AA2G™
(L-Ascorbic Acid 2-Glucoside)
Vitamin C - Physiological Activities -

Physiological Activities of AsA

- Antiscorbutic Activity
  (Enhance Collagen Synthesis)
- Antioxidative Activity
- Inhibition of Melanin Synthesis
- Antiviral Activity
- Enhance Immune System

L-Ascorbic Acid (AsA)
Stable Vitamin C Derivatives

AsA is Unstable to Heat, Light and Oxidation.

Active Site
(Antioxidant Activity etc.)

Vitamin C Active
L-ascorbic acid 2-phosphate (AA-2P)

Vitamin C Not Active
L-ascorbic acid 2-sulfate (AA-2S)

Vitamin C Not Active
2-O-octadecyl-L-ascorbic acid (CV-3611)
Reaction Process of
A Novel Stable Form of Vitamin C

Liquefied Starch

AsA

Enzyme A

L-Ascorbic acid 2-maltooligoside (AA2Gn)

Enzyme B

L-Ascorbic acid 2-glucoside (AA2G)
Characteristics of AA2G
Characteristics of AA2G

Properties
Since C2-hydroxyl group of AsA is masked with glucose, AA2G is nonreducible and very stable to oxidative conditions.
### Physicochemical Properties of AA2G and AsA

<table>
<thead>
<tr>
<th></th>
<th>AA2G</th>
<th>AsA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight</td>
<td>338.27</td>
<td>176.13</td>
</tr>
<tr>
<td>Melting Point</td>
<td>158.5 - 159.5°C</td>
<td>190.0°C</td>
</tr>
<tr>
<td>Heat of Fusion</td>
<td>38.5 kJ/mol</td>
<td>37.9 kJ/mol</td>
</tr>
<tr>
<td>Specific Rotation $[^{20\D}_{\alpha}]$ (c = 5)</td>
<td>$+189.6^\circ$ (at pH 2.0) $+246.3^\circ$ (at pH 7.1)</td>
<td>$+22.1^\circ$ (at pH 2.3) $+119.9^\circ$ (at pH 7.1)</td>
</tr>
<tr>
<td>Water Solubility (at 25°C)</td>
<td>125 g/100 g·H₂O</td>
<td>33 g/100 g·H₂O</td>
</tr>
<tr>
<td>UV Maximum Absorbance</td>
<td>238 nm (at pH 2.0) 260 nm (at pH 7.0)</td>
<td>243 nm (at pH 2.0) 265 nm (at pH 7.0)</td>
</tr>
<tr>
<td>pH (1.0 w/v %)</td>
<td>2.3</td>
<td>2.6</td>
</tr>
<tr>
<td>Dissociation Constant</td>
<td>pK₁ 3.0</td>
<td>pK₂ 4.1</td>
</tr>
<tr>
<td>Reducing Activity</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Stability of AA2G in an Aqueous Solution

Method:

1) Aqueous solutions of AA2G and AsA were each adjusted to pH 5.0 and pH 7.0. These solutions were incubated at 37 or 50 °C.

2) The amount of AA2G or AsA in the aqueous solutions was continuously monitored.
Heat Stability of AsA and AA2G Under Various pH Conditions

<table>
<thead>
<tr>
<th>pH</th>
<th>Color&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Percentage Remaining&lt;sup&gt;2&lt;/sup&gt;(%)</th>
<th>pH</th>
<th>Color&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Percentage Remaining&lt;sup&gt;2&lt;/sup&gt;(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.59</td>
<td>0.186</td>
<td>96.3</td>
<td>2.20</td>
<td>0.122</td>
<td>12.8</td>
</tr>
<tr>
<td>3.03</td>
<td>0.233</td>
<td>92.8</td>
<td>3.01</td>
<td>0.099</td>
<td>68.8</td>
</tr>
<tr>
<td>4.00</td>
<td>0.373</td>
<td>85.1</td>
<td>4.00</td>
<td>0.034</td>
<td>98.6</td>
</tr>
<tr>
<td>5.02</td>
<td>0.237</td>
<td>89.8</td>
<td>5.02</td>
<td>0.034</td>
<td>98.6</td>
</tr>
<tr>
<td>6.15</td>
<td>0.429</td>
<td>87.7</td>
<td>6.05</td>
<td>0.069</td>
<td>99.1</td>
</tr>
<tr>
<td>6.96</td>
<td>0.433</td>
<td>94.9</td>
<td>7.00</td>
<td>0.066</td>
<td>99.1</td>
</tr>
</tbody>
</table>

