

Enhancing Effect of 2-*O*- α -D-Glucopyranosyl-L-ascorbic Acid, a Stable Ascorbic Acid Derivative, on Collagen Synthesis

Yosimaru KUMANO,^a Tetsuo SAKAMOTO,^{*a} Mariko EGAWA,^a Muneo TANAKA,^a and Itaru YAMAMOTO^b

Shiseido Research Center,^a 1050 Nippa-cho, Kouhoku-ku, Yokohama 223-0057, Japan and Department of Immunochemistry, Faculty of Pharmaceutical Science, Okayama University,^b 1-1-1 Tushima-naka, Okayama 700-0082, Japan.

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The objective of the present study was to compare 2-*O*- α -D-glucopyranosyl-L-ascorbic acid (AA-2G) with ascorbic acid (AA) and ascorbic acid 2-phosphate (AA-2P) concerning the promotion of collagen production in human skin fibroblasts.

Though AA-2G was still observed to be promoting collagen synthesis at the same level on the 8th day of the culture, collagen synthesis was seen to decrease on the fifth day of culturing with AA and AA-2P. This sustained collagen synthesis-promoting action is considered to be a major feature of the novel vitamin C derivative, AA-2G by conducting an experiment in which an α -glucosidase inhibitor was present, it was shown that AA-2G exerts its collagen synthesis-promoting action after being decomposed to AA by α -glucosidase. Further, we observed that for AA-2G, even on the 8th day of the culture, the amount of AA in the fibroblasts was virtually unchanged from the beginning of the experiment, whereas, in the case of adding AA and AA-2P, virtually no AA was detectable in the culture medium on the fifth day.

These findings suggest that AA-2G is decomposed to AA by α -glucosidase in the cells. This AA promotes collagen synthesis, which is prolonged through AA-2G's sustained decomposition.

Key words vitamin C; ascorbic acid 2-glucoside; ascorbic acid; collagen synthesis; human skin fibroblast

Vitamin C (ascorbic acid, AA) has been widely used not only as a nutritional supplement but also in pharmaceuticals and cosmetics in expectation of a certain pharmaceutical effect. However, vitamin C is unstable in aqueous solutions, even under normal culture conditions at neutral pH and 37 °C. To solve this problem, a number of stable derivatives, which include L-ascorbic acid 2-sulfate (AA-2S) and L-ascorbic acid 2-phosphate (AA-2P), have been developed.^{1,2)} AA-2P shows antiscorbutic activity in guinea pigs^{3,4)} and has a stimulatory effect on collagen synthesis in cultured human skin fibroblasts,^{5,6)} but AA-2S does not show these effects.^{7,8)} 2-*O*- α -D-Glucopyranosyl-L-ascorbic acid (AA-2G, Fig. 1), is a novel vitamin C derivative synthesized from ascorbic acid and maltose or oligosaccharide by the use of transglucosylation enzymes.⁹⁻¹²⁾

Because AA-2G is stable in neutral solution, several other AA effects which have not been previously observed due to the unstable nature of AA and cell damage caused by high AA concentrations, were demonstrated in cell culture experiments.¹³⁾ Such results have been very useful in clarifying the essential physiological actions of AA. AA-2G is considered to be an ideal vitamin C derivative in terms of stability, safety, physiological activity and feasibility of mass production.

The enhancing effect of AA-2G on collagen synthesis was investigated by Yamamoto *et al.*¹⁴⁾ It was found that the effect of AA-2G in this regard was similar to that of AA at a concentration of 0.1 mM, and that this effect was greater than that of AA at high concentrations. However, this study only compares the collagen synthesis-enhancing action of AA-2G and AA, but does not compare AA-2G with the existing vitamin C derivatives in this respect.

Structurally, the difference between the existing vitamin C derivative, AA-2P, and the novel vitamin C derivative, AA-2G, is that in the 2 position the former has phosphate and the latter glucose. A comparison of the collagen synthesis-en-

hancing actions of AA-2G and AA-2P, therefore, compares the activity of the hydrolases which convert the derivatives into AA, so an understanding of this is essential in determining whether we can expect the pharmacological activity of vitamin C to be manifested when AA-2G is added to cosmetic products.

