Bioorganic & Medicinal Chemistry 18 (2010) 6179–6183

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Intramolecular acyl migration and enzymatic hydrolysis of a novel monoacylated ascorbic acid derivative, 6-*O*-dodecanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid

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ARTICLE INFO

Article history:
Received 12 May 2010
Revised 5 June 2010
Accepted 8 June 2010
Available online 12 June 2010

Keywords:
Acyl migration
6-sDode-AA-2G
Lipophilic ascorbic acid derivative
Stable ascorbic acid derivative
Enzymatic hydrolysis

ABSTRACT

A stable ascorbic acid derivative, 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G), exhibits vitamin C activity in vitro and in vivo after enzymatic hydrolysis to ascorbic acid. AA-2G has been approved by the Japanese Government as a quasi-drug principal ingredient in skin care and as a food additive. In order to achieve efficient action as an ascorbic acid source, a pro-vitamin C agent, on a variety of cells or tissues, we have synthesized a series of monoacyl AA-2G derivatives. Our previous studies indicate that a series of the derivatives is a readily available source of AA activity in vitro and in vivo, and suggested that intramolecular acyl migration of the derivatives might have occurred in a neutral aqueous solution. In this study, intramolecular acyl migration and enzymatic hydrolysis of a monoacyl AA-2G derivative, 6-*O*-dodecanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-sDode-AA-2G), were investigated. 6-sDode-AA-2G underwent an intramolecular acyl migration to yield ca. 10% of an isomer in neutral aqueous solutions, and the acyl-migrated isomer was isolated and characterized as 5-*O*-dodecanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (5-sDode-AA-2G). In some tissue homogenates from guinea pigs as well as in neutral aqueous solutions, 6-sDode-AA-2G underwent partial acyl migration to give 5-sDode-AA-2G. 6-sDode-AA-2G and the resulting 5-sDode-AA-2G were predominantly hydrolyzed with esterase to AA-2G and then with α-glucosidase to ascorbic acid in the tissue homogenates. The results will provide a further basis for its use as an ingredient in skin care, as an effective pharmacological agent and as a promising food additive.

1. Introduction

A stable ascorbic acid derivative, 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G, Fig. 1), developed by Yamamoto et al.,[1–4] exhibits vitamin C activity in vitro and in vivo after enzymatic hydrolysis to ascorbic acid (AA, Fig. 1) by α-glucosidase.[5–7] AA-2G has been approved by the Japanese Government as a quasi-drug principal ingredient in skin care and as a food additive and is now widely used as a medical additive in commercial cosmetics. In order to achieve efficient action as an AA source, a pro-vitamin C agent, on a variety of cells or tissues, we have chemically and enzymatically synthesized a series of monoacylated AA-2G (Fig. 1).[8–11] The monoacyl AA-2G derivatives were identified as 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acids possessing a straight-acyl chain of varying length from C_4 to C_{18} (6-sAcyl-AA-2G) and a branched-acyl chain of varying length from C_6 to C_{16} (6-bAcyl-AA-2G). When the long-term stability of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G was tested in a neutral aqueous solution for 28 days, the amounts of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G sharply decreased during the first day.[10] After a rapid decrease in the first day, the amount of 6-sAcyl-AA-2G gradually decreased up to 28 days of storage, while there was little decrease in 6-bAcyl-AA-2G. A major degradation product of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G was found to be their deacylated form, AA-2G. However, the deacylation (AA-2G release) profile of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G was in conflict with the pattern of decrease in the intact forms, especially in the early stage. These results suggested that intramolecular acyl migration of 6-sAcyl-AA-2G and 6-bAcyl-AA-2G might have occurred.