1: measured at OD420 nm  
2: measured by HPLC

Method:

1) Each solutions containing 2% of AsA and AA2G were adjusted to various pH levels and incubated at 125°C for 60 minutes (autoclave).
2) After incubation, the color and composition of each solutions were measured.
Stability of AA2G in the Presence of Cu$^{2+}$

Method:

1) Solutions 100 $\mu$M each of AsA and AA2G were incubated in the absence and presence of 10 $\mu$M CuSO$_4$ (0.1 M acetate buffer, pH 6.5).

2) The residual AsA and AA2G were determined by measuring absorbance of reaction mixtures at 265 nm for AsA and 260 nm for AA2G, respectively.

[Yamamoto et al., Chem. Pharm. Bull., 38 (11), 3020 (1990)]
Stability Test of AA2G and AA-2P in Various pH Levels

Method:

AA2G and AA-2P (0.20g) were each dissolved in deionized water. Solutions of each substance were made and pH levels of each solution were adjusted by addition of 1 M NaOH or 1 M HCl solutions. The solutions were standardized to exactly 100 ml (0.2%). The aliquot samples of 25 ml were each placed in a 30 ml glass bottle that was tightly sealed. The samples were stored in the dark at 50°C for 20 days. The stored materials were periodically analyzed for the development of color at an absorbance of 420 nm. The concentration of the remaining AA2G and AA-2P was also assayed.

Storage Conditions:
- Temperature 50°C
- Concentration 0.2%
- Period of storage 20 days
Physiological Activities of AA2G
- Prevention of scorbutic activity on guinea pigs
- Enhancement of collagen synthesis in cultured human skin fibroblasts
- Enhancement of immune responses in cultured murine and human lymphocytes
- Inhibition of melanin synthesis in melanoma cells
- Reduction of existing melanin

Exhibition of Physiological Activities of AsA in vivo and in vitro by AA2G

AA2G Releases AsA by the Action of α-Glucosidase
Method:

1) Guinea pigs were fed an AsA-deficient diet for 13 days (Symptoms of scurvy were observed).

2) On the 13th day, the animals were divided into three test groups. The groups were orally administrated either water, water containing AA2G (19.2 mg/body/day), or AsA (10mg/body/day) for 7 days.

3) Body weights were recorded daily.

[Yamamoto et al., J. Pharmacobio-Dyn., 13, 688 (1990)]
Chemical Properties of AA2G

- AA2G has the C-2 hydroxyl group bound to glucose.
- AA2G has excellent stability under the oxidative condition compared to conventional vitamin C (oxygen, heating, copper).
- AA2G is hydrolyzed by $\alpha$-Glucosidase and to release vitamin C which possess biological activity.
Efficacy of AA2G in Cosmetics
Efficacy of AA2G in Cosmetics

1. Intradermal Sustainability of AsA
2. Enhancement of Collagen Synthesis
3. Prevention of Melanin Pigment Formation
4. Decolorization of Existing Melanin
5. Suppression of Skin Damages Caused by UV Radiation
Percutaneous Absorption of AA2G and Its Conversion to AsA in the Skin

Method:
1) Cream containing 2% of AA2G and cream containing 3% of AA-2P were each applied to the legs of five volunteers. Then, covered each with cling film for 14 hours.
2) Placebo cream was applied to one other subject under same conditions.
3) Urine samples were taken every two hours for 26 hours.
4) AsA contents in urine were measured.

Human Subjects

AsA Content in Urine (mg/100 mL)

<table>
<thead>
<tr>
<th>(Hours)</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cream Applied</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cream Removed</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AA-2P Cream
AA2G Cream
Placebo Cream

Intra-dermal Distribution of AsA from AA2G Cream

Method:

1) Cream containing 2% of AA2G and cream containing 3% of AA-2P cream were each applied separately to the forearm of male subject. Then, covered each area with cling film for 12 hours.

