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Permeation and metabolism of a series of novel lipophilic ascorbic acid derivatives, 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acids with a branched-acyl chain, in a human living skin equivalent model

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Abstract—A series of novel lipophilic vitamin C derivatives, 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acids possessing a branched-acyl chain of varying length from C_8 to C_{16} (6-bAcyl-AA-2G), were evaluated as topical prodrugs of ascorbic acid (AA) with transdermal activity in a human living skin equivalent model. The permeability of 6-bAcyl-AA-2G was compared with those of the derivatives having a straight-acyl chain (6-sAcyl-AA-2G). Out of 10 derivatives of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G, 6-sDode-AA-2G and 6-bDode-AA-2G exhibited most excellent permeability in this model. Measurement of the metabolites permeated from the skin model suggested that 6-bDode-AA-2G was mainly hydrolyzed via 6-*O*-acyl AA to AA by tissue enzymes, while 6-sDode-AA-2G was hydrolyzed via 2-*O*-α-D-glucopyranosyl-L-ascorbic acid to AA. The former metabolic pathway seems to be advantageous for a readily available source of AA, because 6-*O*-acyl AA, as well as AA, is able to show vitamin C activity.

2-*O*-α-D-Glucopyranosyl-L-ascorbic acid (AA-2G), a stable ascorbate derivative developed in our laboratory,[1,2] has already been used as a medical additive in the cosmetic field. This hydrophilic vitamin C derivative exhibits vitamin C activity in vitro and in vivo after enzymatic hydrolysis to ascorbic acid (AA) by α-glucosidase.[3–5] Recently, we have synthesized a series of monoacylated derivatives of AA-2G, 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acids with a straight-acyl chain (6-sAcyl-AA-2G), by a chemical or enzymatic processing with the aim of efficient transdermal activity.[6,7] 6-sAcyl-AA-2G has been shown to have radical scavenging activity per se,[8–10] and some of them have satisfactory skin permeability as well as antiscorbutic activity in guinea pigs.[6,11] 6-sAcyl-AA-2G is also susceptible to enzymatic hydrolysis by mammalian tissue esterase and α-glucosidase to produce AA. More recently, we have synthesized branched-acyl chain derivatives of 6-sAcyl-AA-2G (6-bAcyl-AA-2G) to improve the stability in long-term storage in terms of deacylation.[12] 6-bAcyl-AA-2G as well as 6-sAcyl-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, one of the 6-bAcyl-AA-2G derivatives, 6-bDode-AA-2G, exhibited a pronounced therapeutic effect in scorbutic guinea pigs by its repeated oral administrations.[13] However, the skin permeability of 6-bAcyl-AA-2G has not been determined yet. In this paper, we investigated the permeation and metabolism of 6-bAcyl-AA-2G as topical prodrugs of AA in a human living skin equivalent model comparing with that of 6-sAcyl-AA-2G.

A series of novel lipophilic vitamin C derivatives, 6-sAcyl-AA-2G and 6-bAcyl-AA-2G were synthesized with AA-2G and each acid anhydride having an acyl