6-sAcyl-AA-2G and 6-bAcyl-AA-2G had radical scavenging activity per se,[12–15] and some of them with an appropriate length of the acyl chain group exhibited skin permeability superior to that of AA-2G and AA.[9,11] In the series of monoacylated AA-2G, 6-sDode-AA-2G, and 6-bDode-AA-2G showed the best permeability in a skin model. 6-sOcta-AA-2G and 6-bDode-AA-2G showed satisfactory bioavailability as AA supplements in rats or guinea pigs compared with AA-2G.[16,18] 6-sAcyl-AA-2G synergistically enhanced both dibutyrylcyclic AMP- and nerve growth factor-induced neurite outgrowth in PC12 cells, and the synergistic effect was stronger than

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doi:10.1016/j.bmc.2010.06.027

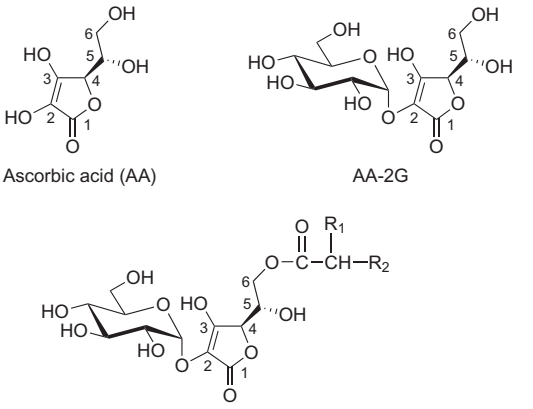


Figure 1. Chemical structures of AA, AA-2G, 6-sAcyl-AA-2G, and 6-bAcyl-AA-2G.

that of AA-2G and AA.[19] In in vitro experiments, 6-sAcyl-AA-2G was hydrolyzed via AA-2G to AA, while 6-bAcyl-AA-2G was mainly hydrolyzed via 6-*O*-acyl AA to AA.[11,16,17]

In this study, we investigated the pH-dependent degradation or intramolecular acyl migration of a monoacyl AA-2G derivative, 6-sDode-AA-2G, and elucidated the chemical structure of an acyl-migrated isomer. We also investigated the hydrolytic susceptibility of the isomer by tissue enzymes from guinea pigs, and we propose the metabolic pathway of 6-sDode-AA-2G under neutral conditions.

2. Materials and methods

2.1. General experimental procedure

^{1}H NMR spectra were recorded on a Varian INOVA-AS600 instrument. Electron spray ionization (ESI) high-resolution mass spectra were obtained on a Bruker Daltonics MicrOTOF II instrument using direct sample injection. 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, and with a system consisting of a Hitachi L-7100 pump, L-7420 detector, L-7300 column oven, and D-2500 chromato-integrator.

2.2. Chemicals

2-*O*-α-D-Glucopyranosyl-L-ascorbic acid (AA-2G) was a gift from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). 6-*O*-Dodecanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-sDode-AA-2G) was chemically synthesized by the method described in our previous report.[9] Reagents were used without further purification. All water used was Milli-Q grade.

2.3. Stability of 6-sDode-AA-2G in aqueous solutions at different pH values

6-sDode-AA-2G (200 mM) dissolved in DMSO was diluted with McIlvaine's citric acid-phosphate buffer (pH 5.0–8.0) to give 1.0 ml of 1 mM solution. The resulting solution was stored at 37 °C for periods up to 12 h, and 50-μl samples were periodically taken. The concentrations of the samples were analyzed by HPLC. The separation of 6-sDode-AA-2G and its isomer was done by isocratic elution from an Inertsil Ph column (4.6 × 250 mm, 5 μm, GL Sciences, Inc., Tokyo, Japan) kept at 40 °C with 70% MeOH–H_2O containing 0.5% formic acid at a flow rate of 0.7 ml/min. The absorbance at 240 nm was monitored. The 6-sDode-AA-2G content was determined from the peak area of the sample with reference to calibration by authentic 6-sDode-AA-2G. The concentration of the isomer was calculated as 6-sDode-AA-2G equivalent. HPLC analysis of AA-2G was carried out as described in our previous report.[20]