In the present study, we demonstrated that AA-2G very effectively enhanced the synthesis of collagen in cultured human skin fibroblasts, and this effect was sustained for longer than that of AA-2P and AA in long-period cultures. It is assumed that the enhancement of collagen synthesis by AA-2P was not sustained because AA is released very rapidly from AA-2P by hydrolysis, and this AA is unstable in the culture medium, so it is assumed that the AA disappears within a short time. However, the rate of hydrolysis for AA-2G in the fibroblasts was slower, enabling the effect of AA to be sustained in this case.

We consider that this is due to specific and sustained hydrolysis by α -glucosidase.

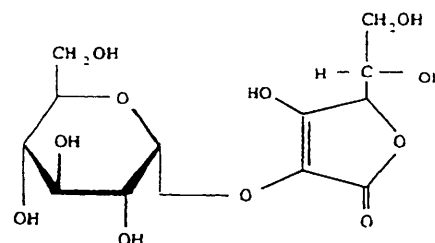


Fig. 1. Chemical Structure of 2-*O*- α -D-Glucopyranosyl L-Ascorbate (AA-2G)

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials Reagents and other materials used in this study were obtained from the following sources: Eagle's minimum essential medium (MEM) from Nissui Pharmaceutical; AA-2G and ^{14}C -AA-2G ($1\text{-}^{14}\text{C}$, 2.6 MBq/mg) from Hayashibara Biochemical Labs; L-ascorbic acid sodium from Wako Pure Chemical Industries; magnesium salt of AA-2P from Showa Denkou Co., Ltd.; castanospermine from Boehringer Mannheim Yamanouchi; ^{14}C -AA-2P ($1\text{-}^{14}\text{C}$, 1.6 MBq/mg), ^{14}C -AA ($1\text{-}^{14}\text{C}$, 1.85 MBq/mg) and ^3H -proline ($2,3\text{-}^3\text{H}$, 3.6 MBq/mg) from Daiichi Pure Chemicals Co., Ltd.

Cell Culture Human dermal skin fibroblasts from the Riken Cell Bank (male, 3 d old) were used. Fibroblasts (10^4 cells) were placed on 35 or 60 mm dishes and grown in Eagle's MEM supplemented with 10% fetal bovine serum (MEM-10) at 37°C for 6–8 d in an atmosphere of 5% CO_2 -air until apparent confluence was obtained.

Collagen Synthesis Collagen synthesis was determined by measuring the amount of labeled proline in collagen protein and non-collagen protein using purified collagenase.¹⁵⁾ The fibroblasts were incubated in 2 ml of MEM-10 containing L-[2,3- ^3H] proline (148 kBq/Petri dish), 0.5 mM β -aminopropionitrile fumarate and L-ascorbic acid derivatives at various concentrations for a fixed time. A certain experiment was carried out in the presence of castanospermine (0.1 mM).

Collected medium was mixed with 1 ml of 1.84 M trichloroacetic acid to precipitate macro-molecular proteins. The precipitate obtained was dissolved in 1 ml of Tris-HCl buffer (pH 7.6, 0.1 M), and cooled ethanol was added to a final concentration of 66%. This was then dried *in vacuo* and dissolved in 300 μl of 0.2 M NaOH, neutralized to pH 6–8 with 0.3 M HCl and then treated with collagenase. The mixture so obtained was exposed to a reaction system consisting of 100 μl of the reagents, 10 μl of collagenase solution (5 units), 10 μl of 25 mM CaCl_2 and 20 μl of 62.5 mM *N*-ethylmaleimide for 90 min at 37°C and mixed with 250 μl of 0.61 M trichloroacetic acid–0.5% tannic acid. The precipitate was treated with 500 μl of 0.31 M trichloroacetic acid–0.25% tannic acid. Acid-soluble fractions were combined and acid-insoluble fractions were solubilized with 500 μl of 0.1 M Tris-HCl buffer (pH 7.6) containing 17.3 mM sodium dodecyl sulfate and 5 mM dithiothreitol at 95°C for 5 min. Both types of fractions were estimated to be collagen and noncollagen proteins, respectively, and their radioactivities were measured using a scintillation counter. The relative rate of collagen synthesis to total protein synthesis was calculated using a rearrangement of the formula of the Diegelmann and Peterkofsky¹⁶⁾ equation as follows:

$$\text{Rate of collagen synthesis (\%)} = \frac{\text{collagen radioactivity} \times 100}{\text{collagen radioactivity} + 5.4 \times \text{non-collagen radioactivity}}$$

