Synthesis and Characterization of 6-*O*-Acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic Acids with a Branched-acyl Chain

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We previously reported the chemical synthesis of a series of novel monoacylated vitamin C derivatives, 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acids (6-Acyl-AA-2G) possessing a straight-acyl chain of varying length from C_4 to C_{18}, as effective skin antioxidants. In this paper, we describe branched type of 6-Acyl-AA-2G derivatives (6-bAcyl-AA-2G) synthesized by use of a 2-branched-chain fatty acid anhydride as an acyl donor. The stability of 6-bAcyl-AA-2G in neutral solution was much higher than that of 6-Acyl-AA-2G, while they were susceptible to enzymatic hydrolysis for exerting vitamin C effect. These branched derivatives as well as 6-Acyl-AA-2G increased the radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl and the lipophilicity in octanol/water-partitioning systems with increasing length of their acyl group. In addition, the 6-bAcyl-AA-2G derivative with an acyl chain of C_{12}, 6-bDode-AA-2G had the excellent solubility to various solvents, suggesting easy handling in cosmetic use. These characteristics of 6-bAcyl-AA-2G may be available for skin care application as an effective antioxidant.

Key words 6-acyl ascorbic acid 2-glucoside; lipophilic ascorbate; stable ascorbate; branched-acyl chain; solubility; skin application

A stable ascorbate derivative, 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G), developed in our laboratory[1—4] has already been utilized as a medical additive in the cosmetic field. This stable hydrophilic vitamin C derivative exhibits vitamin C activity *in vitro* and *in vivo* after enzymatic hydrolysis to ascorbic acid (AA) by α-glucosidase.[5—9] Moreover, we have succeeded in the chemical synthesis of a series of monoacylated AA-2G with an efficient transdermal activity in relatively good yields.[10] The monoacyl AA-2G derivatives were identified as 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acids (6-Acyl-AA-2G) possessing a straight-acyl chain of varying length from C_4 to C_{18}. 6-Acyl-AA-2G was also synthesized by protease-catalyzed regioselective acylation in pyridine.[11] Some of them with an appropriate length acyl chain group exhibited satisfactory stability[10] and radical scavenging activity.[12,13] 6-Acyl-AA-2G was susceptible to enzymatic hydrolysis by mammalian tissue esterase and/or α-glucosidase to produce AA-2G and AA.[10,14]

Extremely high stability in the long-term storage is required in the case of the application of a cosmetic material. 6-Acyl-AA-2G was shown to be very stable in a neutral solution, although its stability was less superior to that of AA-2G.[10] The insufficient stability may be ascribed to the hydrolysis of the ester bond at the C-6 hydroxyl group of AA. Branched fatty acid esters such as cetyl 2-ethylhexanoate, glyceryl tri-2-ethylhexanoate and glyceryl triisopalmitate, which are excellent in the hydrolytic stability, are widely utilized as cosmetic materials.

In this paper, we describe the synthesis and characterization of the improved monoacyl vitamin C derivatives, 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acids possessing a 2-branched-acyl chain. The properties of the derivatives are evaluated by the stability in neutral solution, radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH), susceptibility to enzymatic hydrolysis, partitioning behavior in *n*-octanol/buffer systems, and solubility in a wide variety of solvents.

Results and Discussion

Synthesis of 6-bAcyl-AA-2G Monoacylated ascorbic acid derivatives with high stability in the long-term storage were developed from a requirement for cosmetic application.

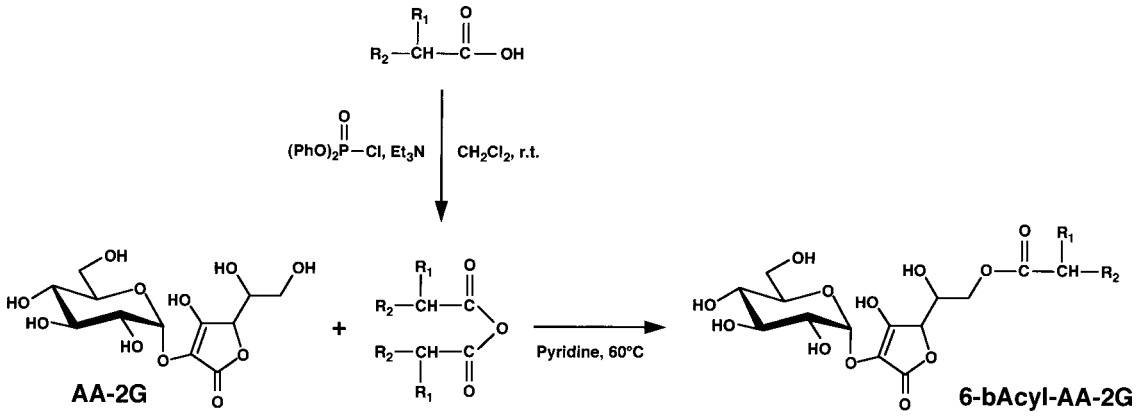

Chart 1

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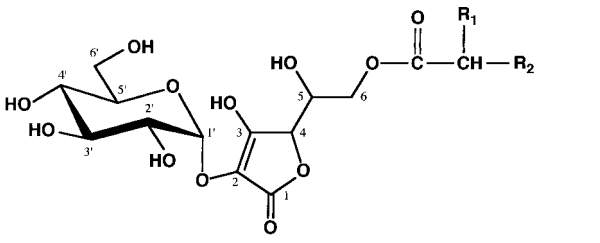

6-Acyl-AA-2G

$R_1 = H$, $R_2 = CH_2(CH_2)_2CH_3$: 6-Hexa-AA-2G
$= CH_2(CH_2)_4CH_3$: 6-Octa-AA-2G
$= CH_2(CH_2)_8CH_3$: 6-Dode-AA-2G