2) Placebo cream was applied to the subject under the same conditions.

3) The skin samples were fixed with formalin and stained by Reticulin Silver Impregnation method on the indicated days.

**Method:**

1) Human skin fibroblasts were each cultured in the presence of $^{14}$C-AsA, $^{14}$C-AA-2P and $^{14}$C-AA2G.

2) On the indicated days, the cells were sonicated. The supernatants obtained by centrifugation were prepared for thin layer chromatography (TLC).

3) Mixture of benzene, methanol, acetone, and acetic acid were used as solvent (9:9:1:2). The Rf values were as same as that of AsA.

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Sustained Vitamin C: Activity of AA2G

How much $\alpha$-glucosidase do human epidermis possess?

Acid Phosphatase: 830
$\alpha$-Glucosidase: 10

(All values are in n mol/one gram per minute of wet wt tissue.)

[P.D. Mier et al., *British Journal of Dermatology*, 94, 443 (1976)]
Method:

1) Human skin fibroblasts were each incubated in the absence and presence of each collagen stimulator (0.25 mM) for 1-8 days.

2) The relative rate of collagen synthesis and total protein synthesis were determined.

Inhibitory Effect of AA2G on Melanin Formation

AA2G Inhibits Melanin Formation Through the Liberation of AsA by $\alpha$-Glucosidase

$\alpha$-Glucosidase \[ \text{AA2G} \]

AsA

Reducing Action

Lightening of Melanin Pigment by Reducing Action

Tyrosinase

L-Tyrosine $\rightarrow$ L-Dopa $\rightarrow$ Dopa-Quinone $\rightarrow$ Melanin (Black) $\rightarrow$ Reduced Melanin

Melanocyte
Method:

1) B16 melanoma cells were each cultured with AA2G and AsA for 12 hours.

2) The cells were treated with theophyline (0.5 mM) for 48 hours.

3) The cells were lysed with TCA and the intracellular melanin contents were measured.

[Miyai and Yamamoto et al., Nishinihon J.Dermatol., 58 (3), 439 (1996)]
**Method:**

1) B16 melanoma cells were each cultured with AsA, AA-2P, and AA2G (2 mM) for hours indicated in hours.

2) The cells were treated with theophyline (0.5 mM) for 24 hours.

3) The cells were lysed with TCA and the intracellular melanin contents were measured.

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**Inhibitory Effect of AA2G on Melanin Formation**

Inhibitory Effect of AA2G on Melanin Formation

Control  AA2G (2.5 mM)

■ Method:

1) B16 melanoma cells were each treated with theophylline (0.5 mM) after 12 hours in the absence and presence of AA2G (2.5 mM).

2) After 48 hours, the cells were reacted with L-DOPA for 1.5 hours and were stained by Nuclear Fast Red.

[Miyai and Yamamoto et al., Nishinihon J.Dermatol., 58 (3), 439 (1996)]
Influence of AsA on Melanin Formation

Polymerization

Reducing Action

Reduced Melanin (Colorless)

Melanin

Vitamin E GSH

Dehydro Ascorbic Acid

Recycling

Dehydro Ascorbic Acid
Reducing Action of AA2G on Existing Melanin (Lightening of Melanin Pigment)

Method:

1) B16 melanoma cells were cultured until confluent (48 hours). Theophyline (0.5 mM) was added to each culture during last 24 hours of culture.

2) The cells were each treated with AsA, AA-2P, and AA2G (2 mM) for 1-2 days.

3) The cells were lysed with TCA and each intracellular melanin contents were measured.