Keywords: Lipophilic ascorbate (6-Acyl-AA-2G); Stable ascorbate (AA-2G); Skin permeation.
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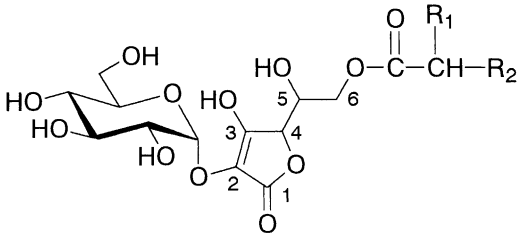
chain of varying length from C_8 to C_{16} by the methods in our previous reports.[6,12] The respective 6-sAcyl-AA-2G and 6-bAcyl-AA-2G used in this study were as follows: 2-*O*-α-D-glucopyranosyl-6-*O*-octanoyl-L-ascorbic acid (6-sOcta-AA-2G), 6-*O*-decanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-sDeca-AA-2G), 6-*O*-dodecanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-sDode-AA-2G), 2-*O*-α-D-glucopyranosyl-6-*O*-tetradecanoyl-L-ascorbic acid (6-sMyri-AA-2G) and 2-*O*-α-D-glucopyranosyl-6-*O*-hexadecanoyl-L-ascorbic acid (6-sPalm-AA-2G); and 2-*O*-α-D-glucopyranosyl-6-*O*-(2-propylpentanoyl)-L-ascorbic acid (6-bOcta-AA-2G), 6-*O*-(2-butylhexanoyl)-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-bDeca-AA-2G),[14] 2-*O*-α-D-glucopyranosyl-6-*O*-(2-pentylheptanoyl)-L-ascorbic acid (6-bDode-AA-2G), 2-*O*-α-D-glucopyranosyl-6-*O*-(2-hexyloctanoyl)-L-ascorbic acid (6-bMyri-AA-2G)[14] and 2-*O*-α-D-glucopyranosyl-6-*O*-(2-heptylnonanoyl)-L-ascorbic acid (6-bPalm-AA-2G).[14] These structures are shown in Figure 1.

Our previous study showed that 6-sDode-AA-2G among 6-sAcyl-AA-2G having an acyl chain of varying length from C_4 to C_{12} was effectively permeated into a human skin model.[6] This result suggested that 6-sDode-AA-2G may be available for skin care application as an effective antioxidant. However, it is not clear whether the 6-sAcyl-AA-2G derivatives with a long chain acyl group, such as 6-sMyri-AA-2G and 6-sPalm-AA-2G, and a series of 6-bAcyl-AA-2G are biologically available for AA supplementation in a human skin model. Permeability of a series of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G possessing an acyl chain of varying length from C_8 to C_{16} was investigated in a human living skin equivalent model.

The human skin equivalent consists of a dermal equivalent, reconstituted with collagen and dermal fibroblasts that are biosynthetically active, and a differentiated epidermis that arises from cultured keratinocytes plated onto the surface of the dermal equivalent.[15] The epidermis possesses a stratum corneum which provides barrier function properties.[16] There are several reports utilized for absorption and metabolism experiments of drugs on this skin model.[17–19] A human living skin equivalent model suitable for the percutaneous absorption test, TESTSKIN™ LSE-high was used in this study. The time-course of permeation amount in the receptor compartment was investigated after each AA derivative was applied to TESTSKIN.[20] The intact form and its metabolites such as AA-2G, 6-*O*-acyl AA and AA were observed in the receptor medium by passing through the skin. The formation of AA-2G and 6-*O*-acyl



6-sAcyl-AA-2G

R_1=H, R_2=$(CH_2)_5CH_3$: 6-sOcta-AA-2G

=$(CH_2)_7CH_3$: 6-sDeca-AA-2G

=$(CH_2)_9CH_3$: 6-sDode-AA-2G

=$(CH_2)_{11}CH_3$: 6-sMyri-AA-2G

=$(CH_2)_{13}CH_3$: 6-sPalm-AA-2G

6-bAcyl-AA-2G

R_1= R_2=$(CH_2)_2CH_3$: 6-bOcta-AA-2G

=$(CH_2)_3CH_3$: 6-bDeca-AA-2G

=$(CH_2)_4CH_3$: 6-bDode-AA-2G

=$(CH_2)_5CH_3$: 6-bMyri-AA-2G

=$(CH_2)_6CH_3$: 6-bPalm-AA-2G

Figure 1. Chemical structures of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G.