AA Amount in Fibroblasts Fibroblasts were incubated in 2 ml of MEM-10 containing 1 mM L-ascorbic acid derivatives and ascorbic acid derivatives labelled with ^{14}C (100–150 kBq/Petri dish) for a fixed time. Once cultured fibroblasts were washed three times with PBS and 1.0 ml of PBS was added. They were then scraped out with a cell scraper and transferred to an Eppendorf tube. The cell suspension (2×10^4 cells/l) in 0.1 M PBS was sonicated and centrifuged at 4000 rpm (4°C) for 10 min to obtain a supernatant. All of the

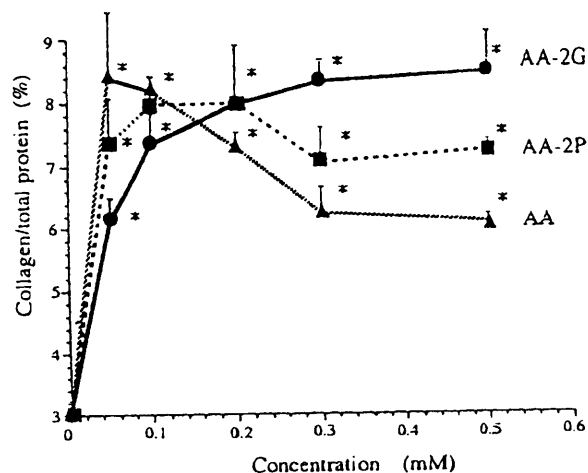


Fig. 2. Effect of Increased Concentrations of AA and Its Derivatives on Collagen Synthesis in Human Skin Fibroblasts

The cells were incubated in 2.0 ml of MEM-10 (minimum essential medium with 10% fetal bovine serum) containing [^3H] proline (148 kBq/dish) and AA, AA-2G or AA-2P (0.05–0.5 mM) for 24 h at 37°C in an atmosphere of 5% CO_2 -air. The relative rate of collagen synthesis to total protein synthesis was determined by the bacterial collagenase method. Points are the means \pm S.D. of triplicate cultures. * Significantly different than value determined in the absence of AA, AA-2P or AA-2G ($p < 0.05$).

supernatant was applied to a silica gel plate, and this was developed with benzene, methanol, acetone and acetic acid in a ratio of 9:9:1:2, respectively. After development of the color with iodine, the silica gel in the fraction (which is equivalent to the R_f value of ascorbic acid) was scraped off and dispersed in a small amount of PBS. The radioactivity in a fixed amount was measured with a scintillation counter.

Statistical Analysis Mean values and standard deviations were analyzed using the Student's *t* test and $p < 0.05$ was taken as indicating significance.

RESULTS

Collagen Synthesis in Human Fibroblasts Figure 2 shows the amount of collagen produced when the vitamin C derivatives were added in various concentrations to a fibroblast culture. Though collagen synthesis was enhanced by the addition of AA and its derivative, AA-2P, this effect was reduced when the amounts added were relatively high, and this occurred at a lower concentration for AA. However, with AA-2G, there was no reduction in the promotion of collagen synthesis.

When the fibroblasts were cultured in the presence of AA or its derivatives, and the amount of collagen produced was measured over time, it was found that collagen production was double that with the control. With AA and AA-2P, the increase in collagen synthesis continued only until the third day and it rapidly decreased thereafter. On the other hand, with AA-2G, the rate of collagen synthesis stayed high throughout the 8 d of the experiment, showing a very different pattern from that of AA and AA-2P (Fig. 3).

