5-hydroxy-2-hydroxymethyl-4*H*-pyran-4-one) is a fungal metabolic product and well recognized for its effective inhibition of tyrosinase in cosmetics.[6] However, because of its instability, kojic acid has limited use, especially in solution. Therefore, its derivatives have been extensively studied to overcome this significant drawback of kojic acid.

Recently, in a continuation of our study on the development of stable derivatives of kojic acid, we have had much interest in 3-aminopropane phosphoric acid because it is reported to stimulate collagen production in cultured human fibroblasts.[7] Furthermore, it is very compatible to skin and 3-aminopropane phosphoric acid is utilized as an active ingredient in cosmetic products.[7] Based on the results, we prepared 5-[(3-aminopropyl)phosphinooxy]-2-(hydroxymethyl)-4*H*-pyran-4-one **2** (Kojyl-APPA) by the reaction of kojic acid and 2-chloro-[1,3,2]oxazaphosphinane 2-oxide **4** in the presence of triethylamine in a mixture of chloroform and ethanol, followed by hydrolysis in acidic condition.

In this study, we evaluated the depigmenting effects of Kojyl-APPA such as tyrosinase inhibition *in vitro* and *in situ*, and the inhibitory activity on melanin synthesis in mouse melanoma and normal humal melnaocytes compared with kojic acid. Skin permeation of Kojyl-APPA and kojic acid was also investigated.

Chemistry

Synthesis of 5-[(3-Aminopropyl)phosphinooxy]-2-(hydroxymethyl)-4*H*-pyran-4-one 2 (Kojyl-APPA) The synthetic scheme of Kojyl-APPA **2** is shown in Chart 1. First, 2-chloro-[1,3,2]oxazaphosphinane 2-oxide **4** was synthesized in 91% yield by the reaction of 3-amino-1-propanol **3** and phosphorus oxychloride in the presence of triethylamine in dichloromethane at 5 °C. This intermediate **4** is very hydroscopic, so very thorough storage under anhydrous condition is required. Second, **4** was reacted with kojic acid in the presence of the organic base such as pyridine or triethylamine in a mixture of chloroform and ethanol to give 2-hydroxymethyl-5-(2-oxo-2λ^5-[1,3,2]oxazaphosphinan-2-yloxy)-pyran-4-one **5** in 82% yield, followed by hydrolysis in acidic condition to give Kojyl-APPA **2** in 98% yield. Interestingly, the OH group in 2-position of kojic acid did not take part in reaction with 2-chloro-[1,3,2]oxazaphosphinane 2-oxide. 2-Chloro-[1,3,2]oxazaphosphinane 2-oxide can be utilized as a very powerful precursor of 3-aminopropane phosphoric acid.

The stability of Kojyl-APPA and kojic acid was investi-

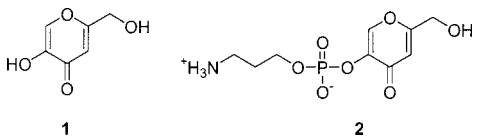
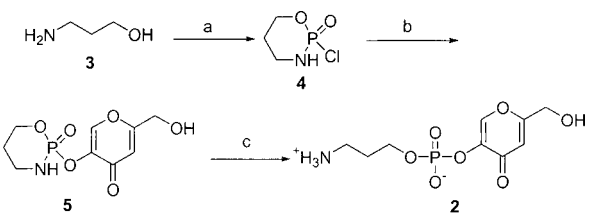


Fig. 1



Reagents: (a) $POCl_3$, TEA, CH_2Cl_2; (b) kojic acid, TEA, $CHCl_3$/EtOH; (c) H^+, H_2O/MeOH

Chart 1

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gated by storing their aquous solutions (pH 2—8) at 50 °C and analyzing each content every week at 254 nm by HPLC (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). To our knowledge, although kojic acid is stable in acidic condition, it is known to be substantially decomposed above pH 6. According to our results, 40% and 52% of kojic acid were decomposed in the solution of pH 7 after 2 and 4 weeks, respectively. In case of pH 8, only 19% of kojic acid survived after 4 weeks. On the contrary, about 90% of Kojyl-APPA remained even after 4 weeks in both solutions (pH 7 and 8). Therefore, Kojyl-APPA is much more stable than kojic acid.

Biological Activity and Discussion

The biological activity of Kojyl-APPA was determined by study on the tyrosinase activity, melanin synthesis in Mel-Ab cells and normal human melanocyte, and *in situ* tyrosinase activity. To determine the mushroom tyrosinase activity, we modified the method described by A. Vanni *et al.*[8] For kojic acid, IC_{50} of 23 μmol/l was reported.[9] However, according to our results, Kojyl-APPA did not inhibit mushroom and melanocyte derived tyrosinase directly. We suspect the result is due to the blocking of OH group in 5-position of kojic acid, which is very important in inhibiting tyrosinase by chelating copper. *In vitro* tyrosinase assays using human melanocyte, Kojyl-APPA showed no inhibition activities, either.

