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2-O-(β-D-Glucopyranosyl)ascorbic Acid, a Novel Ascorbic Acid Analogue Isolated from *Lycium* Fruit

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A novel stable precursor of ascorbic acid (vitamin C), 2-*O*-(β-D-glucopyranosyl)ascorbic acid, was isolated from both the ripe fresh fruit and dried fruit of *Lycium barbarum* L., a plant of the Solanaceae family. The chemical structure was inferred by instrumental analyses and confirmed by chemical synthesis. The dried fruit of *Lycium barbarum* L. contained ca. 0.5% of it, which is comparable to the ascorbic acid content of fresh lemons. It increased the blood ascorbic acid by oral administration to rats, and it was also detected in blood from the portal vein.

KEYWORDS: Vitamin C; ascorbic acid; *Lycium* fruit

INTRODUCTION

The physiological roles of ascorbic acid are well established. It is involved in collagen biosynthesis and is a biological antioxidant and a catalyst of oxidation−reduction reactions of the iron ion in cytochrome *c* (*1*). Ascorbic acid is widely used as a nutrient in the pharmaceutical and cosmetic industries as well as a preservative on the basis of its antioxidant activity (*2*). However, ascorbic acid is extremely unstable on exposure to light, heat, oxygen, and metal ions. Various attempts to modify the structure have been made in order to improve the stability and alter its physical properties.

It has been attempted to introduce substituents at the hydroxyl groups of the 2,3-enediol, which is the unstable and antioxidizing moiety of ascorbic acid, to develop a more stable ascorbic acid, which is easily able to cleave the bond(s) to generate active ascorbic acid in the body, known as "provitamin C". For examples, the 2-*O*-sulfate (*3*) and 2-*O*-phosphate (*4*) have greatly enhanced stability compared to unmodified ascorbic acid and are known to be converted to ascorbic acid in vivo and by intracellular hydrolysis through the action of sulfatases or phosphatases. These compounds are already used in cosmetics and quasi-drugs. Stable forms of ascorbic acid that are glycosylated at the 2- or 3-hydroxyl groups are also known. For example, 2-*O*-(α-D-glucopyranosyl)ascorbic acid was produced by glucosyltransferase (*5*). 2-*O*-(β-D-Galactopyranosyl)ascorbic acid was obtained by galactosidase (*6*), and 3-*O*-(β-D-glucopyranosyl)ascorbic acid was also obtained by chemical synthesis (*7*).

2-*O*-(α-D-Glucopyranosyl)ascorbic acid has been the most thoroughly investigated of the three and is currently used in cosmetics and quasi-drugs and as a food additive in Japan. The α-glucoside is highly stable to acidic and various oxidative conditions. Following oral administration it is hydrolyzed by α-glucosidases present in the digestive organs (saliva, intestinal digestive juices, and the small intestinal tract) to generate active ascorbic acid. It is also hydrolyzed at a moderate rate by enzymes present in the cell membrane of cultured cells, whereby the action of ascorbic acid is exhibited continuously.

Lycium chinense Miller, a plant of the Solanaceae family, is listed in the ancient Chinese medical text "Compendium of Materia Medica". The ripe fruit of this plant known as *lycii fructus* and the leaves known as *lycii folium* are used as foods, while the root, known as *lycii cortex radicis*, is used as a Chinese herbal medicine (*8*). Plants of the *Lycium* species are known to contain betaine, carotene, nicotinic acid, zeaxanthin, and a cerebroside (*9*) and exhibit hypoglycemic, antihypertensive, lipotropic, and hepatic function-protecting effects. The lipotropic and hepatic function-protecting effects are attributed to betaine, which acts as a methyl group donor (*10*). A plant extract of the *Lycium* species has been reported to promote growth and lactic acid production in some bacteria (*11*).