In order to examine the cause of the decrease in collagen synthesis after the fifth day of culture when 0.25 mM AA was added, a small amount of AA (0.07 mM) was added to the fibroblasts every day and the collagen content measured. The result is shown in Fig. 4. When AA was added only once, the inhibition of collagen synthesis on the fifth day of the incu-

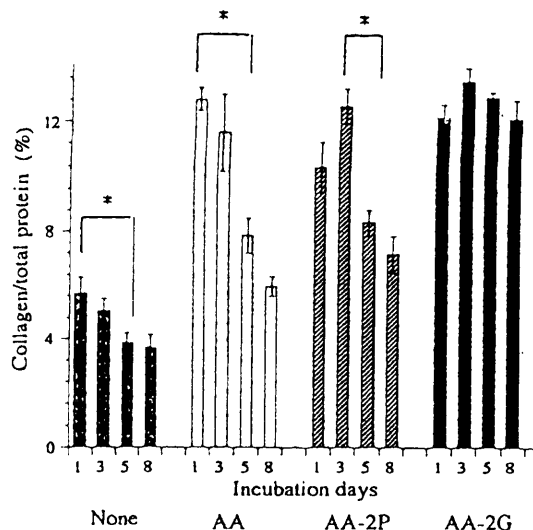


Fig. 3. Longevity of Stimulatory Action of AA and Its Derivatives on Collagen Synthesis in Human Skin Fibroblasts

The cells were incubated in 2.0 ml of MEM-10 containing [³H] proline (148 kBq/dish) and each stimulator (0.25 mM) for 1–8 d. The relative rate of collagen synthesis to total protein synthesis was measured by the bacterial collagenase method. Points are the mean ± S.D. of triplicate cultures. * Significantly different than value in paired group (*p* < 0.05).

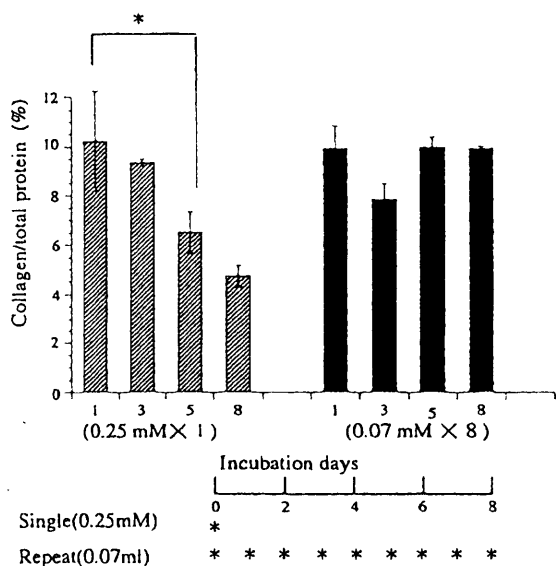


Fig. 4. Effect of Repeated Additions of AA at 24 h Intervals on Collagen Synthesis in Human Skin Fibroblasts

The cells were incubated for 8 d in 2.0 ml of MEM-10 containing [³H] proline (148 kBq/dish). The time schedule for the addition of AA is shown below the figure. A single addition was done at the initial day of culture and repeated additions were done nine times at 12 h intervals. In this experiment, the indicated concentrations of AA were added at every addition. The relative rate of collagen synthesis to total protein synthesis was measured by the bacterial collagenase method. Points are the mean ± S.D. of triplicate cultures. * Significantly different than value in paired group (*p* < 0.05).

tion was observed to be the same as that shown in Fig. 3. When it was added repeatedly, however, the marked inhibition of collagen synthesis was no longer observable by the eighth day of incubation.

The amount of AA in the fibroblasts was then measured over time after ¹⁴C-labelled AA, AA-2P and AA-2G were added to the culture. In the case of AA and AA-2P, the AA content of the fibroblasts was at a maximum on the first day