***De novo* Melanin Synthesis in Mouse Melanoma Cells** The Mel-Ab cell lines are C57BL/6 mouse-derived spontaneously-immortalized melanocyte cell lines.[10] Inhibition of melanin synthesis in Mel-Ab cells treated with different concentrations of Kojyl-APPA or kojic acid was measured (Fig. 2). Kojyl-APPA and kojic acid decreased the melanin content of Mel-Ab cells to similar extent, about 75% of control.

***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[11] during the last 48—96 h of melanocyte treatment as described previously.[12] 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. Inhibition of neo-melanin synthesis in normal human melanocyte treated with different concentrations of Kojyl-APPA or kojic acid was measured. According to the results, kojic acid decreased the melanin content of melanocytes to 38% and 35% of control at 2× 10^{-3} M and 4×10^{-3} M, respectively (Fig. 3). On the contrary, Kojyl-APPA decreased the melanin content of melanocytes to 43% of control at 4×10^{-3} M (Fig. 3).

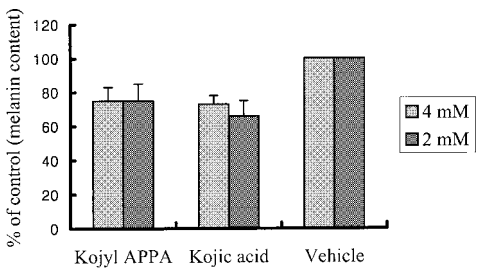

Fig. 2. Inhibition of Melanin Synthesis in Mel-Ab Cells Treated with Different Concentrations of Kojyl-APPA or Kojic Acid

Results were expressed as percentages of control, and data are means±S.D.

***In Situ* Tyrosinase Assays** *In situ* tyrosinase activity was measured in normal human melanocyte cultured for 12 and 24 h. The inhibitory effects were evident at 24 h after Kojyl-APPA and kojic acid treatment. Tyrosinase activity was reduced to about 30% by Kojyl-APPA treatment and 33% by kojic acid (Fig. 4). We inferred that tyrosinase inhibition activity of Kojyl-APPA is due to decomposition to 3-aminopropane phosphoric acid and kojic acid by bioenzymes in cells, but not by Kojyl-APPA itself.

***In Vitro* Skin Permeation Test** In order to show its functions properly, Kojyl-APPA must permeate through the stratum corneum and reach viable epidermal and dermal layers or viable subcutaneous layers. However, the barrier property of skin acts as a major obstacle for the transdermal delivery. Figure 5 shows the *in vitro* skin permeation of Kojyl-APPA and kojic acid performed with abdominal skin of female hairless guinea (strain IAF/HA-hrBR) using Franz diffusion cells. The cumulative amounts of permeated Kojyl-APPA and kojic acid in the receptor compartment after 24 h were plotted respectively. According to the results, there was significant increase (about 8 times) in the skin permeation of Kojyl-APPA compared with kojic acid. It indicates that applied to the skin, the former is more effective than the latter.

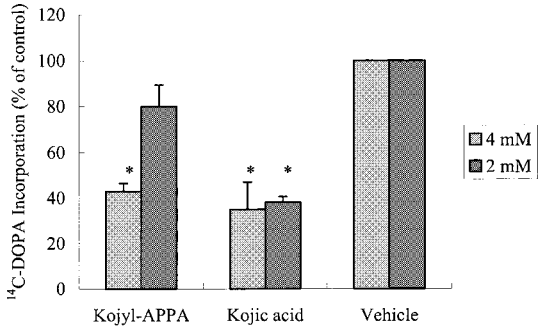

Fig. 3. Inhibition of Neomelanin Synthesis in Normal Human Melanocytes

Test materials were treated for 48 h. Results were expressed as percentages of control, and data are means±S.D. An asterisk indicates values significantly different from the control group as determined by the two-tailed *t*-test. $*p<0.05$.

