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Design and efficient synthesis of novel ascorbyl conjugated peptide with high collagen biosynthesis stimulating effects

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ABSTRACT

Collagen is critical for skin strength and elasticity, and its degradation leads to wrinkles that accompany aging. Based emphasis on the aesthetics, we tried to make a new compound that can highly stimulate collagen biosynthesis and synthesized ascorbyl conjugated peptide that is a complex form connected by succinoyl linker. We conducted several in vitro and in vivo experiments to identify if the compound has a potent activity, comparing to the ascorbic acid only for collagen biosynthesis. Our in vitro and in vivo result identified that ascorbyl conjugated peptide can stimulate collagen biosynthesis in human dermis and is assumably stable in the rat skin extracts. In conclusion, we strongly suggest that ascorbyl conjugated peptide can be used as a main ingredient for cosmetic products as well as wound healing agents.

Collagen is one of the long, fibrous proteins in structural connective tissues. Bundles of collagen and collagen fibers are major components of the extracellular matrix that serve many functions, such as providing support and anchorage sites for cells and separating tissues from one another. Therefore, the amount of collagen plays a critical role in determining the natural aging and photoaging. It has been known that the amount of collagen decreases by 10–50% from the age of 30 to 80.[1–4] With the finding that activated collagen synthesis in the body increases the dermal matrix, resulting in the effects of improving wound healing, skin elasticity, wrinkle reduction, etc., collagen has been utilized in the nutrient supplements and therapeutic agents. Some of oligopeptides having less than 10 amino acids, which exist in collagen, can be the smallest activation units which relate to functions as messenger, stimulator, and neurotransmitter, and take part in such physiological activities as growth control, nursing, immunity, digestion, blood pressure, and healing. Particularly, effective peptides in skin regeneration are disclosed.[5] For example, a tri-, tetra-, or hexa-peptide selected from the group consisting Gly-Lys-His, Gly-His-Lys, Gly-Pro-Hyp(hydroxyproline), and Glu-Glu-Met-Gln-Arg-Arg promotes the synthesis of collagen and glycosaminoglycan in the dermis, thereby increasing moisture retention ability and elasticity of the dermis and improving wrinkle problems. In 1993, Katayama et al. demonstrated that a penta-peptide from type I procollagen promotes extracellular matrix production in the human lung fibroblasts.[6] The group observed that 80% of an enhancement in the collagen production was found with penta-peptide Lys-Thr-Thr-Lys-Ser alone, which is the minimal size necessary for stimulating ECM biosynthesis.[7]

However, typically peptides tend to be cleared from the bloodstream from minutes to hours by enzymatic digestion, thereby greatly reducing sufficient effects. Therefore, the development of peptide with improved safety and stability and superior skin permeability are needed. Peptides can be modified in many ways to prevent degradation by endopeptidases and exopeptidases. These include acetylation and glycosylation at N-terminals or amidation at C-terminals and the use of unnatural amino acids at specifically labile sites within a complete peptide.[8,9] Besides modifications above, peptides can be also engineered with incorporation of D-amino acids and cyclization, which reduce the conformational flexibility of linear peptides and substantially increase the stability against the proteolysis.[8] Especially palmitated penta-peptide, a major ingredient of Matrixyl™ from Sederma, is one of the most famous materials that succeeded in enhancing its stability and permeability by using lipid molecules. With the respect to the efficient synthesis, solubility, and original activity, we selected the ascorbic acid as a coupling agent to penta-peptide, which has been known to have strong anti-oxidative activity, collagen biosynthesis, and whitening effects. Moreover, we could expect that the activity of collagen production would be improved by dual effects of ascorbic acid and peptide in HDF (human dermal fibroblast) cells. In this letter, regarding these facts, we have designed an ascorbyl conjugated penta-peptide (Fig. 1) Lys-Thr-Thr-Lys-Ser identified by Katayama et al. In order to attach ascorbic acid to penta-peptide, we introduced a succinoyl linker between two molecules. We described

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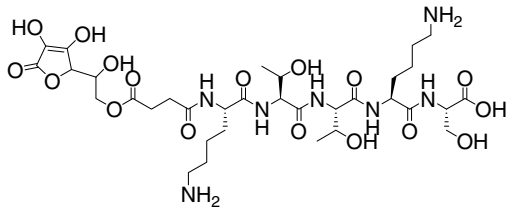
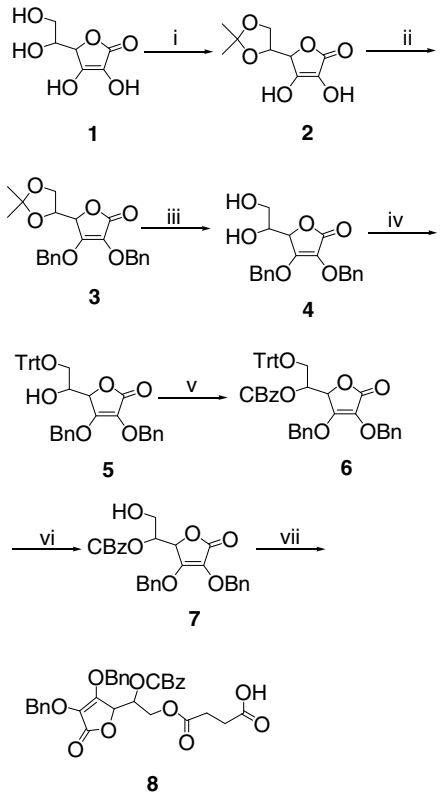