2.4. Preparation and purification of acyl-migrated isomer

6-sDode-AA-2G (416.0 mg) was dissolved in 4 ml of DMSO. The solution was diluted with 0.1 M sodium phosphate buffer (pH 7.0) to give 80 ml of 10 mM 6-sDode-AA-2G aqueous solution. The aqueous solution was incubated for 3 h at 37 °C. The reaction mixture was adjusted to pH 2.3 with formic acid and chromatographed on a Diaion HP20 column (4.6 × 6 cm, Mitsubishi Chemical Co., Tokyo) eluted with H_2O and MeOH/H_2O (9:1, v/v). The methanolic eluate was concentrated to dryness. The residue was recrystallized from methanol to give 6-sDode-AA-2G (275.6 mg). The resulting filtrate containing an acyl-migrated isomer was concentrated to dryness. The residue was suspended in MeOH/formic acid/H_2O (60:0.5:39.5, v/v) and centrifuged at 10,000*g* for 10 min. After centrifugation, the precipitate was dried in vacuo to give 6-sDode-AA-2G (59.8 mg) and the supernatant was repeatedly purified by semi-preparative HPLC to yield an acyl-migrated isomer (15.9 mg) and 6-sDode-AA-2G (11.7 mg) under the following conditions: column, Inertsil Ph-3 (10 × 250 mm, 5 μm, GL Sciences, Inc.); solvent, 60% MeOH/H_2O containing 0.5% formic acid; flow rate, 3.3 ml/min; detection wavelength, 240 nm. Finally, an acyl-migrated isomer and 6-sDode-AA-2G were obtained in yields of 15.9 mg and 347.1 mg, respectively.

2.4.1. 5-*O*-Dodecanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (acyl-migrated isomer)

^{1}H NMR (600 MHz, CD_3OD) δ_H: 0.94 (3H, t, *J* = 7.2 Hz), 1.33 (16H, br s), 1.61 (2H, br qui, *J* = 7.2 Hz), 2.35 (2H, t, *J* = 7.2 Hz), 3.47 (1H, dd, *J* = 9.0, 10.2 Hz, H-4′), 3.55 (1H, dd, *J* = 3.6, 9.6 Hz, H-2′), 3.78–3.82 (5H, m), 4.13 (1H, ddd, *J* = 3.0, 4.2, 10.2 Hz, H-5′), 5.05 (1H, d, *J* = 1.8 Hz, H-4), 5.27 (1H, d, *J* = 3.6 Hz, H-1′), 5.32 (1H, ddd, *J* = 1.8, 6.6, 6.6 Hz, H-5). ESI-HRMS *m*/*z* [M−H]$^-$: calcd for $C_{24}H_{39}O_{12}$: 519.2447, found: 519.2443.

2.5. Intramolecular acyl migration and enzymatic hydrolysis of 6-sDode-AA-2G in tissue homogenates

Hartley guinea pigs (male, 6 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The care and use of the animals in this study followed the guidelines of Okayama University. Guinea pigs that had been fed ad libitum on a stock diet of Oriental RC4 (Oriental Yeast Co., Tokyo, Japan) and water were starved for 24 h before use and then killed by decapitation under diethyl ether anesthesia. All of the subsequent manipulations were done at around 4 °C. The liver, small intestine, and kidney were removed, rinsed with cold saline, and homogenized in four volumes of a 0.1 M potassium phosphate buffer (pH 7.0) with an Ultra-Turrax T 25 basic disperser (IKA Labortechnik, Germany). Each tissue homogenate was centrifuged at 700*g* for 10 min, and the supernatants were further centrifuged at 12,000*g* for 20 min. The resulting supernatants were dialyzed overnight against 10 mM potassium phosphate buffer (pH 7.0), before being centrifuged at 12,000*g* for 20 min for use as enzyme sources. Each reaction mixture was

composed of 160 μl of 1.25 mM 6-sDode-AA-2G dissolved in 0.1 M potassium phosphate buffer (pH 7.0) and 40 μl of the enzyme solution just described. After incubation for 1 or 12 h at 37 °C, the reaction mixture was diluted 10 times with 75% MeOH/H_2O containing 1% acetic acid and then centrifuged at 8000*g* for 10 min. Intramolecular acyl migration and enzymatic hydrolysis of 6-sDode-AA-2G in the supernatant were measured by HPLC analysis. The separation of AA and AA-2G was done by isocratic elution from an Inertsil ODS-3 column (4.6 × 250 mm, 5 μm, GL Sciences, Inc.) 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*-dodecanoyl AA (6-sDode-AA), 5-*O*-dodecanoyl AA (5-sDode-AA), 6-sDode-AA-2G, and 5-sDode-AA-2G was done by isocratic elution from an Inertsil Ph column (4.6 × 250 mm, 5 μm) kept at 40 °C with 70% MeOH/H_2O containing 1% acetic acid at a flow rate of 0.7 ml/min. The absorbance at 240 nm was again monitored. The peak of 6-sDode-AA and 5-sDode-AA was identified by comparison with the retention time of a complete α-glucosidase hydrolysate of 6-sDode-AA-2G in 0.1 M phosphate buffer (pH 7.0).