6-bAcyl-AA-2G

$R_1 = R_2 = CH_2CH_3$: 6-bHexa-AA-2G
$= CH_2CH_2CH_3$: 6-bOcta-AA-2G
$= CH_2(CH_2)_3CH_3$: 6-bDode-AA-2G

Fig. 1. Structure of 6-*O*-Acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic Acids with a Straight- or Branched-Acyl Chain

We expected that increasing the substitution at the α position to the carbonyl in the acyl moiety might inhibit the deacylation. 2-*O*-α-D-Glucopyranosyl-L-ascorbic acid (AA-2G) was coupled with acid anhydrides with various branched-chain lengths in pyridine to give 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid having a 2-branched-acyl chain (6-bAcyl-AA-2G) as shown in a Chart. They were identified as 6-*O*-(2-ethylbutyryl)-2-*O*-α-D-glucopyranosyl-, 2-*O*-α-D-glucopyranosyl-6-*O*-(2-propylpentanoyl)- and 2-*O*-α-D-glucopyranosyl-6-*O*-(2-pentylheptanoyl)-L-ascorbic acid (abbreviations: 6-bHexa-AA-2G, 6-bOcta-AA-2G, and 6-bDode-AA-2G, respectively) by UV spectra, elemental analyses and nuclear magnetic resonance spectroscopy (Fig. 1). In order to avoid the possibility of different biological effects by the enantiomer, an acyl group that has the same length of aliphatic groups attached to the C-2 was employed as a model for a 2-branched-acyl chain. 6-bAcyl-AA-2G had superior yields when the molar ratio of AA-2G to acid anhydrides was essentially a 1 : 2 ratio, while the synthesis of 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid having a straight-acyl chain (6-Acyl-AA-2G, Fig. 1) needed only 1.2 equivalent of acid anhydrides.[10] The detailed examination of molar ratio permitted producing 6-bAcyl-AA-2G as a major product, although monoacylation without the use of any protective group was seemed to be difficult. However, the reason that one hydroxyl group at the C-6 of AA was regioselectively acylated from two primary hydroxyl groups of the whole molecule is unclear.

Stability of 6-bAcyl-AA-2G in Aqueous Solution The stability of 6-bAcyl-AA-2G in a neutral aqueous solution (10 mM) at 50 °C was evaluated on the basis of remaining ratio measured by HPLC (Fig. 2). AA-2G, the deacylation form from 6-bAcyl-AA-2G was also analyzed by HPLC. It was found that 82—84% of 6-bAcyl-AA-2G remained intact after 28 d (Fig. 2a). 6-bAcyl-AA-2G sharply decreased during the first one day, after which was kept approximately constant. The difference in stability among their derivatives was not observed. The very small releasing amounts (1—2%) of AA-2G indicated that 6-bAcyl-AA-2G avoided the hydrolytic cleavage of the ester bond at the C-6 of AA (Fig. 3). On the other hand, 61—68% of 6-Acyl-AA-2G was found to remain intact after 28 days (Fig. 2b). Although the significant difference in stability among their derivatives was not observed, the remaining amount of 6-Acyl-AA-2G tended to increase with increasing length of their acyl group. A major

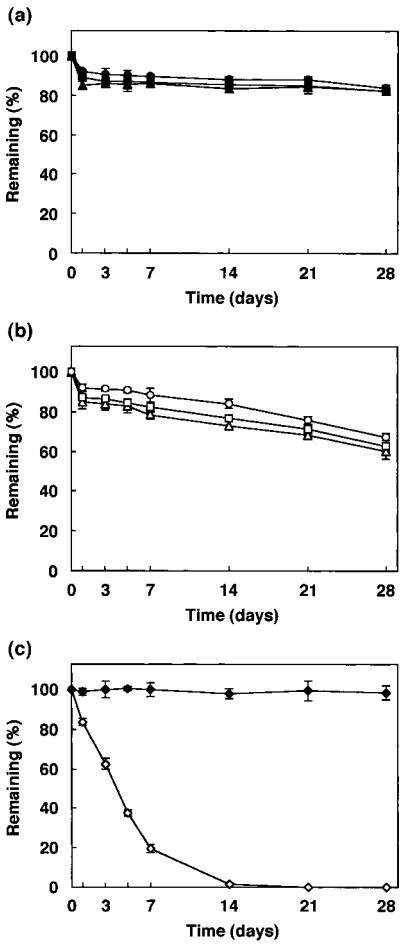

Fig. 2. Stability of 6-bAcyl-AA-2G (a) and 6-Acyl-AA-2G (b) in Aqueous Solution

Ascorbic acid (c, ◇), AA-2G (c, ◆), 6-Hexa-AA-2G (△), 6-bHexa-AA-2G (▲), 6-Octa-AA-2G (□), 6-bOcta-AA-2G (■), 6-Dode-AA-2G (○) and 6-bDode-AA-2G (●) were dissolved in 100 mM potassium phosphate buffer (pH 6.5) to give 10 mM solution, and then incubated at 50 °C for the indicated time. The concentration of the test compounds was analyzed by HPLC. Each value represents the mean±S.D. (n=6).