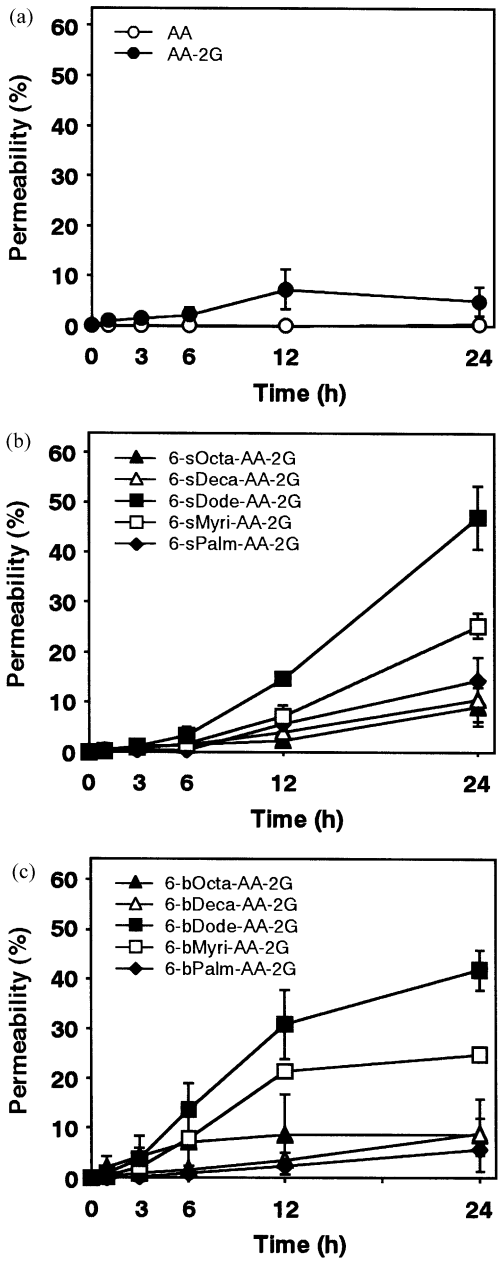


Figure 2. Total permeability of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G in TESTSKIN: (a) Permeability of AA and AA-2G; (b) Permeability of 6-sAcyl-AA-2G; (c) Permeability of 6-bAcyl-AA-2G. Each value represents the mean ± SEM (n = 5).

AA from 6-sAcyl-AA-2G (or 6-bAcyl-AA-2G) was respectively attributed to the esterase and α-glucosidase activities in TESTSKIN. The formation of AA also resulted from the hydrolysis by both esterase and α-glucosidase. In order to compare the ability as AA source, the permeability was evaluated as the ratio of the total amount of the compounds permeated as the intact form and its metabolites to the applied amount of the test compound (Fig. 2). The permeation amount of 6-sDode-AA-2G and 6-bDode-AA-2G rapidly increased after the beginning, and was still gaining when the experiment was terminated 24 h later. The maximal penetration ratio of 6-sDode-AA-2G and 6-bDode-AA-2G was 47 and 41%, respectively. The permeability of AA-2G did not reach 10% after 24 h incubation. AA was scarcely permeable into the receptor medium. Thus, 6-sDode-AA-2G and 6-bDode-AA-2G showed the skin permeability superior to those of AA-2G and AA. The permeation levels of 6-sOcta-, 6-bOcta-, 6-sDeca-, 6-bDeca-, 6-sPalm- and 6-bPalm-AA-2G were similar to that of AA-2G. 6-sAcyl-AA-2G and 6-bAcyl-AA-2G derivatives possessing an acyl chain length from C_8 to C_{12} tended to increase the permeation activity with increasing length of their acyl group, and from C_{12} to C_{16} tended to decrease with increasing length of their acyl group. In addition, no difference was observed in the permeability at 24 h between 6-sAcyl-AA-2G and 6-bAcyl-AA-2G with an acyl chain of the same length. Bonina et al. described that an effective dermal prodrug must possess an increased lipophilicity, compared with the parent drug, together with adequate aqueous solubility.[21] These results suggested that 6-sDode-AA-2G and 6-bDode-AA-2G had suitable lipophilicity and water solubility for effective skin permeation. Therefore, the penetration ability of 6-sDode-AA-2G and 6-bDode-AA-2G was superior to that of the other derivatives.