3. Results and discussion

The effect of pH on stability of 6-sDode-AA-2G in aqueous solution (1 mM) at 37 °C was evaluated on the basis of remaining concentration measured by HPLC (Fig. 2). The concentration of 6-sDode-AA-2G tended to decrease with increase in pH from 5 to 8. At pH 8 and 7, the concentration of 6-sDode-AA-2G sharply decreased during the first 30 min and 3 h, respectively, after which the concentration remained almost constant. AA-2G, the deacylated form from 6-sDode-AA-2G, was also analyzed by HPLC. The amount of AA-2G released was 14.8 ± 2.9 μM (n = 9) even at pH 8 after 12 h. The concentration of AA-2G was not in agreement with the decreased concentration (162 μM) of 6-sDode-AA-2G at pH 8 after 12 h. Similar results were obtained in our previous study.[10] These results indicated that the initial decrease was not caused by hydrolytic cleavage of the ester bond at C-6 of the AA moiety in 6-sDode-AA-2G.

A new peak, with the remarkable decrease, was observed by HPLC analysis, and the peak area ratio of 6-sDode-AA-2G to the new compound remained almost constant throughout the experiment (data not shown). A typical chromatogram of 6-sDode-AA-2G incubated in pH 8 buffer at 37 °C for 12 h is shown in Figure 3. The retention time of the new peak was not identical to that of a complete α-glucosidase hydrolysate of 6-sDode-AA-2G. These results indicated that the ether bond at C-2 of the AA moiety in 6-sDode-AA-2G was not cleaved. Intramolecular acyl migrations

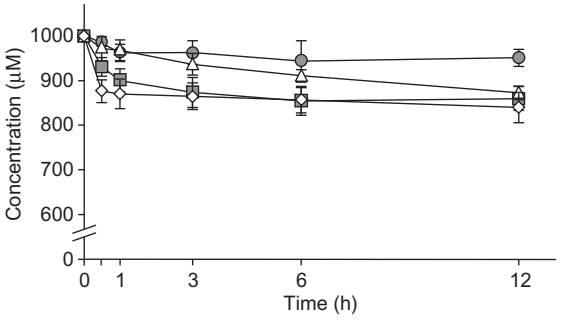


Figure 2. Effect of pH on stability of 6-sDode-AA-2G in aqueous solution. 6-sDode-AA-2G was dissolved in McIlvaine's citric acid-phosphate buffer to give 1 mM solution and was then incubated at 37 °C for the indicated time. The concentration of 6-sDode-AA-2G was measured by HPLC. The pH values were 5 (●), 6 (△), 7 (■), and 8 (◇). Each value represents the means ± SD (n = 9).

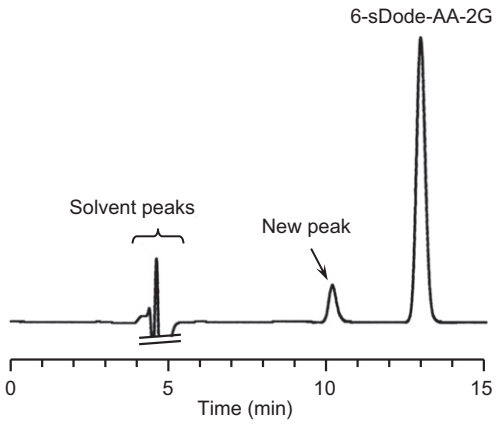


Figure 3. A typical chromatogram of 6-sDode-AA-2G incubated in McIlvaine's citric acid-phosphate buffer (pH 8) at 37 °C for 12 h.