Figure 1. Structure of ascorbyl conjugated peptide **11**(ACP **11**).

the synthetic progress of ascorbyl conjugated peptide **11** (ACP **11**) and were able to recapitulate its biological activity in HDF cells and stability in the rat skin extracts, comparing to the original activity of ascorbic acid and penta-peptide alone.

There are three parts of synthesis in ACP **11**. The first part is the synthesis of succinoyl ascorbate **8**. Second part is peptide synthesis in solid phase chemistry. And third part is the conjugation of succinoyl ascorbate and peptide.

Scheme 1 shows the synthesis of succinoyl ascorbate in part I. To begin with, original vitamin C must be blocked for linking between vitamin C and peptide at a carbon position 5 or 6. First acetal **2** was synthesized by reaction with acetyl chloride, which was alkylated to obtain the 2,3-di-*O*-protected derivative. After cleavage of the isopropylidene-protecting group, tritylation was carried out using trityl chloride to produce compound **5**. Succinoyl ascorbate **8** was synthesized by protection and deprotecion step of compound **5** followed by acylation of 6-OH using succinic anhydride.



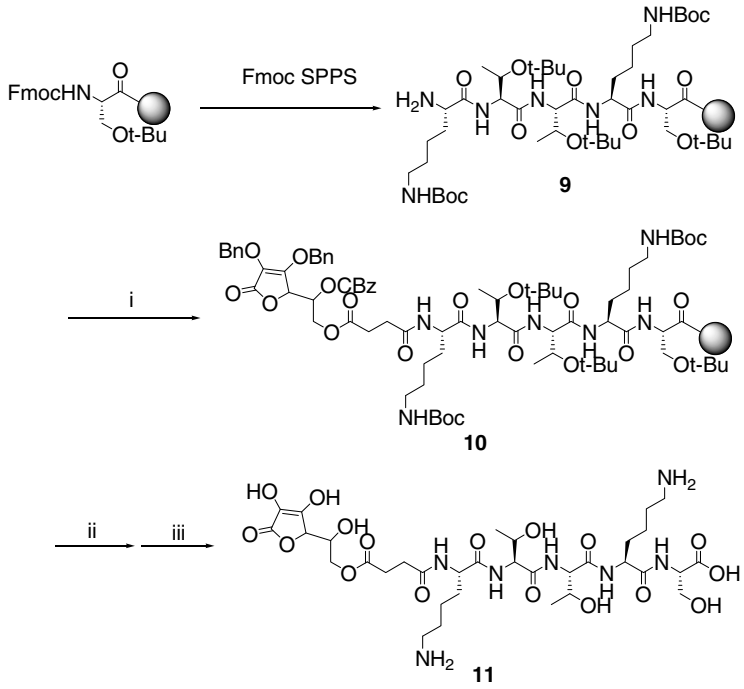
Scheme 1. Reagents and conditions: (i) L-ascorbic acid, acetone, acetyl chloride (60%); (ii) K_2CO_3, DMF, BnBr, 50 °C (90%); (iii) 3 N HCl, THF, 50 °C (90%); (iv) trityl chloride, CH_2Cl_2, TEA (90%); (v) carbobenzyloxy chloride, dimethylaminopyridine, CH_3CN (80%); (vi) 1.5 N HCl, CH_3CN, 50 °C; (vii) succinic anhydride, DMF, K_2CO_3 (2 steps, 80%).