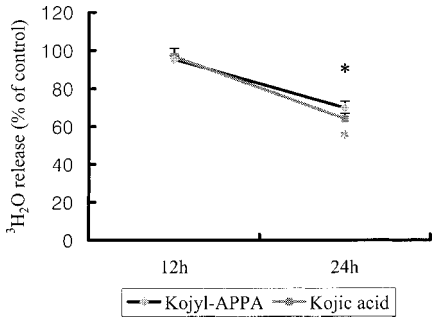

Fig. 4. *In Situ* Tyrosine Hydroxylase Activity

Melanocytes were seeded on 48 well culture dishes at 2×10^5 cells per well and were treated with 2 mM Kojyl-APPA and kojic acid containing 2 μCi [^{3}H]tyrosine per ml. Results were expressed as percentages of control, and data are means±S.D. An asterisk indicates values significantly different from the control group as determined by the two-tailed *t*-test. $*p<0.05$.

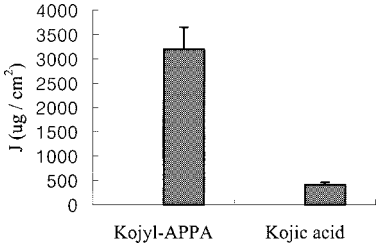

Fig. 5. The Cumulative Amounts of Kojyl-APPA and Kojic Acid Permeated through Excised Guinea Pig Skin After 24 h

Data are means±S.D.

Conclusion

We have continued the study on the development of novel stable derivatives of kojic acid with other bioactive compounds. Kojyl-APPA was easily prepared by the reaction of kojic acid with 2-chloro-[1,3,2]oxazaphosphinane 2-oxide, which is a powerful precursor of 3-aminopropane phosphoric acid, followed by hydrolysis in acidic condition. To our surprise, OH group in 2-position of kojic acid did not take part in reaction with 2-chloro-[1,3,2]oxazaphosphinane 2-oxide. Kojyl-APPA showed much increased stability and its extent of permeation through skin was much greater than kojic acid. Though Kojyl-APPA had no tyrosinase inhibition effect compared with kojic acid *in vitro*, it showed inhibition effect *in situ*. It means that Kojyl-APPA is converted to kojic acid and 3-aminopropane phosphoric acid enzymatically in cells. In addition, Kojyl-APPA showed the inhibitory activity to a same extent as kojic acid on melanin synthesis in mouse melanoma and normal human melnaocytes.

Experimental

All melting points were determined on a Fisher John's melting point apparatus and were uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Varian GEMINI-300 BB (300 MHz) spectrometer (with tetramethylsilane as an internal standard). Infrared (IR) absorption spectra were recorded on a JASCO IR-810. Solvents were laboratory grade or better. The following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad.

2-Chloro-[1,3,2]oxazaphosphinane 2-Oxide 4 To a solution of 3-amino-1-propanol (2.95 g, 39.3 mmol) and triethylamine (7.40 g, 73.1 mmol) in dichloromethane (20 ml), a solution of phosphorus oxychloride (5.69 g, 37.1 mmol) was added dropwise at 5 °C for 2 h. After filtration to remove triethylamine hydrochloride salt, the filtrate was dried over $MgSO_4$ followed by filtration and concentration *in vacuo*. The residue was precipitated by addition of toluene to give 2-chloro-[1,3,2]oxazaphosphinane 2-oxide **4** (5.30 g, 91%) as a white solid. mp 79—82 °C. IR ν_{max} (KBr) cm^{-1}: 3254, 1477, 1274, 1092, 1036, and 996. ^{1}H-NMR ($CDCl_3$) δ: 1.61—1.80 (1H, m), 2.00—2.20 (1H, m), 3.20—3.42 (2H, m), 4.30—4.55 (2H, m), 4.90 (1H, br).

5-[(3-Aminopropyl)phosphinooxy]-2-(hydroxymethyl)-4*H*-pyran-4-one 2 (Kojyl-APPA) To a solution of kojic acid (3.73 g, 26.2 mmol) and triethylamine (6.10 g, 60.3 mmol) in ethanol (100 ml), 2-chloro-[1,3,2]oxazaphosphinane 2-oxide (5.30 g, 34.1 mmol) in chloroform (100 ml) was added dropwise at room temperature for 1 h. While the reaction mixture was further stirred for 12 h, 2-hydroxymethyl-5-(2-oxo-2λ^5-[1,3,2]oxazaphosphinan-2-yloxy)-pyran-4-one **5** was precipitated as a white solid. The intermediate **5** was filtered, dried *in vacuo* and used in the following hydrolysis reaction without further purification (5.61 g, 82%). IR ν_{max} (KBr) cm^{-1}: 3286, 3155, 1659, 1592, 1454, 1429, 1335, 1255, 1202, 1112, 1043, and 996. ^{1}H-NMR (D_2O) δ: 1.70—1.81 (1H, m), 2.00—2.18 (1H, m), 2.91 (2H, t, J=8.4 Hz), 3.25—3.40 (2H, m), 4.42—4.60 (2H, m), 6.68 (1H, s), 8.41 (1H, s). Then, 2-hydroxymethyl-5-(2-oxo-2λ^5-[1,3,2]oxazaphosphinan-2-yloxy)-pyran-4-one (5.61 g, 21.5 mmol) was dissolved in water at pH 3—4 and stirred at 40 °C for 5 h. Methanol (150 ml) was added to the reaction mixture to precipitate Kojyl-APPA (5.88 g, 98%) which was obtained by filtration and drying *in vacuo.* mp 118—128 °C (decomp.). IR ν_{max} (KBr) cm^{-1}: 3446, 3322, 2904, 1658, 1616, 1250, 1090, and 863. ^{1}H-NMR (D_2O) δ: 1.82—1.98 (2H, m), 3.05 (2H, t, J=8.4 Hz), 3.95—4.08 (2H, m), 4.42 (2H, s), 6.64 (1H, s), 8.27 (1H, s).