In view of the metabolism of the derivatives in the skin, the data of 6-sDode-AA-2G and 6-bDode-AA-2G in Figure 2 was reevaluated at each amount of the permeated compounds into the receptor compartment. Figure 3 shows the time-course of permeation and metabolism after application of 6-sDode-AA-2G and 6-bDode-AA-2G to TESTSKIN. In the application of 6-sDode-AA-2G, it was found that both AA and AA-2G levels were gradually increased (Fig. 3a). The amount of AA-2G was about 3-fold greater than that of AA after 24 h incubation. A small amount of 6-sDode-AA-2G was detected in the receptor compartment. 6-sDode-AA-2G level was slightly increased for periods up to 12 h, after which it was maintained. In contrast, 6-*O*-dodecanoyl ascorbic acid (6-sDode-AA) was observed in the trace amounts. The profiles suggested that 6-sDode-AA-2G was difficult to penetrate TESTSKIN as the intact form and that 6-sDode-AA-2G absorbed into the skin was hydrolyzed via AA-2G to AA in situ and released as its metabolites in the receptor medium. In the application of 6-bDode-AA-2G, it was found that 6-bDode-AA-2G level was rapidly increased, reached a maximum at 12 h after, and then decreased (Fig. 3b). The decrease may be attributed to the hydrolysis by enzymes leaked from the skin and/or to the reabsorption by the skin tissue. 6-*O*-(2-Pentylheptanoyl)ascorbic acid (6-bDode-AA) and AA levels were gradually increased throughout the incubation. In contrast, AA-2G was slightly detectable in the receptor compartment. It is noteworthy that the AA releasing amount from 6-bDode-AA-2G was less than that from 6-sDode-AA-2G. It seems that the prevention of enzymatic degradation of the ester bond could be attributed to the steric hindrance by the 2-alkyl group of the acyl moiety. The profiles suggested that 6-bDode-AA-2G was able to penetrate TESTSKIN as the intact form and that 6-bDode-AA-2G retained in the skin was hydrolyzed via 6-bDode-AA to AA in situ and released as its metabolites. The metabolic pathway of 6-sDode-AA-2G and 6-bDode-AA-2G agrees well with the hydrolysis pattern of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G with various tissue homogenates from guinea pigs described previouly.[13] Murine spleen homogenate also showed a similar hydrolysis pattern of 6-sDode-AA-2G and 6-bDode-AA-2G (data not shown). It was reported that 6-*O*-palmitoyl ascorbate

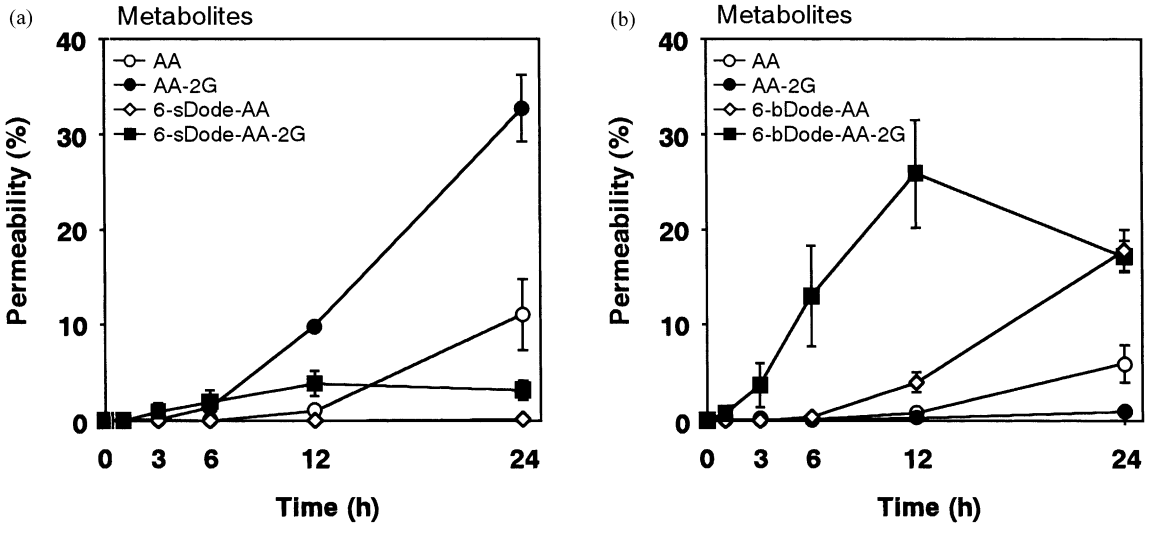


Figure 3. Time-course of permeation and metabolism after application of 6-sDode-AA-2G and 6-bDode-AA-2G to TESTSKIN: (a) Permeation and metabolism of 6-sDode-AA-2G; (b) Permeation and metabolism of 6-bDode-AA-2G. Each value represents the mean ± SEM ($n = 5$).