The linear peptide KTTKS was synthesized on 2-chlorotrityl resin preloaded with Fmoc-serine using standard Fmoc chemistry employing HBTU/HOBt as coupling reagents. To the peptide synthesized, coupled up to a N-terminal amino acid, a 20% (piperidine/*N*-methylpyrolidone) solution was added to remove the Fmoc groups. Then, the peptide was washed with *N*-methylpyrolidone and dichloromethane, and coupled with succinoyl ascorbate **8** derivative synthesized in Scheme 1. The resulting peptide-coupled vitamin C derivative was incubated in a special cleavage cocktail to remove the peptide protection groups. Eliminating the benzyl groups protecting the alcohol groups at carbon positions 2 and 3 of vitamin C affords a peptide-coupled vitamin C derivative **11** (Scheme 2).[10]

In addition to synthesis of ACP **11**, it is also important to identify the original activity of ECM production for the development of cosmetic materials. We examined the effect of ACP **11** in collagen biosynthesis by using primary cultured neonatal human dermal fibroblasts (HDFs). The quantity of collagen produced by HDFs was measured by the modified method described by Martens, Gut, 1992, 33, 1664–1670. In case of the cells not treated with any test substance, their collagen levels were designed '100%'. This assay will be described in detail below.

HDFs were seeded onto 96-well plates at the number of 3000 cells and grown in DMEM containing 5% fetal bovine serum (Invitrogen, Maryland, USA) for 24 h. After changing with fresh assay media including 0.1% FBS, test samples were treated into HDFs to stimulate the collagen production. After incubation for 72 h, a bit of supernatant was added into the plate which is coated with human collagen type I antibody. Then, the plate was placed at room temperature for 2 h to allow the reaction of antibody and antigen. The plate was washed with phosphate buffered saline with 0.5% Tween 20 three times to remove unbound collagen, and then biotin-labeled human collagen type I antibody was added into each well. About an hour later at room temperature, streptavidin–horseradish peroxidase (St. Louis, Sigma) was treated into each well to detect biotin-labeled antibodies bound to type I collagen. The quantity of collagen in the plate was transformed into OD value by treating tetramethylbenzidine (St. Louis, Sigma), as a substrate of HRP. The reaction HRP and TMB were stopped by 1 N HCl, and then OD value was measured at 450 nm. As shown in Figure 2, expectedly ACP **11** was found to have the strong stimulatory activity on collagen synthesis than ascorbic acid alone at every concentration. Actually, we could notice that the cytotoxicity of Pal-KTTKS was found at more than 100 μM of concentration, even if it had the highest collagen inducing effects at 100 μM of concentration.

In this experiment, the ACP **11** was synthesized efficiently and showed a high activity in collagen biosynthesis from 1 to 1000 μM concentration, comparing others. According to the experimental results in vitro, the conjugation form with ascorbic acid and penta-peptide has better abilities for stimulating collagen biosynthesis than any others. Although ACP **11** is potentially effective in collagen biosynthesis, it does not clearly demonstrate whether the linker is required to induce collagen biosynthesis in human fibroblast cells. To further access about the linker effect of ACP **11**, we treated each of ascorbic acid and penta-peptide(KTTKS) into human fibroblast cells. As shown above the data, each of ascorbic acid and penta-peptide, which is a loss of linker, is not so much significant active as ACP **11**. This result can indicate that the linker is indispensible to make the strong collagen expression in human fibroblast cells. However, we could not clarify how the chemical works in stimulating collagen biosynthesis at the cell based system still. One possibility that can explain why ACP **11** showed the highest collagen synthetic effect at every concentration is that the synergic effects of linkage between ascorbic acid and penta-peptide would contribute to the collagen biosynthesis as well as the



Scheme 2. Reagents: (i) **8**, HBTU, HOBt, DIPEA, DMF; (ii) TFA/thioanisole/H_2O/triisopropylsialne (92.5:2.5:2.5:2.5 (v/v)); (iii) 10% Pd/C, H_2.

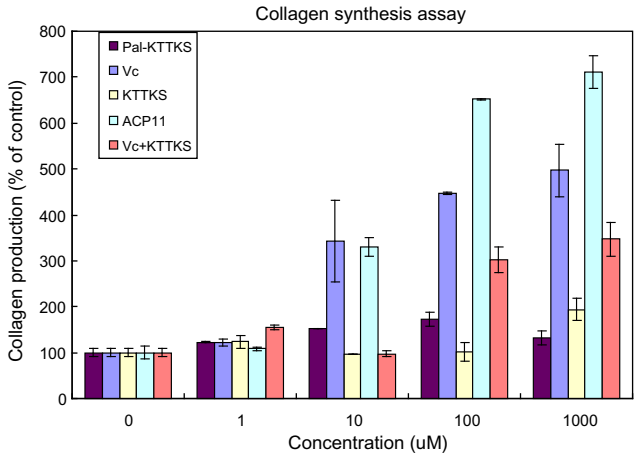


Figure 2. Collagen biosynthesis induced by ascorbyl conjugated peptide **11** (ACP **11**). Equal amounts of human normal fibroblast cells were seeded in 96-well plates including 0.1% FBS, and were treated with various concentrations of sample. After 72 h incubation, cells were collected and tested using ELISA ($p < 0.01$).