Inhibitory Effect of AA2G on Tyrosinase Activity

Separation of B-16 melanoma cells (possessing α-glucosidase on cell membrane) into supernatant and residue by centrifugation. Supernatant contains cell content without α-glucosidase but residue contains α-glucosidase.
Inhibitory Effect of AA2G on Tyrosinase Activity

Method:
Enzyme reaction

Tyrosinase solution
(Supernatant or residue of B16 Melanoma cell Lysate) 0.25 mL

50 mM Phosphate buffer (pH 6.8) 0.25 mL

Sample soln. (AA2G or AsA; Final 3.3 mM) 0.5 mL

Substrate soln. (0.05% L-Dopa) 0.5 mL

Incubated at 37°C

Measured Absorbance at 475 nm

The vertical axis represent level of colorization by Dopa, pigmented by tyrosinase activity (Dopa-Quinone converted from L-dopa). With the supernatent, AsA is not released from AA2G and unable to prevent tyrosinase activity. On the other hand, the residue with α-glucosidase, inhibited tyrosinase activity.

[Miyai and Yamamoto et al., Nishinihon J.Dermatol., 58 (3), 439 (1996)]
**Suppressive Effect of AA2G on UV-Induced Skin Damage**

Method:

1) Human skin keratinocytes were each cultured with AA2G, AA-2P, or AsA (0.2 mM) for 24 hr.

2) Each cells were radiated with UVB at 17 mJ/cm², then cultured for 24 hr.

3) The number of viable cells were determined by using a hematocytometer.

Method:

1) Human skin fibroblasts were cultured until confluent.

2) The cells were each radiated with UVB at 292 mJ/cm², then AA2G, AA-2P, or AsA (0.2 mM) was added to each culture.

3) After 7 days, the number of viable cells were determined by DNA assay method.

Suppressive Effect of AA2G on UV-Induced Skin Damage

Method:
1) Human keratinocyte cells (SCC) were pretreated with various antioxidants for 9 hours.
2) The cells were radiated with UVB at 20 mJ/cm², then cultured for 24 hr.
3) The number of viable cells were determined by neutral red assay method.

Suppression of UV-Induced Death of Cells by Various Anti-Oxidants

<table>
<thead>
<tr>
<th>Concentrations of various antioxidants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
</tr>
<tr>
<td>Glutathione (GSH)</td>
</tr>
<tr>
<td>Butylated hydroxytoluene (BHT)</td>
</tr>
<tr>
<td>Superoxide dismutase (SOD)</td>
</tr>
<tr>
<td>1,3-Dimethyl 2-thiourea (DMTU)</td>
</tr>
<tr>
<td>α-Tocopherol (VE)</td>
</tr>
<tr>
<td>AA2G</td>
</tr>
</tbody>
</table>

Human Keratinocyte Cell Line

[Suppressive Effect of AA2G on UV-Induced Skin Damage]

**Anti-Oxidative Effect of AA2G**

( Suppression of Hydroxyl Radicals )

**Method:**

1) Human keratinocyte cells (SCC) were each pretreated with AA2G, AsA, or catalase for 7 hours.

2) Hydrogen peroxide (0.4 mM) was added to each culture.

3) After 2 hours, number of viable cells were determined by neutral red assay method.

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**Human Keratinocyte Cells**

**UV Irradiation**

**OH Radicals Production**

**Lipid Peroxidation, Damage of Cell Membrane**

**Cell Death**

[AsA (mM) 0 100 0.1 1.0 1.0
AA2G (mM) 0 0.1 1.0
Catalase (U/mL) 0 1 2 3 4 5
Non-Treatment**

**Number of Viable Cells (x 10^4/well)**

**H₂O₂ Treatment**

AA2G Suppression of UV-Induced Lipid Peroxidation
(Hydroxy Radicals Production)

Human Skin Keratinocytes

Peroxidized Lipid (Abs 586 nm)

<table>
<thead>
<tr>
<th>No Irradiation</th>
<th>None</th>
<th>AA2G</th>
<th>VE</th>
<th>AA2G + VE</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV Radiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Method:

1) AA2G (3 mM), \( \alpha \)-tocopherol (Vitamin E <VE> / 3 mM), or AA2G (3 mM) + \( \alpha \)-tocopherol (3 mM) was added to a suspension of human skin keratinocytes.

2) After incubation for an hour, the suspension was radiated with UVB at 3 J/cm\(^2\).

3) The quantity of peroxidized lipids were determined by Phenol Indoleamine method.

Method:

1) Cream containing 2% of AA2G and placebo were each applied to the inner upper-arm once a day for 20 days.