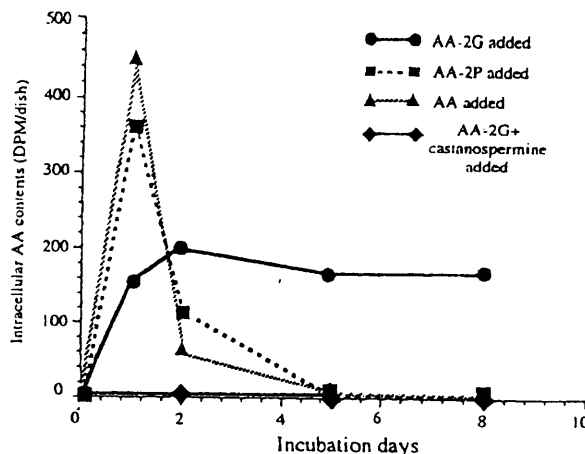


Fig. 5. Intracellular AA Content in Human Skin Fibroblasts

Human skin fibroblasts were cultured in 2.0 ml of MEM-10 in the presence of ¹⁴C-AA, ¹⁴C-AA-2P or ¹⁴C-AA-2G at 37 °C for 8 d in an atmosphere of 5 % CO₂-air. After a fixed period of time, the cell suspension (2 × 10⁶ cells/l) in 0.1 M PBS was sonicated and centrifuged at 4000 rpm (4 °C) for 10 min to obtain a supernatant. All of the supernatant was applied to a silica gel plate, and this was developed with benzene, methanol, acetone and acetic acid in a ratio of 9:9:1:2, respectively. After development of the color with iodine, the silica gel in the fraction (which is equivalent to the R_f value of ascorbic acid) was scraped off and dispersed in a small amount of PBS. The radioactivity in a fixed amount was measured with a scintillation counter.

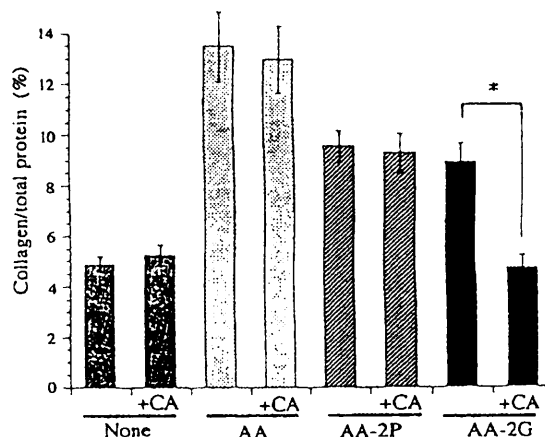


Fig. 6. Abolishment of AA-2G-Induced Collagen Synthesis in Human Skin Fibroblasts by Castanospermine

Cells were incubated in 2.0 ml of MEM-10 containing [³H] proline (148 KBq/dish) and AA, AA-2P or AA-2G (0.1 mM) for 24 h in the presence or absence of castanospermine (0.1 mM) for 24 h. The relative rate of collagen synthesis to total protein synthesis was determined by the collagenase method. Values are the mean ± S.D. of triplicate cultures. * Significantly different than value in paired group (*p* < 0.05).

of incubation and decreased rapidly after that. In the case of AA-2G, however, it was clear that the amount of AA on the eighth day of incubation was at the same level as on the first day (Fig. 5).

The following experiments were carried out with the intention of clarifying that the enhancing effect of AA-2G on collagen synthesis is caused, not by AA-2G, but by AA which is produced by hydrolysis with α-glucosidase existing in the fibroblasts. AA, or the vitamin C derivatives, and castanospermine (an inhibitor of α-glucosidase) were added to the fibroblasts, and the amount of collagen synthesis on the first day of culture was measured. It was found that the addition of castanospermine had no effect on the enhancement of collagen synthesis by AA or AA-2P. On the other hand, this effect for AA-2G was almost completely abrogated in the pres-

ence of castanospermine (Fig. 6).

DISCUSSION

The present study thus demonstrated that the novel vitamin C derivative, AA-2G, promotes collagen synthesis in a human skin fibroblast and that this action is sustained much longer than that with AA and the existing vitamin C derivative, AA-2P. Although Yamamoto *et al.* compared AA-2G with AA for collagen synthesis promotion,¹⁴⁾ they did not compare it with AA-2P, the most commonly used vitamin C derivative, up till now. The primary objective of the present study was therefore to compare AA-2G with AA-2P concerning the promotion of collagen synthesis.

The concentrations of the vitamin C derivatives were varied to see how this would affect the promotion of collagen synthesis. For AA, it was found that collagen synthesis reached a maximum at 0.05 mM but that its promotion tended to drop off when the concentration became higher. Hata *et al.* have reported that the promotion of collagen synthesis and cell proliferation of AA were highest at 0.1 mM and that these effects were less with a concentration of 1.0 mM.¹⁷⁾ This is because in the culture medium AA is easily oxidized to dehydroascorbic acid (an oxidized form of vitamin C), and it is assumed that high AA concentrations are cytotoxic, due to the formation of hydrogen peroxide in this case. Though AA is known to be toxic at high concentrations, of 200 mM, and upwards, there has so far been no report of toxicity at concentrations down to 1 mM. Murakami *et al.* reported that, on measuring the toxicity of AA and AA-2G at 1 mM with respect to fibroblasts, while there was absolutely no toxicity with AA-2G, AA showed very high toxicity.¹³⁾ However, there has been no report of any toxicity at such a low concentration as 0.3 mM, the one used in our study. We therefore assume that the reduction in the collagen synthesis-promoting action observed when AA and AA-2P were added at a concentration of 0.3 mM is due to their stability in the culture medium rather than toxicity.