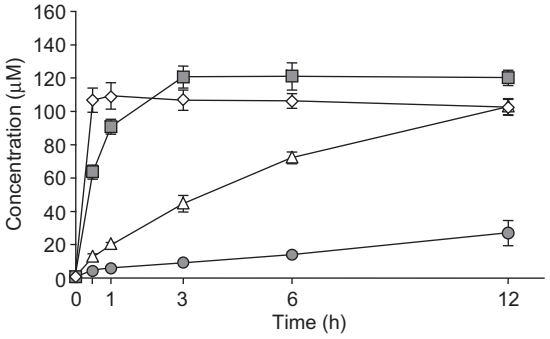
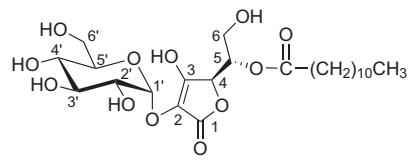


Figure 4. Effect of pH on acyl migration of 6-sDode-AA-2G in aqueous solution. This experiment was carried out with the experiment for which results are shown in Figure 2. The amount of 6-sDode-AA-2G isomer was measured by HPLC. The concentration of the isomer was calculated for 6-sDode-AA-2G equivalent. The pH values were 5 (●), 6 (△), 7 (■), and 8 (◇). Each value represents the means ± SD (n = 9).

have been previously reported to occur for ascorbic acid esters,[21,22] and they are well-known reactions of glycerol esters.[23] Thus, it seems likely that 6-sDode-AA-2G underwent an intramolecular acyl migration to yield the isomer and was in equilibrium with the isomer.

The effect of pH on intramolecular acyl migration of 6-sDode-AA-2G in aqueous solution at 37 °C was investigated by HPLC. The concentration of the acyl-migrated isomer was calculated as 6-sDode-AA-2G equivalent. The degree of acyl migration clearly depended on pH of the solution (Fig. 4). The concentration of the acyl-migrated isomer sharply increased with increase in pH from 5 to 8. At pH 8, the isomer level reached a maximum after 1 h and slightly decreased for periods up to 12 h. At pH 7, the isomer level reached a plateau after 3 h and then remained fairly constant until the experiment ended at 12 h. The highest migration level



5-*O*-Dodecanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid
(5-sDode-AA-2G)

Figure 5. Chemical structure of a 6-sDode-AA-2G isomer, 5-*O*-dodecanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid.

was 120 μM in pH 7 McIlvaine's buffer at 3–12 h. The remaining concentration of 6-sDode-AA-2G was in the range of 850–870 μM in pH 7 McIlvaine's buffer at 3–12 h (Fig. 2). The sum of the isomer and the remaining 6-sDode-AA-2G concentrations agreed with the initial concentration of 6-sDode-AA-2G (1 mM), indicating that a rapid decrease of 6-sDode-AA-2G in neutral aqueous solutions was caused by intramolecular acyl migration.

In order to purify the acyl-migrated isomer, 6-sDode-AA-2G (416.0 mg) was incubated in 80 ml of sodium phosphate buffer (pH 7.0) at 37 °C for 3 h. The reaction mixture was chromatographed on a Diaion HP20 column. 6-sDode-AA-2G- and its isomer-containing fraction was concentrated to dryness. The residue was recrystallized to remove 6-sDode-AA-2G as a crystal. The resulting filtrate containing an acyl-migrated isomer was repeatedly purified by semi-preparative HPLC. Finally, the acyl-migrated isomer and 6-sDode-AA-2G were obtained in yields of 15.9 and 347.1 mg, respectively. The acyl-migrated isomer was determined to be 5-*O*-dodecanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (5-sDode-AA-2G, Fig. 5) by the mass spectrum and ^{1}H NMR spectrum. Recently, when we synthesized 5-sDode-AA-2G from AA-2G and vinyl laurate with a protease from *Bacillus subtilis* in DMF with 3% (v/v) of water, 5-sDode-AA-2G was fully characterized and identified by the mass spectrum and several NMR spectra (^{1}H, ^{13}C, ^{1}H–^{1}H COSY, HSQC, and HMBC).[24] The ^{1}H NMR data agreed well with our previous data for 5-sDode-AA-2G synthesized enzymatically.