degradation product of 6-Acyl-AA-2G was found to be AA-2G (Fig. 3). AA-2G was scarcely degraded during the tested period, while AA disappeared after 21 d (Fig. 2c). These results indicate that 6-bAcyl-AA-2G was more stable than 6-Acyl-AA-2G. However, the deacylation (AA-2G release) profile of 6-bAcyl-AA-2G as shown in Fig. 3 was in conflict with the initial remarkable decrease of the intact form as shown in Fig. 2a. The similar results were observed in 6-Acyl-AA-2G (Figs. 2b, 3). A new peak, with the remarkable decrease, was observed by HPLC analysis, and the peak area ratio of 6-bAcyl-AA-2G (or 6-Acyl-AA-2G) to the new compound was kept approximately constant through the experiment (data not shown). It is difficult to suppose the unidentified compound as 6-*O*-acyl AA, because the remaining amount of AA-2G revealed the high stability of the ether linkage at the C-2 of AA. Intramolecular acyl migrations have been previously reported to occur for ascorbic acid esters,[15,16] and they are well-known reactions of glycerol esters.[17] Thus it seems likely that 6-bAcyl-AA-2G underwent an intramolecular acyl migration to yield the isomer and was in equilibrium with the isomer within one day. Therefore, these findings lead to a conclusion that 6-bAcyl-AA-2G had a good ability of avoiding the hydrolysis comparable to that

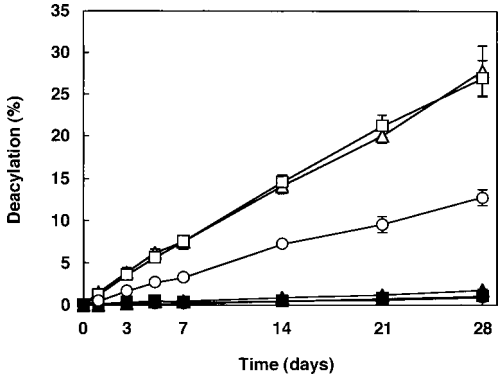

Fig. 3. Release of AA-2G from 6-bAcyl-AA-2G and 6-Acyl-AA-2G by the Hydrolytic Cleavage of the Ester Bond

The deacylation of 6-Hexa-AA-2G (△), 6-bHexa-AA-2G (▲), 6-Octa-AA-2G (□), 6-bOcta-AA-2G (■), 6-Dode-AA-2G (○) and 6-bDode-AA-2G (●) was determined by measurement of AA-2G release. This experiment was carried out with the experiment on Fig. 2. The concentration of AA-2G was analyzed by HPLC. Each value represents the mean±S.D. (n=6).

Table 1. Radical Scavenging Activity of 6-bAcyl-AA-2G against DPPH[a)]

Sample	EC_{50} ($\times10^{-5}$ M)
AA	2.4
AA-2G	4.5
6-Hexa-AA-2G	3.7
6-Octa-AA-2G	3.6
6-Dode-AA-2G	3.3
6-bHexa-AA-2G	3.7
6-bOcta-AA-2G	3.6
6-bDode-AA-2G	3.1

a) After the reaction was carried out at 25 °C for 20 min, the decolorization of DPPH was measured at 516 nm. The EC_{50} value was determined as the concentration of each sample required to give 50% of the absorbance shown by a blank. Each EC_{50} value represents the mean of three separate experiments.

of AA-2G under the nonenzymatic condition.

Radical Scavenging Activity of 6-bAcyl-AA-2G A model for scavenging the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) has been used extensively to predict the antioxidant activities. The radical scavenging activity of AA derivatives has been estimated by the colorimetric method using DPPH.[18—20)] We previously showed that 6-Acyl-AA-2G was slightly superior as a radical scavenger to AA-2G, although the scavenging activity was lower than that of AA.[10,12)] Stoichiometric evaluation also indicated that the quantity of radicals quenched by AA-2G and 6-Acyl-AA-2G was superior to that of AA in a long-term reaction.[13)]

Whether the branched-acyl group affects the reactivity with DPPH radical was investigated. Table 1 shows the 50% effective concentration (EC_{50}) calculated from the dose response curve of radical scavenging activity. The EC_{50} of each 6-bAcyl-AA-2G ($3.1—3.7\times10^{-5}$ M) was smaller than that of AA-2G (4.5×10^{-5} M), indicating that 6-bAcyl-AA-2G is more efficient than AA-2G in terms of the radical scavenging potency. The scavenging activity of both 6-Acyl-AA-2G and 6-bAcyl-AA-2G tended to increase with increasing length of their acyl group. In addition, the values of 6-bHexa-, 6-bOcta-, and 6-bDode-AA-2G were nearly identical to those of 6-Hexa-, 6-Octa-, and 6-Dode-AA-2G, respectively. These results indicate that the conversion of a straight-acyl chain into a branched-acyl chain had no effect on the radical scavenging.

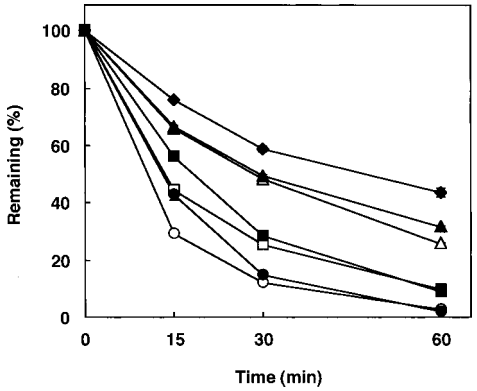

Fig. 4. Hydrolysis of 6-bAcyl-AA-2G and 6-Acyl-AA-2G by Rice Seed α-Glucosidase

Each sample (1.0 mM) of AA-2G (◆), 6-Hexa-AA-2G (△), 6-bHexa-AA-2G (▲), 6-Octa-AA-2G (□), 6-bOcta-AA-2G (■), 6-Dode-AA-2G (○) and 6-bDode-AA-2G (●) was incubated with rice seed α-glucosidase (1.0 unit/ml) at 37 °C for the indicated time. Remaining amount of the derivatives was determined by HPLC analysis. Each value represents the mean±S.D. (n=3).