Studies by Hata *et al.*⁶⁾ and Yamamoto *et al.*¹⁸⁾ have already shown that AA is unstable in culture media, whereas Hata *et al.* reported AA-2P to be stable.⁶⁾ They dissolved AA and AA-2P in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at a concentration of 10 mM. After sterilizing by filtration, they kept the samples in capped tubes in order to determine the stability. At 37 °C, not more than 50% of the AA remained after 1 d and, after 3 d, the detectable amount was insignificant. On the other hand, 85% of the AA-2P remained after 1 week.

In the present study, we added AA, AA-2P and AA-2G to Eagle's medium containing 10% fetal bovine serum, and inoculated it with fibroblasts after sterilizing it by filtration. The media were then cultured at 37 °C in an atmosphere containing 5% CO₂, and the fibroblast AA content was measured. In the case of AA and AA-2P, the AA content was at a maximum on the first day of the culture. On the second day, however, it was close to zero. For AA-2G, the fibroblast AA content was maintained at virtually the same level from day 1 to day 8 of the culture.

Drawing a conclusion from these results and those obtained by Hata *et al.*⁶⁾ AA-2P is decomposed in a short time to AA by the alkaliphosphatase in the fibroblasts and, as

pointed out by Hata *et al.*,⁶⁾ this AA is unstable in the culture medium so it is assumed that because of this the AA disappears in a short time. The experimental result that, when AA was added in small amounts every day, there was no reduction in the collagen synthesis-promoting action, even on the 8th day, is considered to back up this assumption.

Thus, when the collagen synthesis-promoting action was studied in a prolonged culture, AA and AA-2P were found to be effective until the 3rd day but, after that, the amount of collagen synthesis dropped off rapidly. In contrast, the collagen synthesis-promotion action of AA-2G was sustained, and it was still effective in this respect on the 8th day. This action tended to closely follow the fibroblast AA content, strongly suggesting the possibility that AA-2G's collagen synthesis-promotion action arises from the action of AA derived from it through hydrolysis by α -glucosidase. This was tested by adding the α -glucosidase inhibitor castanospermine as well as AA-2G, in which case the collagen synthesis-promoting action was observed to virtually cease altogether.

It has been reported that AA-2G itself is unlikely to be absorbed through the intestine, considering its poor transport into the cells.¹⁹⁾ In the present study, the presence of AA-2G in the cultured fibroblasts could not be confirmed, but instead, the intracellular amount of AA was observed to increase. Therefore, to exhibit its vitamin C activity, AA-2G needs to be hydrolyzed before membrane transport. In fact, the addition of an α -glucosidase inhibitor in the culture medium completely abolished the stimulatory effect of AA-2G. Because the existence of α -glucosidase in bovine serum has been reported,²⁰⁾ we determined the stability of AA-2G in a culture medium containing fetal bovine serum. However, the participation of such a serum-derived enzyme can be ruled out, because AA-2G was not hydrolyzed under these conditions. From these findings, we assume the α -glucosidase bound to fibroblast cell membranes and was involved in the hydrolysis of AA-2G.

Figure 5 shows the results of culturing for 5 d with AA-2P. Notwithstanding the observations that virtually no AA was detectable in the fibroblasts, and the collagen synthesis-promoting action was decreasing, the results indicate that only an extremely small amount of AA is used for collagen synthesis in cells.

Blanck and Peterkofsky have reported that AA is active even at very low concentrations.²¹⁾ They observed that, in guinea pigs kept on an AA deficient diet, the hydroxylation of proline was proceeding unaffected despite the extremely low AA concentration. Yamamoto *et al.* have suggested the possibility that AA is recycled in cell enzymatic reactions.¹⁴⁾

In the present study, we have demonstrated that a novel vitamin C derivative, AA-2G, has a collagen synthesis promoting action in a human skin fibroblast and that a major feature of AA-2G is that this action is sustained over a long period of time.

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