increased stability of ACP **11** from peptidases. Thus, we additionally examined the stability of ACP **11** using rat skin extracts indirectly as an ex vivo test. In this stability test, we observed ACP **11** appear to be maintained for more than 6 h, comparing to the penta-peptide. It is thus possible that ACP **11** has more stable and highly effective in the collagen biosynthesis than each of penta-peptide and ascorbic acid. Since many peptides have had troubles in solving the problem of skin permeation because of the rapid degradation by proteases and the hydrophilic properties, we thought ACP **11** was required to be tested for its stability. To give an answer to this, we just followed the method from the Yamamoto group.[11] After preparing for the rat skin with hair-clipping step, it was cut into small pieces. Ten percent homogenates were made with PBS by homogenizing the skin, using a POLYTRON homogenizer (Kinematica, GmbH, Switzerland). After centrifuged for 5 min at 3000g at 4 °C, the supernatant was required to have one more centrifuge step to get pure supernatant for 30 min at 20,000g at 4 °C. Then, the supernatant was collected and adjusted with PBS to a protein concentration of 4 mg/ml determined by the method of Lowry et al.[12] Test samples at the indicated concentrations were added into the 4 mg/ml of homogenate solution by 1–1 v/v ratio. The mixture was preincubated at 37 °C for 10 min, and then 50 μl of the sample was taken out of the whole mixture at every 10 min. Each of samples was treated with 100 μl of methanol to stop the reaction and centrifuged for 5 min at 10,000g at 4 °C for the removal of precipitated proteins and the analysis using HPLC as well.

In Figure 3, we could observe ACP **11** was maintained for more than 6 h, even if its content was less than 10%. The HPLC peak of penta-peptide, however, was lost in the mixed sample and even ascorbic acid could not be detected because of its instability (data not shown). Based on the result, we are not a hundred percent sure, but we can expect ACP **11** is more stable than others, because the linkage of peptide and ascorbic acid could be protected from some kinds of proteases in the rat skin extracts.

In conclusion, here we demonstrated that ACP **11** has the strong effects in collagen biosynthesis and is significantly stable in the rat

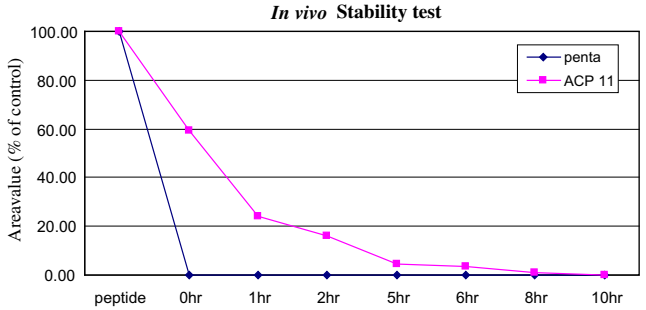


Figure 3. Stability test of ascorbyl conjugated peptide **11** (ACP **11**) in rat skin extracts. Each of samples was analyzed by a Shimadzu reversed phase HPLC in Thermo Hypersil Gold C18 packed columns (250 × 4.6 mm, 5 μm sized particle) using a water (0.1% TFA) acetonitrile (0.1% TFA) gradient.

skin extracts. For the further study, ACP **11** was needed to check the level of permeation in the in vivo model for the industrial field. Besides ACP **11** was required to verify what the mechanism is or how it works in collagen biosynthesis for the scientific filed. Finally, we hopefully want to suggest that ACP **11** can be used as a therapeutic agent for the wound healing as well as functional materials for cosmetics, when the intracellular mechanism of this chemical is defined.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.10.112.

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10. *Synthesis of compound **11**:* The dried product **10** (100 mmol) was incubated in a mixture of trifluoroacetic acid: thioanisole/water/triisopropylsilane at a ratio of 92.5:2.5:2.5:2.5 (v/v) for 3 h to remove the peptide protection groups and resin. Removed resin by filtration and the reaction mixture was precipitated with ether, was collected by filtration, washed with ether and dried in vacuo. The precipitated peptide was treated with 10% Pd/C (20 g) in methanol with stirring for 4 h at room temperature under a hydrogen atmosphere. Thereafter, the solution was filtrated over Celite to remove the Pd/C, concentrated under pressure, and purified by passage through reverse phase high performance liquid chromatography using a isocratic of 0.1% TFA in 5% acetonitrile, freezing dried to give **11** (41 g, 50% 2 step yield) ^{1}H NMR (CD_3OD): 4.78 (m, 3H), 4.31 (m, 10H), 3.90 (m, 2H), 2.96 (q, 4H), 2.65 (m, 4H), 1.92 (m, 2H), 1.74 (m, 6H), 1.52 (m, 4H), 1.23 (q, 6H). MS *m/z* 822.3 (M^+).
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