Susceptibility of 6-bAcyl-AA-2G to Enzymatic Hydrolysis Ascorbic acid 2-phosphate (AA-2P) and ascorbic acid 2-sulfate (AA-2S) are well known as stable derivatives. AA-2P was demonstrated to exert an antiscorbutic activity in guinea pigs and rhesus monkeys, and the activity is explained by phosphatase-catalyzed hydrolysis to AA.[21,22)] However, AA-2S was devoid of a substantial vitamin C activity.[23,24)] The oral administration of AA-2P in rats increased the serum AA levels, but the treatment of AA-2S caused only a slight increase.[5)] In addition, ascorbic acid 2-*O*-methyl ether without enzymatic degradation had no vitamin C activity.[25)] In contrast, 6-bromo-6-deoxy-L-ascorbic acid possessed an antiscorbutic activity in guinea pigs.[26)] These facts demonstrate that the free 2- and 3-enolic hydroxyl groups of AA are essential to exert the antiscorbutic activity.

In order to get the free 2- and 3-enolic hydroxyl groups of AA, the hydrolysis by α-glucosidase of 6-bAcyl-AA-2G was investigated. Rice seed α-glucosidase, which effectively released AA from AA-2G in our previous research,[2)] was used in this experiment. Hydrolysis of 6-bAcyl-AA-2G to 6-*O*-acyl AA was determined by HPLC analysis. The remaining amounts of 6-bAcyl-AA-2G were the same levels as those of 6-Acyl-AA-2G (Fig. 4). These results indicate that the conversion of a straight-acyl chain into a branched-acyl chain had no effect on the hydrolysis by α-glucosidase. The hydrolytic cleavage rate of AA-2G was less than that of 6-Acyl- and 6-bAcyl-AA-2G. In addition, the susceptibility to α-glucosidase of 6-Acyl- and 6-bAcyl-AA-2G tended to increase with increasing length of their acyl group. The increased susceptibility seems to result from the high affinity of α-glucosidase for their derivatives with increasing length of their acyl group. 6-Acyl-AA-2G was hydrolyzed with mammalian tissue esterase and/or α-glucosidase to produce AA-2G and AA.[10,14)] In contrast, 6-bAcyl-AA-2G was predominantly hydrolyzed to 6-*O*-acyl AA with tissue homogenates from guinea pigs (data not shown), suggesting that the prevented enzymatic degradation of the ester bonds was attributed to the steric hindrance by the 2-alkyl groups of their acyl moieties. It was reported that 6-*O*-palmitoyl ascorbate stimulated collagen synthesis in cultured human fibroblasts at lower

doses than does AA.[27] Uesato *et al.* reported that some of 6-*O*-acylated ascorbic acids possessing a straight- and a branched-acyl chain displayed the marked anti-tumor promoting activities.[28] It appears that the 6-*O*-acylated AA derivatives efficiently penetrated into the hydrophobic region of lipid bilayer to exert the activity. Thus, various physiological and pharmacological actions of 6-bAcyl-AA-2G could be effectively elicited by the hydrolysis not to AA but to 6-*O*-acyl AA.

In addition, the intact form of 6-bAcyl-AA-2G possessed the radical scavenging activity as described above, while the radical scavenging activity of ascorbyl 2,6-dipalmitate and AA-2P is extremely weak.[10] Ascorbyl 2,6-dipalmitate and AA-2P are relatively stable *in vitro* and exhibit antioxidative activity after enzymatic hydrolysis to AA by esterase, or phosphatase *in vivo*. Therefore, the prominent effectiveness of 6-bAcyl-AA-2G as an antioxidant may be shown not only by the fact that the intact form possessed the radical scavenging activity, but also by the possibility that 6-bAcyl-AA-2G seems to be enzymatically metabolized and to play an important part in antioxidation as AA or 6-*O*-acyl AA *in vivo*. These results indicate that 6-bAcyl-AA-2G may be usable as an effective pharmacological agent and as a promising antioxidant *in vivo*.

Partitioning Behavior of 6-bAcyl-AA-2G in *n*-Octanol/Buffer Systems The partition between *n*-octanol and water is generally accepted to be a suitable model for studying affinity on cell membranes or on the corneal layer of epidermis.[29] Our previous research showed that partitioning behavior in an *n*-octanol/buffer system agreed with skin permeability in a human skin model.[10] The lipophilicity of 6-bAcyl-AA-2G was investigated with an *n*-octanol/10 mM sodium acetate buffer (pH 5.0) system at 37 °C, because the acidity of human skin surface varies within the pH range of about 4—6.[30—32] The distribution percent of 6-bAcyl-AA-2G in octanol increased with increasing length of their acyl group (Table 2). 6-bHexa-, 6-bOcta-, and 6-bDode-AA-2G were found to be 12.6, 53.2, and 99.3% in octanol, respectively. The distribution percent of 6-Acyl-AA-2G also increased with increasing length of their acyl group. The lipophilicity of 6-bDode-AA-2G was equivalent to that of 6-Dode-AA-2G, while the lipophilicity was slightly decreased by the substitution from the straight-acyl chain to the branched-acyl chain of C_6 or C_8 carbon atoms. In contrast, AA and AA-2G were not distributed into octanol.