Some effects cited in "Compendium of Materia Medica" cannot be explained by the known components listed above, such as the ability to benefit complexion and maintain beauty, and antiaging properties. These effects of the *Lycium* fruits could be attributed to the actions of ascorbic acid. However, no evidence could be attained for the presence of ascorbic acid in either the fresh or dried fruit of *Lycium barbarum* L. It was proposed that a provitamin C could be present, and research was consequently undertaken to isolate such a substance. Thus, we carefully examined water-soluble fractions of the dried fruits, and found a new analogue of ascorbic acid, 2-*O*-(β-D-glucopyranosyl)ascorbic acid (**1**) (Figure 3).

MATERIALS AND METHODS

Plant Materials. Fresh and dried fruits of *Lycium* species (*Lycium barbarum* L. harvested in Ningxia and Neimonggol and *Lycium*

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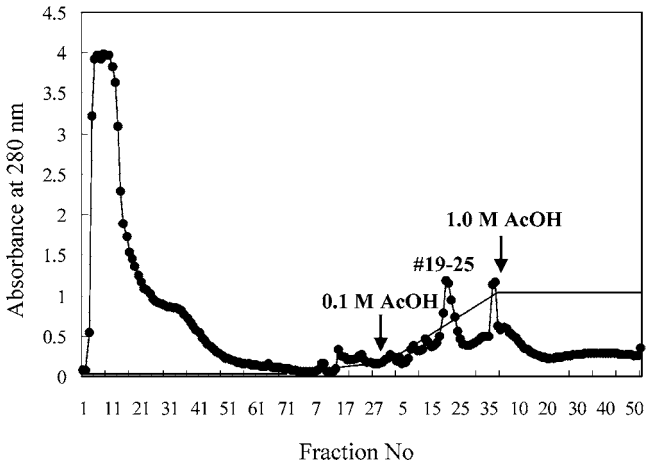

Figure 1. Elution pattern of the extract of dried fruit of *Lycium barbarum* L. on Dowex 1-X8 column chromatography.

chinense Miller harvested in Hebei in China) were imported by Shinwa Bussan Co., Ltd.

Chemicals. Chemicals used were of reagent grade.

Animals. Guidelines from the Prime Minister's Office of Japan (no. 6 of 27 March 1980) were followed for the care and use of laboratory animals. Ten-week-old male Wistar rats were purchased from Nihon Charles River Co.

Instrumentation. NMR [^{1}H, ^{13}C, double quantum filtered correlated spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence spectroscopy (HSQC), and heteronuclear multiple bond correlation spectroscopy (HMBC)] were recorded on a Bruker DRX 750 spectrometer. ^{1}H NMR were also recorded on a Bruker DRX 400 spectrometer. High-resolution mass and fast atom bombardment (FAB) mass spectra (negative and positive mode) were recorded on a JEOL JMS HX-110/110A spectrometer. The infrared spectrum was recorded on a Mattson FT-IR. High-performance liquid chromatography (HPLC) for purification was performed with a type 305 master pump equipped with a type 116 UV detector and FC-203 type B fraction collector (Gilson). Analytical HPLC for measuring the content of **1** in plants and blood concentration of ascorbic acid and **1** were performed by a LC-10Ai System (Shimadzu Co., Ltd.).