Tyrosinase Assay We used 1.5 ml of the following 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 a solution of the test material has been added. Tyrosinase derived from normal human melanocytes was also studied as follows. Normal human melanocytes were inoculated in 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 12000×***g*** for 10 min to obtain a supernatant as a crude tyrosinase enzyme. The activity of tyrosinase was measured using [^{3}H]tyrosine by the Pomerantz method.[14)]

***De novo* Melanin Synthesis in Mouse Melanoma Cells** The Mel-Ab cell lines are C57BL/6 mouse-derived spontaneously-immortalized melanocyte cell lines. These melanocytes were grown on plastic tissue culture flasks. Media for Mel-Ab cells were 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 10000 U/l penicillin. Confluent Mel-Ab cultures were removed from the plastic flasks using 0.25% trypsin/EDTA. Cells were placed into Falcon 24 well plastic culture plates at a density of 10^5 cells/well and incubated for 24 h in media prior to treatment with the designated compound. After 24 h, the media are replaced with 990 μl of fresh medium. Then 10 μl of the test compounds (dissolved in a vehicle which was composed of 50% propylene glycol, 30% ethanol and 20% water) were added to duplicate wells. Control wells were treated with the same vehicle alone. The feeding and agent treatment was repeated daily for three days with no additional treatment on the fourth day. Following four days of treatment, the remaining adherent cells were assayed. One well of the duplicate wells was used for melanin content determination and the other was for cell viability observation. The melanin content of melanocytes after treatment was determined by the addition of 1 ml/well of 1 N NaOH to cells. The crude cell extracts were assayed at 400 nm using a spectrophotometer (ELX 800, Bio-TEK Instrument Inc., U.S.A.). Cell viability was measured by dyeing live cells with crystal violet.

***De novo* Melanin Synthesis in Normal Human Melanocytes** Neomelanin synthesis was measured by the rate of incorporation of L-[3-^{14}C]3,4-dihydroxyphenylalanine into newly synthesized melanins[11)] during the last 48—96 h of melanocyte treatment as described previously.[12)]

***In Situ* Tyrosinase Assays** The early rate-limiting step of the biosynthetic pathway of melanin (hydroxylation of tyrosine) 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[13)] of the methods of Pomerantz[14)] and Oikawa *et al.*[15)] as described previously.[12)] Cells were seeded into 48 well culture plate at 2×10^5 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 ^3H_2O.

***In Vitro* Skin Permeation Test** Female hairless guinea pigs (strain IAF/HA-hrBR) were sacrificed for *in-vitro* skin permeation test. All were 8 weeks old. Abdominal skin was excised and divided to mount on Franz diffusion cells (Lab Fine instruments, Korea). The diameter of each diffusion cell was 0.9 cm and the compartment volume was 5 ml. The receptor compartment was filled with 10 wt.% glycerin aqueous solution. The receptor compartment was kept at 32 °C by circulating water through an external jacket and stirring constantly with a magnetic bar. In this study, 2 wt.% of Kojyl-APPA and Kojic acid were used, respectively. HPLC analysis was carried out taking 5 ml receptor solution after 24 h. All samples were filtered with 13 mm disk filter (pore size 0.45 μm) before injection. The analysis was performed with Agilent 1100 series (Agilent Technologies) equipped with diode array detector. YMC-Pack NH_2 (YMC Co., Ltd., Japan) column was used for the stationary phase and the mobile phase was solution of 50% aqueous CH_3CN solution including 50 mM $(NH_4)H_2PO_4$ at a flow rate of 0.8 ml/min. Kojyl-APPA and kojic acid were detected at a wavelength of 254 nm.

Statistical Analysis Data were presented as mean±S.D. from three independent experiments. Statistical comparison between different treatments was done by two-tailed *t*-test.

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