2) Five hours after the final application of the cream, the arms were radiated with UVB.

3) Twenty-four hours after radiation, skin samples were taken from the radiated areas by shave biopsy, then were fixed in formaldehyde solution of 2%.

4) The number of sunburn cells of the skin specimens were calculated under a microscope.

Simultaneous Effect of AA2G and Vitamin E

**Method:**

1) Human Keratinocyte cells (SCC) were pre-cultured for 9 hours with: 1.0mM of AA2G, 1.0mM and 2.5mM of \( \alpha \)-Tocopherol, combination of 1mM of AA2G+ 1.0mM of \( \alpha \)-Tocopherol, and (another) combination of 1.0mM of AA2G + 2.5mM of \( \alpha \)-Tocopherol.

2) After radiating UVB (20 mJ/cm\(^2\)), the test cells were cultured for another 24 hours.

3) The number of viable cells were determined by neutral red assay.

**Suppressive Effect of AA2G on UV-Induced Skin Damage**

UV-Induced Erythema and Pigmentation in Human Skin

- **Effective**
- **Moderately Effective**
- **Not Different**
- **Moderately Reverse**
- **Reverse**

Number of Cases

<table>
<thead>
<tr>
<th>Erythema</th>
<th>Pigmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow</td>
<td>Blue</td>
</tr>
</tbody>
</table>

**Method:**

1) Creams containing 2% of AA2G and control were each applied to part the inner right arm of 15 healthy volunteers.

2) Each cream were applied 3 times a day for 6 consecutive days.

3) During the experiment, tested areas were each radiated with UVA and UVB once a day for a total of 3 days.

[Miyai and Yamamoto et al., *Nishinihon J. Dermatol.*, 58 (3), 439 (1996)]
Efficacy of AA2G: Reduction of Existing Wrinkles

**Method:**

1) Sixteen female subjects (ages 37-55) participated in 90-day experiment and cream containing 2% of AA2G were applied around their eyes.

2) Silicone replicas were taken from the tested areas at initiation, 45th day, and 90th day of the experiment.

3) The depth of the wrinkles and amount of roughness at the tested areas were measured by using those silicone replicas.
Efficacy of AA2G in Cosmetics

- AA2G gradually convert into active vitamin C by α-glucosidase and has greater sustainable effect than conventional vitamin C.
- AA2G promote collagen synthesis by acting on human skin fibroblasts.
- AA2G suppress melanin synthesis in melanocytes, and prevent undesirable pigmentation.
- AA2G reduce existing melanin, resulting in brighter pigmentation.
- AA2G capture free radicals and inhibit inflammatory activities of the skin.
- AA2G suppress UV-induced erythema and pigmentation.
- AA2G reduce development of wrinkles.
Potential Use of AA2G
Potential Use of AA2G

1. Maintenance of the Skin Resilience
   - Enhancement of Collagen Synthesis

2. Brightening Effect
   - Inhibition of Tyrosinase Activity
   - Antioxidative Activity (Reducing Action)

3. Prevention of Inflammation and Photoaging in the Skin
   - Antioxidative Activity
   (Scavenging Action of Hydroxyl Radicals)
Conclusion
Conclusion

• AA2G was originally developed as a pharmaceutical (quasi-drugs) agent for suppressing melanin in Japan for “brightening” cosmetics.

• AA2G possess effectiveness in maintaining skin resilience and suppression of skin cells damaged by UV radiation (one of the major causes of skin aging).

• AA2G has displayed dramatic effects on fundamental biological activities. Other possible applications besides active ingredient for daily brightening cosmetics include sunscreen, anti-photoaging cosmetics, and wrinkle repairing cosmetics (which efficacies are proven by several studies).
Inhibitory Effectors Against UV-induced Damages

Promoters of Melanin exclusion (α-hydroxy acid Placenta Protein)

Inducers of Metallothionein (Zinc Gluconate and Myristate)

UVB

Induction of Active Oxygen Species

Immune Suppression

Formation of Sunburn Cells

Sunscreen

Promotion of Melanogenesis

Sunscreen

Inhibitors of Melanoocyte Stimulators (Matricaria chamomilla L.)

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