Isolation and Purification of 1 from *Lycium barbarum* L. After 100 g of dried fruit of Ningxia *Lycium barbarum* L. was pulverized with a model TS-10M tablet pulverizer (Tosho Co., Ltd.), the material was immersed in 800 mL of 30% EtOH at room temperature for 6 days, followed by filtration, concentration under reduced pressure, and lyophilization to obtain 65.7 g of extract. A 1.94 g portion of the extract was dissolved in distilled water to make 40 mL of the sample solution (pH 4.5, electric conductivity 1.7 mS/cm). It was passed through a 1.5 × 12 cm Dowex 1-X8 column (acetate form,) at SV (space velocity) = 1. It was then washed with 200 mL of distilled water and subjected to linear gradient elution (100 mL × 2) with 0−0.1 M acetic acid (AcOH), subjected to linear gradient elution (100 mL × 2) with 0.1−1.0 M AcOH, and then eluted with 1.0 M AcOH. The fractions were monitored at 280 nm (Figure 1). Fractions 19−25 with 0.1−1.0 M AcOH linear gradient elution were collected and lyophilized to give 26 mg of powder. A portion was further purified by HPLC. The conditions were as follows. column, 4.6 × 250 mm i.d., 5 μm, ODS-UG-5 (Nomura Chemical Co., Ltd.); mobile phase, 5% MeOH/20 mM ammonium formate/5 mM di-*n*-butylamine acetate; flow rate, 0.5 mL/min; detection wavelength, 254 nm; fractionation at 0.5 min increments. The fraction detected at 254 nm was concentrated under reduced pressure and lyophilized to obtain a high-purity product, which was analyzed by FAB mass spectrometry and NMR spectrometry.

Synthesis of 1. After 5,6-*O*-isopropylideneascorbic acid, **2** (2.0 g, 9.3 mmol) was dissolved in DMSO (20 mL), potassium carbonate (1.3 g, 9.4 mmol) and benzyl bromide (1.1 mL, 9.3 mmol) were added and the mixture was stirred at 50 °C for 4 h. Water was added to the reaction solution, which was then acidified with 1 N HCl, extracted with ethyl acetate (EtOAc), washed with water and then with saturated NaCl, dried with anhydrous $MgSO_4$, concentrated under reduced pressure, and purified by silica gel chromatography (EtOAc/*n*-hexane 3:1) to obtain 1.1 g of 3-*O*-benzyl-5,6-*O*-isopropylideneascorbic acid, **3** (39% yield).

A mixture of **3** (0.6 g, 2.0 mmol) and (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)carbonic acid 2,2,2-trichloroethyl ester (**4**, 2.1 g, 4.0 mmol) was heated at 120−130 °C to melting. After 3 h of reaction, the reaction solution was purified by column chromatography (gradient from 25% to 50% EtOAc/*n*-hexane) to obtain 850 mg of 2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-3-*O*-benzyl-5,6-*O*-isopropylideneascorbic acid, **5** (67% yield).

Compound **5** (850 mg, 1.3 mmol) was dissolved in EtOAc (40 mL), 10% Pd−C (200 mg) was added, and the mixture was stirred under hydrogen atmosphere. After 2 h, the catalyst was filtered off and the filtrate was concentrated to yield approximately 750 mg of 2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-5,6-*O*-isopropylideneascorbic acid.

The debenzylated compound (500 mg, 0.9 mmol) was dissolved in AcOH (5 mL), water (5 mL) was added, and the mixture was heated at 50−60 °C for 1.5 h while being stirred. After concentration of the reaction solution, the residue obtained was extracted with EtOAc, washed with water and then with saturated NaCl, dried with anhydrous $MgSO_4$, and concentrated under reduced pressure, and then the residue obtained was recrystallized from EtOAc/*n*-hexane to obtain 320 mg of 2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)ascorbic acid, **6** (48% yield). ^{1}H NMR [δ (parts per million, ppm), DMSO-d_6] 1.94−2.01 (12H), 3.42 (3H, m), 3.7−4.3 (4H, m), 4.7−5.1 (4H, m), 5.3−5.4 (2H, m), 12.00 (1H, br s). FABMS $(M + H)^+$ *m*/*z* 507.

After **6** (300 mg, 0.6 mmol) was dissolved in MeOH (10 mL), a solution of K_2CO_3 (600 mg) in water (9 mL) was added and the mixture was stirred for 30 min. The reaction solution was neutralized with IR-120 (H^+), the resin was filtered off, and washing was performed with MeOH and 50% aq MeOH. The filtrate and washing solution were combined and concentrated, and then water was added and the mixture was lyophilized to obtain **1** as amorphous crystals (190 mg, 100% yield). ^{1}H NMR [δ (ppm), D_2O] 3.1−3.3 (4H, m), 3.4−3.5 (3H, m), 3.58 (1H, d), 3.80 (1H, t), 4.61 (1H, d), 4.66 (1H, d). FABMS $(M - H)^-$ *m*/*z* 337.