Table 2. Partitioning Behavior of 6-bAcyl-AA-2G in *n*-Octanol/Buffer Systems[a]

Sample	Distribution percent in octanol	
	pH 5.0	pH 7.0
AA	0	0
AA-2G	0	0
6-Hexa-AA-2G	21.0±0.6	7.3±0.6
6-Octa-AA-2G	73.4±0.7	43.1±0.7
6-Dode-AA-2G	96.1±1.8	98.8±0.2
6-bHexa-AA-2G	12.6±0.6	5.1±0.9
6-bOcta-AA-2G	53.2±0.5	25.2±0.2
6-bDode-AA-2G	99.3±0.5	95.0±0.2

a) 6-bAcyl-AA-2G was dissolved in sodium acetate buffer (pH 5.0) or potassium phosphate buffer (pH 7.0) to give 5 ml of 1 mM solution. Five ml of *n*-octanol was added to the resulting solution, vigorously mixed and partitioned at 37 °C for 30 min. The amount of 6-bAcyl-AA-2G in octanol was analyzed by HPLC. Each value represents the mean±S.D. (n=3).

The lipophilicity of 6-bAcyl-AA-2G was also investigated with an *n*-octanol/10 mM potassium phosphate buffer (pH 7.0) system in consideration of the penetration into the cell membrane after skin permeation (Table 2). The same tendency as the above results was observed in this system, although the distribution percent of 6-bHexa-, 6-Hexa-, 6-bOcta-, and 6-Octa-AA-2G was found to decrease slightly with increase in pH. The decrease was assumed to be caused by deprotonation at the C-3 hydroxyl group of AA. In contrast, the major portion of 6-bDode- and 6-Dode-AA-2G was distributed into octanol under both conditions, suggesting that these compounds have high affinity for cell membranes and for the corneal layer of epidermis. These results indicated that the modification of the acyl group exerted little or no effect on the partitioning behavior in the *n*-octanol/buffer systems. We previously reported that 6-Dode-AA-2G of 6-Acyl-AA-2G having a straight-acyl chain was effectively permeated into a human skin model.[10] Therefore, it is expected that 6-bDode-AA-2G may be applicable to skin care field as a cosmetic material.

Solubility of 6-bDode-AA-2G in a Wide Variety of Solvents Considering the application to a cosmetic material, it is desired to easily dissolve in solvents generally employed as a cosmetic base. Table 3 shows the solubility of the promising 6-bDode-AA-2G in various solvents including cosmetic bases such as water, ethanol, glyceryl tri-2-ethylhexanoate and squalane. 6-bDode-AA-2G was readily dissolved in water and ethanol at a concentration of above 250 mM, although the partitioning behavior in Table 2 showed the high lipophilicity. The solubility in acetone, ethyl acetate and glyceryl tri-2-ethylhexanoate was 46.0, 2.40 and 0.090 mM, respectively. 6-bDode-AA-2G was dissolved in a wide variety of solvents, but not in squalane. In contrast, 6-Dode-AA-

Table 3. Solubility of 6-bDode-AA-2G in Various Solvents[a]

Sample	Solubility (mM)					
	Water	Ethanol	Acetone	Ethyl acetate	Glyceryl tri-2-ethylhexanoate	Squalane
AA-2G	>250	ND	ND	ND	ND	ND
6-Dode-AA-2G	0.102±0.013	14.8±1.1	1.27±0.12	0.073±0.004	0.001±0.001	ND
6-bDode-AA-2G	>250	>250	46.0±4.5[b]	2.40±0.08[b]	0.090±0.004[b]	ND

a) Sample (10—30 mg) added 200 μl of each solvent was stirred for 6 h at 25 °C. The resulting mixture was transferred into a centifugal ultrafiltration tube and centrifuged. The amount of AA derivative in the filtrate was determined by HPLC. Each value represents the mean±S.D. (n=3). ND, not detectable. The statistical significance of differences in the mean of each data was calculated with Student's *t*-test. *b*) $p<0.001$ as compared with 6-Dode-AA-2G.

2G that had the same partitioning behavior in Table 2 as 6-bDode-AA-2G was difficult to be dissolved in the all tested solvents. The solubility was about 15 mM even in ethanol in which 6-Dode-AA-2G was most well dissolved. AA-2G, a stable hydrophilic vitamin C derivative was dissolved only in water at a concentration of above 250 mM.

It is noteworthy that 6-bDode-AA-2G, as well as AA-2G, was easily dissolved in water utilized as a main solvent in cosmetic field. The characteristic that 6-bDode-AA-2G with the possibility of high skin permeability showed the high solubility in water seemed to be advantageous for cosmetic materials. These findings indicated that the conversion of a straight-acyl chain into a branched-acyl chain gave excellent results in the solubility. 6-bDode-AA-2G could be dissolved in a wide variety of solvents, suggesting easy handling in cosmetic use. Therefore, 6-bDode-AA-2G seems to be superior for practical use to AA-2G which has been widely utilized as a cosmetic material.

Conclusion

We synthesized 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acids having a 2-branched-acyl chain, 6-bAcyl-AA-2G. The acyl moiety that has the same length of aliphatic groups attached to the C-2 was employed as a model for a 2-branched-acyl chain. 6-bAcyl-AA-2G exhibited a higher stability than 6-Acyl-AA-2G possessing a straight-acyl chain under a nonenzymatic condition, and the stability was comparable to that of AA-2G. The high stability in the long-term storage satisfied a requirement for the application to a cosmetic material. The derivatives showed the radical scavenging activity, and the hydrolysate by α-glucosidase was also considered to exhibit antioxidative activity *in vivo*. In addition, 6-bAcyl-AA-2G increased the lipophilicity in skin permeation model with increasing length of their acyl group. Of 6-bAcyl-AA-2G derivatives, the promising 6-bDode-AA-2G could be dissolved in a wide variety of solvents, suggesting easy handling in cosmetic use. These findings indicate that 6-bAcyl-AA-2G may be an effective antioxidant in skin care. In closing, we expect that 6-bAcyl-AA-2G with the characteristic properties is utilized in not only cosmetic field but also various fields.