stimulated collagen synthesis in human fibroblasts and in human intestinal smooth muscle cells at lower doses than does AA.[22,23] Uesato et al. reported that some of 6-*O*-acylated ascorbic acids with a straight- and a branched-acyl chain displayed marked anti-tumor promoting activities.[24] It appears that the 6-*O*-acylated AA derivatives efficiently penetrated into the hydrophobic region of lipid bilayers to have the activity. These results suggested that 6-bDode-AA-2G might have AA activity by one step hydrolysis to 6-bDode-AA before AA release, whereas 6-sDode-AA-2G needed two hydrolytic steps for AA activity. Thus, various physiological and pharmacological actions of 6-bDode-AA-2G could be effectively elicited by the hydrolysis not to AA but to 6-bDode-AA. Furthermore, the sum (23.7%) of 6-bDode-AA and AA released from 6-bDode-AA-2G was larger than the level (11.1%) of AA from 6-sDode-AA-2G after 24 h. These results indicated that 6-bDode-AA-2G was superior to 6-sDode-AA-2G as a source of vitamin C activity in the skin model.

We prepared a series of novel lipophilic vitamin C derivatives possessing a straight- or branched-acyl chain of varying length from C_8 to C_{16}, 6-sAcyl-AA-2G and 6-bAcyl-AA-2G. The permeability of 6-sDode-AA-2G and 6-bDode-AA-2G was superior to that of AA-2G used as a medical additive, AA and the other 6-sAcyl-AA-2G and 6-bAcyl-AA-2G in a human living skin equivalent model. 6-bDode-AA-2G was mainly hydrolyzed via 6-*O*-acyl AA to AA, while 6-sDode-AA-2G was hydrolyzed via AA-2G to AA. The former metabolic pathway seems to be of advantage, because 6-*O*-acyl AA and AA have vitamin C activity. Our previous study showed that the stability in neutral solution and the solubility to various solvents of 6-bDode-AA-2G was much higher than those of 6-sDode-AA-2G.[12] Therefore, these findings indicate that 6-bDode-AA-2G may be used as a topical prodrug of AA with transdermal activity in skin care.