Measurement of Content of 1 in Plants of *Lycium* Species. Extracts obtained by immersing 3 g of different dry plants in a 10-fold volume of 70% EtOH at room temperature for 7 days were diluted 10-fold with 1.5% metaphosphoric acid/5 M KOH (pH 3.5) and used as test samples for identification of naturally occurring **1**, based on the retention time of 2.63 min in analytical HPLC of chemically synthesized **1** (using a 4.6 × 150 mm i.d., 5 μm Inertsil ODS-3 column (GL Science Co., Ltd.), mobile phase 20% MeOH−20 mM H_3PO_4−5 mM tetra-*n*-amylammonium bromide; flow rate 1.0 mL/min; column temperature 35 °C; detection wavelength 254 nm.

Intestinal Absorption of 1 in Rat. A solution of **1** in Milli-Q ultrapure water (Millipore Corporation) (100 mg/4 mL) was orally administered at a dose of 100 mg/kg by using a feeding tube to overnight-starved and awake test rats ($n = 3$). After 0, 0.5, 2, and 4 h, heparinized blood samples were collected from the portal vein. Each sample was separated by centrifugation (6000*g*, 10 min), and after addition of an equivalent of ice-cooled 10% metaphosphoric acid, containing 40 mM deferoxamine mesylate, the mixture was centrifuged (10000*g*, 10 min) to obtain protein-removed portal vein plasma, of which the unchanged **1** and ascorbic acid concentrations were measured by HPLC on a 4.6 × 150 mm i.d., 5 μm Inertsil ODS-3 column (GL Science Co., Ltd.), mobile phase 15% MeOH−17 mM KH_2PO_4/H_3PO_4 (pH 3.5)−5 mM tetra-*n*-amylammonium bromide; flow rate 0.3 mL/min; column temperature 35 °C; detection wavelength 254 nm.

RESULTS AND DISCUSSION

Isolation and Purification of 1 from *Lycium barbarum* L. The hot water extract of the dried fruit (*lycii fructus*) from *Lycium barbarum* L. was analyzed by HPLC to evaluate ascorbic acid. UV detection (254 nm) revealed a peak that could not be attributed to ascorbic acid. The extract was also found to contain some organic acids such as citric, malonic, succinic, maleic, and lactic acids as well as glucose and fructose. From