Experimental

General Experimental Procedure ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian VXR-500 Instrument with TMS. Melting points were determined on a Yanagimoto micro-melting point apparatus, and are uncorrected. Elemental analyses were performed on a Yanagimoto MT-5 CHN Corder elemental analyzer. UV spectra were obtained on a Shimadzu UV-1200 spectrophotometer. The HPLC analyses were carried out with a system consisting of a Shimadzu SCL-10A system controller, LC-10AD pump, SPD-10AV UV-Vis spectrophotometric detector, SIL-10AD auto injector, CTO-6A column oven, and C-R7A chromatopac.

Chemicals 2-*O*-α-D-Glucopyranosyl-L-ascorbic acid (AA-2G) was obtained from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). 1,1-Diphenyl-2-picrylhydrazyl and diphenyl phosphorochloridate were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Ascorbic acid was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). α-Glucosidase from rice seed and 2-propylpentanoic acid (Sigma) were commercially available. 2-Ethylbutyric acid and Sephadex LH-20 were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Amersham Biosciences, respectively. 2-*O*-α-D-Glucopyranosyl-6-*O*-hexanoyl-L-ascorbic acid (6-Hexa-AA-2G), 2-*O*-α-D-glucopyranosyl-6-*O*-octanoyl-L-ascorbic acid (6-Octa-AA-2G) and 6-*O*-dodecanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-Dode-AA-2G) were synthesized with AA-2G and each acid anhydride in pyridine. The products were purified by recrystallization as described previously.[10]

Synthesis of 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic Acids with a Branched-acyl Chain (6-bAcyl-AA-2G). 6-*O*-(2-Ethylbutyryl)-2-*O*-α-D-glucopyranosyl-L-ascorbic Acid (6-bHexa-AA-2G) The preparation of acid anhydride was carried out according to the procedure of Mestres and Palomo.[33] Diphenyl phosphorochloridate (10.3 ml, 50 mmol) was added to a solution of 2-ethylbutyric acid (12.6 ml, 100 mmol) and triethylamine (14.0 ml, 100 mmol) in dichloromethane (30 ml) and the mixture was stirred at room temperature for 15 min. The solution was washed with cold water (3×200 ml), the organic layer was separated and dried over anhydrous sodium sulfate. Evaporation of the solvent gave 2-ethylbutyric anhydride (10.5 g, 98.2%): ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.97 (12H, t, J=7.5 Hz, C<u>H</u>$_3$CH$_2$×4), 1.54—1.64 and 1.64—1.74 (each 4H, each m, CH$_2$C<u>H</u>C<u>H</u>$_2$×2), 2.31 (2H, tt, J=5.5, 8.2 Hz, C<u>H</u>CO×2). The acid anhydride was used for the following reaction without further purification.

A mixture of AA-2G (4.0 g, 11.8 mmol) and 2-ethylbutyric anhydride (5.5 ml, 24.1 mmol) in pyridine (40 ml) was stirred for 2 h at 60 °C. The reaction mixture was concentrated *in vacuo*. The oil residue was dissolved in water (80 ml), and then partitioned with EtOAc (4×80 ml). The water layer was concentrated and chromatographed on a Sephadex LH-20 column (ϕ 4.0×31 cm) eluted with MeOH–H_2O–AcOH (40 : 59 : 1, v/v). Recrystallization of the product from benzene–isopropanol (4 : 1, v/v) gave 6-bHexa-AA-2G (1.28 g, 24.9%). UV λ_{max} (MeOH+HCl) nm (ε): 234 (9700); UV λ_{max} (MeOH+NaOH) nm (ε): 261 (14800). ^{1}H-NMR (500 MHz, CD_3OD) δ: 0.91 (6H, t, J=7.5 Hz), 1.51—1.59 (2H, m), 1.59—1.68 (2H, m), 2.28 (1H, tt, J=5.3, 8.7 Hz), 3.40 (1H, dd, J=9.4, 10.1 Hz, 4′-H), 3.53 (1H, dd, J=3.7, 9.4 Hz, 2′-H), 3.71 (1H, dd, J=4.9, 11.9 Hz, 6′-Ha), 3.78 (1H, t, J=9.4 Hz, 3′-H), 3.79 (1H, dd, J=2.4, 11.9 Hz, 6′-Hb), 4.03 (1H, ddd, J=2.4, 4.9, 10.1 Hz, 5′-H), 4.14 (1H, ddd, J=1.8, 6.1, 7.0 Hz, 5-H), 4.20 (1H, dd, J=6.1, 11.0 Hz, 6-Ha), 4.26 (1H, dd, J=7.0, 11.0 Hz, 6-Hb), 4.82 (1H, d, J=1.8 Hz, 4-H), 5.37 (1H, d, J=3.7 Hz, 1′-H). ^{13}C-NMR (125 MHz, CD_3OD) δ: 12.11 (×2), 26.03, 26.08, 50.09, 62.06 (6′-C), 65.40 (6-C), 67.88 (5-C), 71.00 (4′-C), 73.32 (2′-C), 74.40 (3′-C), 74.77 (5′-C), 77.28 (4-C), 101.68 (1′-C), 120.32 (2-C), 161.99 (3-C), 172.07, 177.39 (1-C). *Anal.* Calcd for $C_{18}H_{28}O_{12}$: C, 49.540; H, 6.467. Found: C, 49.561; H, 6.396. mp 159—161 °C.