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14. 6-bDeca-AA-2G: ^{1}H NMR (600 MHz, CD_3OD) δ: 0.90 (6H, t, J = 7.2 Hz), 1.22–1.38 (8H, m), 1.46–1.52 (2H, m), 1.58–1.65 (2H, m), 2.40 (1H, tt, J = 5.3, 9.0 Hz), 3.40 (1H, dd, J = 9.4, 10.0 Hz, 4′-H), 3.52 (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 = 2.3, 11.9 Hz, 6′-Hb), 4.03 (1H, ddd, J = 2.3, 4.9, 10.0 Hz, 5′-H), 4.12 (1H, dt, J = 2.0, 6.7 Hz, 5-H), 4.18 (1H, dd, J = 6.7, 11.0 Hz, 6-Ha), 4.26 (1H, dd, J = 6.7, 11.0 Hz, 6-Hb), 4.81 (1H, d, J = 2.0 Hz, 4-H), 5.38 (1H, d, J = 3.7 Hz, 1′-H). HRFABMS m/z $[M+H]^+$: calcd for $C_{22}H_{37}O_{12}$: 493.2285, Found: 493.2275. 6-bMyri-AA-2G: ^{1}H NMR (600 MHz, CD_3OD) δ: 0.89 (6H, t, J = 7.0 Hz), 1.23–1.35 (16H, m), 1.44–1.52 (2H, m), 1.57–1.65 (2H, m), 2.41 (1H, tt, J = 5.2, 9.0 Hz), 3.41 (1H, dd, J = 9.4, 10.3 Hz, 4′-H), 3.53 (1H, dd, J = 3.7, 9.4 Hz, 2′-H), 3.71 (1H, dd, J = 4.8, 11.8 Hz, 6′-Ha), 3.78 (1H, t, J = 9.4 Hz, 3′-H), 3.79 (1H, dd, J = 2.3, 11.8 Hz, 6′-Hb), 4.03 (1H, ddd, J = 2.3, 4.8, 10.3 Hz, 5′-H), 4.12 (1H, dt, J = 2.0, 6.7 Hz, 5-H), 4.18 (1H, dd, J = 6.7, 11.0 Hz, 6-Ha), 4.25 (1H, dd, J = 6.7, 11.0 Hz, 6-Hb), 4.80 (1H, d, J = 2.0 Hz, 4-H), 5.37 (1H, d, J = 3.7 Hz, 1′-H). HRFABMS m/z $[M+H]^+$: calcd for $C_{26}H_{45}O_{12}$: 549.2911, Found: 549.2897. 6-bPalm-AA-2G: ^{1}H NMR (600 MHz, CD_3OD) δ: 0.89 (6H, t, J = 7.1 Hz), 1.23–1.35 (20H, m), 1.44–1.52 (2H, m), 1.57–1.65 (2H, m), 2.41 (1H, tt, J = 5.3, 9.0 Hz), 3.40 (1H, dd, J = 9.4, 10.0 Hz, 4′-H), 3.52 (1H, dd, J = 3.7, 9.4 Hz, 2′-H), 3.71 (1H, dd, J = 4.9, 11.8 Hz, 6′-Ha), 3.78 (1H, t, J = 9.4 Hz, 3′-H), 3.80 (1H, dd, J = 2.4, 11.8 Hz, 6′-Hb), 4.04 (1H, ddd, J = 2.4, 4.9, 10.0 Hz, 5′-H), 4.12 (1H, dt, J = 2.0, 6.6 Hz, 5-H), 4.18 (1H, dd, J = 6.6, 11.0 Hz, 6-Ha), 4.25 (1H, dd, J = 6.6, 11.0 Hz, 6-Hb), 4.80 (1H, d, J = 2.0 Hz, 4-H), 5.37 (1H, d, J = 3.7 Hz, 1′-H). HRFABMS m/z $[M+H]^+$: calcd for $C_{28}H_{49}O_{12}$: 577.3224, Found: 577.3236.
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20. Skin permeation assay: TESTSKIN™ Living skin equivalent-high (LSE-high), which was a three-dimensional model of human skin developed for percutaneous absorption, was purchased from TOYOBO Co (Osaka, Japan). The dermal portion of the LSE-high was rested on a polycarbonate membrane in contact with 1.2 mL of an assay medium (DMEM/Ham's F-12, 1:1) on a six-well plate. A polyethylene ring (10 mm internal diameter) was affixed to the epidermal surface with silicone sealant, and then 200 μL of 10 mM 6-sAcyl-AA-2G or 6-bAcyl-AA-2G solution dissolved in 50 mM sodium phosphate buffer (pH 7.0) was added to the interior of each ring. 6-sPalm- and 6-bPalm-AA-2G were dissolved in DMSO and diluted with phosphate buffer to give 10 mM solution (DMSO 1%). Aliquots of 100 μL were withdrawn from the receptor compartment periodically and replaced with equal volumes of the assay medium maintained at 37 °C. The experiments were performed for 24 h at 37 °C in a humidified incubator gassed with 5% CO_2. The resulting sam-

ples were directly subjected to HPLC analyses. 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-*O*-acyl AA, 6-sAcyl-AA-2G and 6-bAcyl-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 70 or 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-sAcyl-AA-2G and 6-bAcyl-AA-2G contents were determined from the peak area of the samples with reference to the calibration of authentic AA, AA-2G, 6-sAcyl-AA-2G and 6-bAcyl-AA-2G, respectively. The amount of 6-*O*-acyl AA was determined as ascorbyl 6-palmitate equivalent.

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