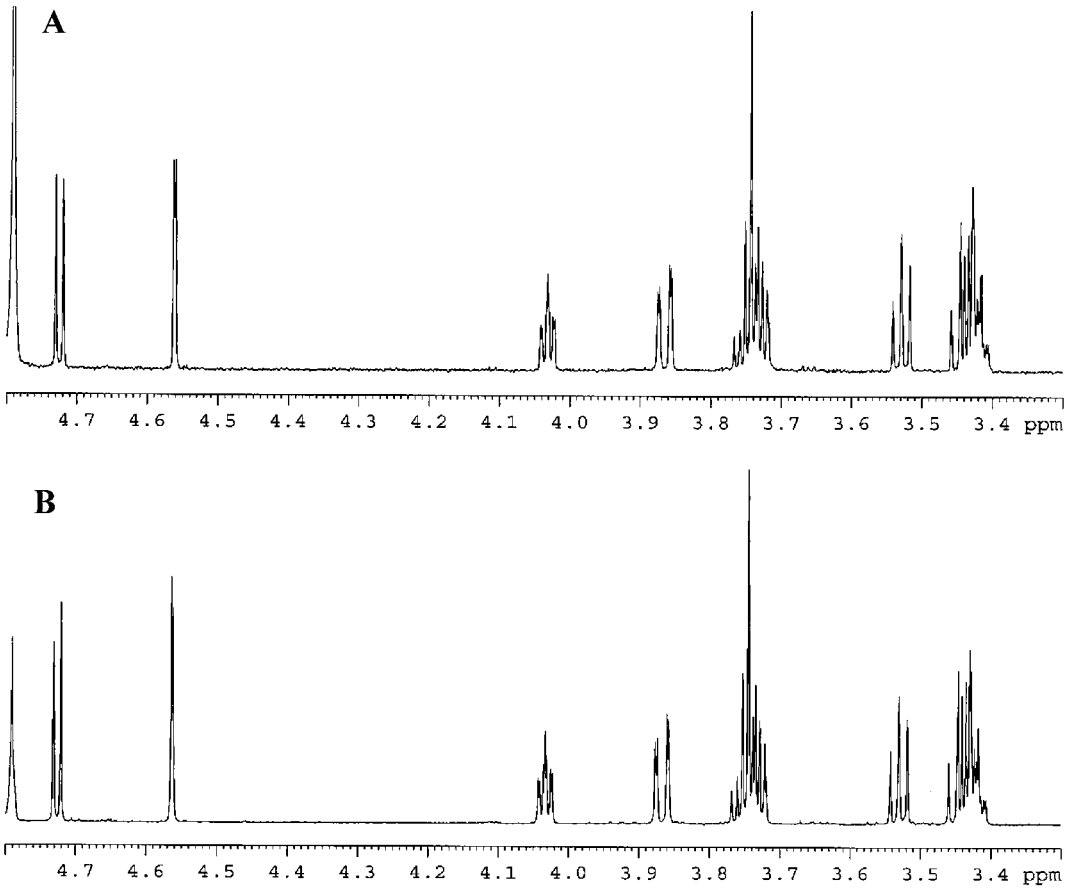

Figure 2. ^{1}H NMR spectra from a 750 MHz NMR spectrometer. (A) Natural product isolated from fruit of *Lycium barbarum* L.; (B) synthetic product.

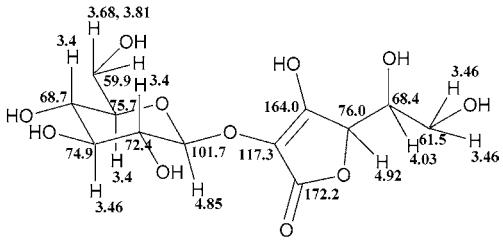

Figure 3. Assignment of ^{1}H and ^{13}C chemical shifts of the natural product.

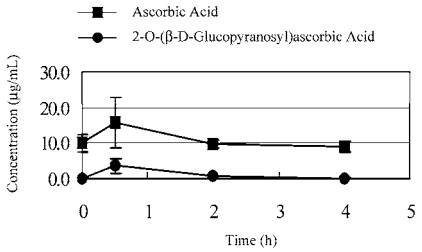

Figure 4. Time course of the blood concentration of **1** and vitamin C. An aqueous solution of **1** was orally administered at a dose of 100 mg/kg. Data represent the mean ± SD of three independent measurements.

the results, we undertook to isolate the substance detected at 254 nm from the extract.

The extract was chromatographed on the anion-exchange resin Dowex 1-X8 eluted with aq AcOH and monitored at 280 nm (Figure 1) to isolate the substance detected at 280 nm. The UV-detected fractions were eluted by a linear gradient of 0.1−1.0 M AcOH and collected in 1.4% yield. A quantity was subsequently purified by HPLC and the product was analyzed to elucidate the chemical structure.