2-*O*-α-D-Glucopyranosyl-6-*O*-(2-propylpentanoyl)-L-ascorbic Acid (6-bOcta-AA-2G) From 2-propylpentanoic acid (15.9 ml, 100 mmol) and diphenyl phosphorochloridate (10.3 ml, 50 mmol), 2-propylpentanoic anhydride (13.1 g, 97.0%) was prepared in a similar manner as 2-ethylbutyric anhydride. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.92 (12H, t, J=7.3 Hz, C<u>H</u>$_3$CH$_2$CH$_2$×4), 1.31—1.43 (8H, m, CH$_3$C<u>H</u>$_2$CH$_2$×4), 1.44—1.51 and 1.61—1.69 (each 4H, each m, C<u>H</u>$_2$CHC<u>H</u>$_2$×2), 2.45 (2H, tt, J=5.2, 8.5 Hz, C<u>H</u>CO×2).

The synthetic reaction of 6-bOcta-AA-2G was essentially the same as that of 6-bHexa-AA-2G. From AA-2G (4.0 g, 11.8 mmol) and 2-propylpentanoic anhydride (7.0 ml, 23.6 mmol), 6-bOcta-AA-2G was obtained as a crystal (1.17 g, 21.4% yield). UV λ_{max} (MeOH+HCl) nm (ε): 234 (9500); UV λ_{max} (MeOH+NaOH) nm (ε): 261 (14500). ^{1}H-NMR (500 MHz, CD_3OD) δ: 0.91 (6H, t, J=7.3 Hz), 1.27—1.36 (4H, m), 1.41—1.49 (2H, m), 1.56—1.64 (2H, m), 2.44 (1H, tt, J=5.2, 9.0 Hz), 3.40 (1H, dd, J=9.4, 10.4 Hz, 4′-H), 3.53 (1H, dd, J=3.7, 9.4 Hz, 2′-H), 3.71 (1H, dd, J=4.9, 11.9 Hz, 6′-Ha), 3.78 (1H, t, J=9.4 Hz, 3′-H), 3.80 (1H, dd, J=2.3, 11.9 Hz, 6′-Hb), 4.03 (1H, ddd, J=2.3, 4.9, 10.4 Hz, 5′-H), 4.12 (1H, ddd, J=1.8, 6.1, 7.0 Hz, 5-H), 4.18 (1H, dd, J=6.1, 11.0 Hz, 6-Ha), 4.25 (1H, dd, J=7.0, 11.0 Hz, 6-Hb), 4.80 (1H, d, J=1.8 Hz, 4-H), 5.37 (1H, d, J=3.7 Hz, 1′-H). ^{13}C-NMR (125 MHz, CD_3OD) δ: 14.32 (×2), 21.61 (×2), 35.74, 35.77, 46.43, 62.07 (6′-C), 65.41 (6-C), 67.85 (5-C), 71.02 (4′-C), 73.33 (2′-C), 74.41 (3′-C), 74.78 (5′-C), 77.27 (4-C), 101.68 (1′-C), 120.33 (2-C), 161.96 (3-C), 172.07, 177.69 (1-C). *Anal.* Calcd for $C_{20}H_{32}O_{12}\cdot 1/5H_2O$: C, 51.321; H, 6.977. Found: C, 51.167; H, 6.936. mp 98—101 °C.

2-*O*-α-D-Glucopyranosyl-6-*O*-(2-pentylheptanoyl)-L-ascorbic Acid (6-bDode-AA-2G) 2-Pentylheptanoic acid was synthesized according to the procedure of the malonic ester synthesis, because it was not commercially available. Diethyl malonate was alkylated by *n*-amyl bromide in EtONa/EtOH, and the product was alkylated again. The dialkylmalonic ester was hydrolyzed to 2,2-dipentylmalonic acid. Decarboxylation of the substituted malonic acid gave 2-pentylheptanoic acid: ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.88 (6H, br t, J=7.0 Hz, pentyl-5-H×2), 1.22—1.36 (12H, m, pentyl-2,3,4-H×2), 1.42—1.51 and 1.58—1.67 (each 2H, each m, C<u>H</u>$_2$CHC<u>H</u>$_2$), 2.31—2.37 (1H, m, C<u>H</u>CO$_2$H), 10.36 (1H, br, D_2O exchangeable, CO$_2$<u>H</u>); FAB-MS m/z 401 ($2MH^+$), 201 (MH^+).

From 2-pentylheptanoic acid (11.4 ml, 50 mmol) and diphenyl phosphorochloridate (5.2 ml, 25 mmol), 2-pentylheptanoic anhydride (8.9 g, 93.7%)

was prepared in a similar manner as 2-ethylbutyric anhydride. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.88 (12H, br t, J=7.0 Hz, pentyl-5-H×4), 1.22—1.38 (24H, m, pentyl-2,3,4-H×4), 1.45—1.53 and 1.61—1.69 (each 4H, each m, C<u>H</u>$_2$CHC<u>H</u>$_2$×2), 2.41 (2H, tt, J=5.5, 8.5 Hz, C<u>H</u>CO×2).