Intramolecular acyl migration and enzymatic hydrolysis of 6-sDode-AA-2G in the liver, small intestine, and kidney homogenates were examined (Fig. 6). The hydrolysates from 6-sDode-AA-2G were measured by HPLC analysis. Each tissue homogenate from guinea pigs was thoroughly dialyzed to remove tissue-intrinsic AA before use as an enzyme source. After 1 h of incubation, 6-sDode-AA-2G was hydrolyzed by the liver, small intestine, and kidney homogenates to give two hydrolysates, AA-2G and AA. These results agree well with the hydrolysis pattern of 6-sAcyl-AA-2G with various tissue homogenates and with a human living skin equivalent model described previously.[11,16] The amount of AA-2G was much larger than that of AA. 6-*O*-Dodecanoyl AA (6-sDode-AA) and 5-*O*-dodecanoyl AA (5-sDode-AA) as hydrolysates were not observed. 6-sDode-AA-2G also underwent partial acyl migration to give 5-sDode-AA-2G. The concentration ratios of 6-sDode-AA-2G to 5-sDode-AA-2G in the liver, small intestine and kidney homogenates were 552:46.4, 157:36.0, and 595:51.4, respectively. After 12 h of incubation, 6-sDode-AA-2G was almost completely hydrolyzed by all of the tissue homogenates. The hydrolysis of 5-sDode-AA-2G also proceeded with passage of time. In the liver and kidney homogenates, increase in AA and AA-2G was observed with decrease in 6-sDode-AA-2G and 5-sDode-AA-2G. In the small intestine homogenate, increase in AA was observed with decrease in 6-sDode-AA-2G, 5-sDode-AA-2G and AA-2G. 6-sDode-AA and 5-sDode-AA were not observed even after 12 h of incubation. These results indicated that 6-sDode-AA-2G underwent acyl migration to give ca. 10% of 5-sDode-AA-2G under neutral conditions and that 6-sDode-AA-2G and the resulting 5-sDode-AA-2G were predominantly hydrolyzed with esterase to AA-2G and then with α-glucosidase to AA (Fig. 7).

AA, also known as vitamin C, has various physiological and pharmacological functions in collagen synthesis,[25] intestinal absorption of iron,[26] and drug-metabolizing enzymes,[27] and acts as an important biological antioxidant.[28] Since humans, monkeys, and guinea pigs cannot synthesize AA due to a lack of L-gulono-γ-lactone oxidase, they must take this essential nutrient from foods. In our previous studies, 6-sAcyl-AA-2G and 6-bAcyl-AA-2G (Fig. 1) showed satisfactory bioavailability as an AA source, a pro-vitamin C agent, in in vitro and in vivo experiments.[9,11,16–19] This study showed that one of the 6-sAcyl-AA-2G compounds, 6-sDode-AA-2G, underwent an intramolecular acyl migration to yield the isomer in neutral aqueous solutions, and the acyl-migrated isomer was isolated and characterized as 5-sDode-AA-2G. 5-sDode-AA-2G, as well as 6-sDode-AA-2G, was predominantly hydrolyzed with esterase to AA-2G and then with α-glucosidase to AA. Therefore, 6-sDode-AA-2G is considered to be an available source of vitamin C activity.