Chemical Structure Determination of the Compound Isolated from Dried Fruits of *Lycium barbarum* L. Mass spectrometry revealed the $(M - H)^-$ peak at *m*/*z* 337.1 in a negative-ion FAB spectrum. On this basis it was speculated that the molecular weight of this compound was 338, and high-resolution mass spectrometry (HRMS) indicated the molecular formula to be $C_{12}H_{18}O_{11}$. Evidence from both TOCSY and COSY ^{1}H NMR spectra suggested that the molecule consisted of a D-glucose moiety and β-glucoside linkage, based on the coupling constant of the anomeric proton (δ_H 4.85, d, $J = 8.0$ Hz), and H_3 (δ_H 3.46, t, $J = 9.1$ Hz), respectively. The ^{1}H NMR spectrum is shown in Figure 2A.

The TOCSY and COSY spectra revealed that the aglycon portion had a structural moiety of -CH-CH-CH_2- (δ_H 4.92, d, $J = 1.86$ Hz; δ_H 4.03, ddd, $J = 1.86$, 6.07, and 7.55 Hz; and δ_H 3.46, 2H, respectively). The chemical shifts of the protons led to the proposal that the carbons were directly bound to oxygen atoms. The infrared spectrum showed carbonyl stretching bands at 1681, 1600, and 1366 cm^{-1} and hydroxyl stretching bands at 3350 and 2955 cm^{-1}. On the basis of this evidence, the aglycon moiety was proposed to be ascorbic acid. The NMR spectra of this moiety are very similar to those of ascorbic acid calcium salt hydrate.

Following identification of ascorbic acid as the aglycon moiety, it was necessary to establish the position in which ascorbic acid was bound to D-glucose. The HMBC spectrum confirmed the bond position, as a correlation between the anomeric proton (δ_H 4.85) of D-glucose and the carbon in the 2-position of ascorbic acid (δ_C 117.3) was evident. Therefore, the structure was proposed to be 2-*O*-(β-D-glucopyranosyl)-ascorbic acid, **1**. The chemical shifts of both ^{1}H NMR and ^{13}C NMR were assigned as shown in Figure 3.

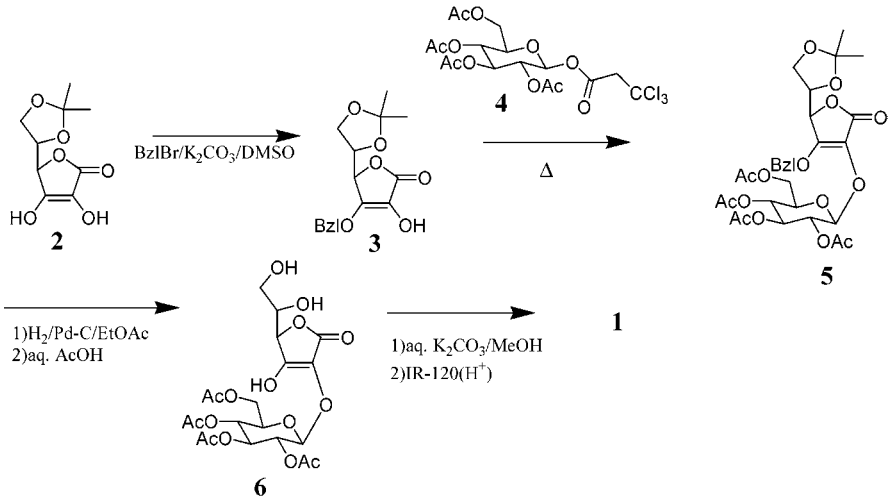

Figure 5. Synthesis of 2-O-(β-D-glucopyranosyl)ascorbic acid (**1**).

Synthesis of 2-O-(β-D-Glucopyranosyl)ascorbic Acid (1). Confirmation of the structure of **1** was achieved by chemical synthesis of the natural product as shown in Figure 5.