The synthetic reaction of 6-bDode-AA-2G was essentially the same as that of 6-bHexa-AA-2G. From AA-2G (4.0 g, 11.8 mmol) and 2-pentylheptanoic anhydride (10.0 ml, 22.5 mmol), 6-bDode-AA-2G was obtained as a crystal (0.88 g, 14.3% yield). UV λ_{max} (MeOH+HCl) nm (ε): 233 (9900); UV λ_{max} (MeOH+NaOH) nm (ε): 261 (15000). ^{1}H-NMR (500 MHz, CD_3OD) δ: 0.89 (6H, t, J=7.0 Hz), 1.21—1.37 (12H, m), 1.43—1.52 (2H, m), 1.56—1.65 (2H, m), 2.40 (1H, tt, J=5.2, 9.0 Hz), 3.41 (1H, dd, J=9.4, 10.1 Hz, 4′-H), 3.53 (1H, dd, J=3.7, 9.4 Hz, 2′-H), 3.71 (1H, dd, J=4.9, 11.9 Hz, 6′-Ha), 3.78 (1H, t, J=9.4 Hz, 3′-H), 3.79 (1H, dd, J=2.4, 11.9 Hz, 6′-Hb), 4.03 (1H, ddd, J=2.4, 4.9, 10.1 Hz, 5′-H), 4.12 (1H, dt, J=1.8, 6.6 Hz, 5-H), 4.18 (1H, dd, J=6.6, 11.0 Hz, 6-Ha), 4.26 (1H, dd, J=6.6, 11.0 Hz, 6-Hb), 4.80 (1H, d, J=1.8 Hz, 4-H), 5.37 (1H, d, J=3.7 Hz, 1′-H). ^{13}C-NMR (125 MHz, CD_3OD) δ: 14.36 (×2), 23.50 (×2), 28.16 (×2), 32.85 (×2), 33.52, 33.56, 46.89, 62.05 (6′-C), 65.29 (6-C), 67.81 (5-C), 70.99 (4′-C), 73.34 (2′-C), 74.41 (3′-C), 74.77 (5′-C), 77.19 (4-C), 101.71 (1′-C), 120.32 (2-C), 161.99 (3-C), 172.05, 177.66 (1-C). *Anal.* Calcd for $C_{24}H_{40}O_{12}$: C, 55.374; H, 7.745. Found: C, 55.176; H, 7.695. mp 158—160 °C.

Stability of 6-bAcyl-AA-2G in Aqueous Solution The test compounds were dissolved in 100 mM potassium phosphate buffer (pH 6.5) to give 10 ml of 10 mM solution. The resulting solution was stored at 50 °C for periods up to 28 d, and 100 μl samples were periodically taken. The concentration of the test compounds was analyzed by HPLC. The difference from initial concentration was taken as the remaining ratio. AA-2G, the deacylation form was also analyzed by HPLC.

Free-Radical Scavenging Activity of 6-bAcyl-AA-2G Free-radical scavenging activity of 6-bAcyl-AA-2G was assayed using a relatively stable free radical, DPPH, according to the method of Blois.[34] The reaction mixture contained 1 ml of 0.5 mM DPPH in ethanol and 4 ml of 1.25×10^{-6}, 1.25×10^{-5}, 6.25×10^{-5}, and 1.25×10^{-4} M antioxidant in ethanol/H_2O (1 : 1, v/v). After the reaction was carried out at 25 °C for 20 min, the decolorization of DPPH was measured at 516 nm. The EC_{50} value was determined as the concentration of each sample required to give 50% of the absorbance shown by a blank.

Hydrolysis of 6-bAcyl-AA-2G by Rice Seed α-Glucosidase Reaction mixtures were composed of 800 μl of 1.25 mM sample dissolved in 100 mM potassium phosphate buffer (pH 7.0) and 200 μl of enzyme solution (1.0 unit/ml final concentration). The hydrolytic reaction was carried out at 37 °C, and 50 μl of sample was periodically taken. The reaction mixture was diluted 10 times with 75% MeOH–H_2O containing 1% acetic acid, and then centrifuged at 8000×$\boldsymbol{g}$ for 10 min. Remaining amount of 6-bAcyl-AA-2G in the supernatant was determined by HPLC analysis.

Partition of 6-bAcyl-AA-2G between *n*-Octanol and Water 6-bAcyl-AA-2G was dissolved in 10 mM sodium acetate buffer (pH 5.0) or 10 mM potassium phosphate buffer (pH 7.0) to give 5 ml of 1 mM solution. Five ml of *n*-octanol was added to the resulting solution, vigorously mixed and partitioned at 37 °C for 30 min. The amount of 6-bAcyl-AA-2G in octanol was analyzed by HPLC. The difference from the total amount was taken as distribution % of 6-bAcyl-AA-2G in octanol.

Solubility of 6-bDode-AA-2G Sample (10—30 mg) was put into a 1.5 ml Eppendorf tube, after which 200 μl of each solvent was added. After sonication for 5 min and incubation at 50 °C for 5 min, the mixture was placed in a thermoregulated shaker (M·BR-022, TAITEC, Saitama, Japan), and was stirred at 500 r/min for 6 h at 25 °C. The resulting mixture was transferred into a centifugal ultrafiltration tube and centrifuged at 2000×$\boldsymbol{g}$ for 10 min. The amount of AA derivative in the filtrate was determined by HPLC.

HPLC Conditions The separation of AA and AA-2G was achieved by isocratic elution from an Inertsil ODS-3 column (ϕ 4.6×250 mm, 5 μm, GL Sciences Inc., Tokyo, Japan) kept at 40 °C with 0.1 M potassium phosphate-phosphoric acid buffer (pH 2.1, containing 10 mg/l of EDTA) at a flow rate of 0.7 ml/min. The absorbance at 240 nm was monitored. The separation of 6-bAcyl-AA-2G and 6-Acyl-AA-2G was carried out by isocratic elution from an Inertsil Ph column (ϕ 4.6×250 mm, 5 μm, GL Sciences Inc.) kept at 40 °C with 75% MeOH–H_2O containing 1% acetic acid at a flow rate of 0.7 ml/min. The absorbance at 240 nm was monitored. AA, AA-2G, 6-bAcyl-AA-2G and 6-Acyl-AA-2G contents were determined from the peak area of the samples with reference to the calibration of authentic AA, AA-2G, 6-bAcyl-AA-2G and 6-Acyl-AA-2G, respectively.

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