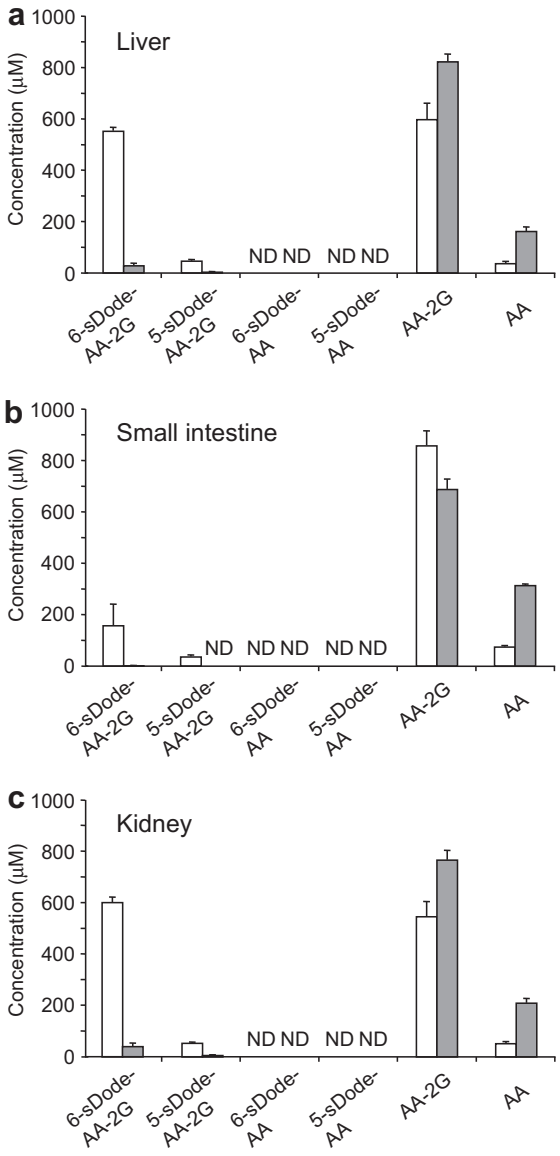


Figure 6. Intramolecular acyl migration and enzymatic hydrolysis of 6-sDode-AA-2G in various tissue homogenates from guinea pigs. Homogenates from the liver (a), small intestine (b), and kidney (c) were used as crude enzymes. Each enzyme and 6-sDode-AA-2G were incubated for 1 h (open column) and 12 h (gray column) at 37 °C. Each hydrolysate in the reaction was analyzed by HPLC. Each value is the means + SEM (n = 6). ND, not detectable.

We have investigated, by a skin model, the characteristics of 6-sAcyl-AA-2G compounds as skin antioxidants.[9] In a series of 6-sAcyl-AA-2G compounds, 6-sDode-AA-2G showed the best permeability in the skin model.[9,11] Because pH of the skin surface is ca. 5,[29] it seems that 6-sDode-AA-2G is not converted to 5-sDode-AA-2G but is mainly metabolized via AA-2G to AA in the skin. In the other tissues under neutral conditions, 6-sDode-AA-2G seems to be metabolized by a pathway shown in Figure 7. These aspects of 6-sDode-AA-2G will provide a further basis for its use not only

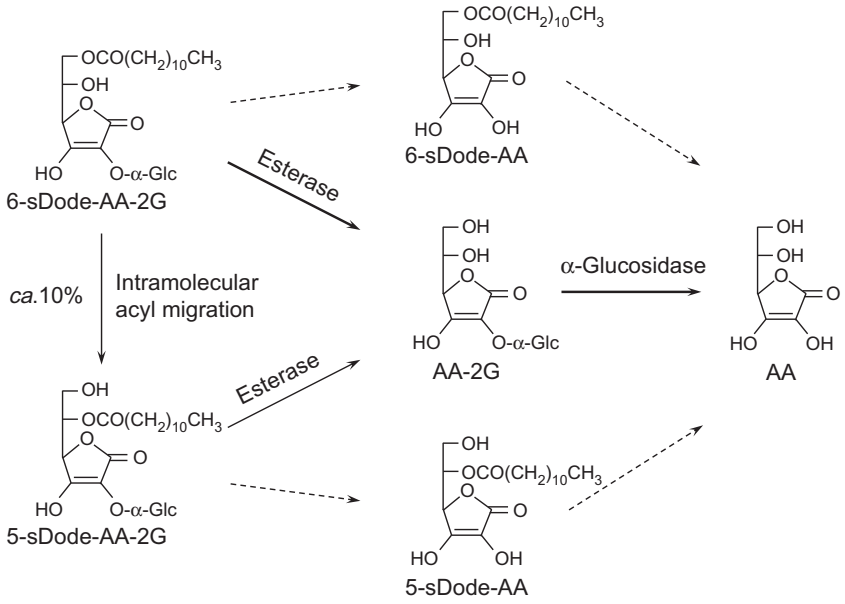


Figure 7. Proposed metabolic pathway of 6-sDode-AA-2G under neutral conditions. Heavy arrows, major pathway; light arrows, minor pathway; dashed arrows, trace pathway.

as an ingredient in skin care but also as an effective pharmacological agent and as a promising food additive.

Acknowledgments

The authors thank Hayashibara Biochemical Laboratories, Inc., Okayama, Japan for the supply of AA-2G. The authors are grateful to the MS Laboratory of Faculty of Agriculture, Okayama University.

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