The intermediate **6** was synthesized in the following manner. Specifically, commercially available **2** was selectively benzylated at the 3-hydroxyl position by a known method (*12*), to produce **3**. The compound **3** as the aglycon was reacted with **4** (*13*) by heating at 120−30 °C without a solvent to obtain the condensed product, **5**. According to Komura (*13*), **2** in which both the 2- and 3-hydroxyl group are free was glycosylated with **4** under the same condition to generate the 3-*O*-glucoside, not the 2-*O*-glucoside because the 3-hydroxyl group of **2** is more acidic and reactive than the 2-hydroxyl group. The benzyl group of **5** was removed by ordinary hydrogenolysis in the presence of Pd−C, followed by deisopropylidenation with aq AcOH at 50 °C to obtain **6**. Compound **6** was treated with K_2CO_3 to remove the acetyl groups, and then the reaction solution was neutralized with a cation-exchange resin, IR-120 (H^+). The neutralized solution was lyophilized to obtain **1**.

Chemical Structure Confirmation of the Isolated Product by Synthetic 1. Synthetic **1** was confirmed by NMR and negative-ion FAB (*m*/*z* 337). The ^{1}H NMR spectrum of **1** as well as other NMR spectra (data not shown) proved identical to that of the isolated natural product, as shown in Figure 2. Thus, the natural product in *Lycium* fruit was confirmed as 2-*O*-(β-D-glucopyranosyl)ascorbic acid.

Measurement of Content of 1 in a Plant of *Lycium* Species. A peak corresponding to **1** was found in extracts of *lycii fructus* (dried fruit) from Neimonggol *Lycium barbarum* L., Hebei *Lycium chinense* Miller, and Ningxia *Lycium barbarum* L., although it was not detected in either leaf (*lycii folium*) and root (*lycii cortex radicis*). It was also not found in other plants of the Solanaceae family such as green pepper and tomato, nor other fruits such as barbados cherry, lemon, camu-camu, sea buckthorn, and grapefruit, which contain plenty of ascorbic acid. Therefore, **1** was localized only in fruit of the *Lycium* species. **1** might be synthesized and accumulated in the fruit. The content of **1** of each extract was analyzed by use of the calibration curve obtained from chemically synthesized **1** by HPLC. As the results, the contents in the extracts were determined to be from 0.86% to 1.2% in all of them, that is, the content in dried fruits is about 0.5%.

Intestinal Absorption of 1 in Rats. It is a question whether **1** is useful as a provitamin C or not. β-Glucosidase is known to be present in membrane-bound form in small intestine tissue and in cytoplasmic form in hepatic and renal tissue (*14*). α-Glucosidase is widely distributed in the digestive organs, where it presumably hydrolyzed 2-*O*-(α-D-glucopyranosyl)-ascorbic acid to ascorbic acid on oral uptake. Yamamoto et al. (*15*) describes that only ascorbic acid is detected in the blood in an experiment with oral administration of 2-*O*-(α-D-glucopyranosyl)ascorbic acid to rats, thus suggesting that instead of activation by α-glucosidase, which is widely distributed in the body, hydrolysis and activation of **1** by β-glucosidase, which is less widely distributed, would be more advantageous in terms of the transport into tissues and long-lasting action.

As shown in Figure 4, the unchanged **1** and the metabolite (ascorbic acid) were found to be present in maximum amounts at 30 min in blood after oral administration of **1** to rats. The result indicates that it can be absorbed in unchanged form through the intestinal tract. On the other hand, as mentioned above, 2-*O*-(α-D-glucopyranosyl)ascorbic acid is not detected in blood on oral administration, therefore it is almost completely hydrolyzed in the digestive organs to exist in blood as ascorbic acid. Thus, **1** existing in blood could migrate into the tissue, and then it is more likely activated to ascorbic acid in the tissues and cells.

1 was contained in dried fruits of *Lycium barbarum* L. in about 0.5%, and in fresh fruits in 0.2−0.3%, which is comparable to the ascorbic acid content of fresh lemons. Some of the healthy effects of fresh fruits or dried fruits of *Lycium* species may originate from **1**.

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