Radical Scavenging Activity against 1,1-Diphenyl-2-picrylhydrazyl of Ascorbic Acid 2-Glucoside (AA-2G) and 6-Acyl-AA-2G

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The radical scavenging activity of the stable derivatives, which are *O*-substituted at the C-2 position of ascorbic acid (AA), against 1,1-diphenyl-2-picrylhydrazyl (DPPH) was evaluated in buffer under different pH conditions, and compared with those of AA and α-tocopherol. AA was shown to have 50% radical scavenging ability (EC_{50}) at a concentration of 2.2×10^{-5} M against 0.1 mM DPPH in 60% ethanol. Ascorbyl 6-palmitate, a lipophilic AA derivative which has a free endiol group and is therefore unstable, also showed potent radical scavenging activity with EC_{50} of 2.9×10^{-5} M. A typical lipophilic antioxidant, α-tocopherol gave a similar EC_{50} value as that of AA. In contrast, ascorbyl 2,6-dipalmitate, AA 2-phosphate and AA 2-sulfate exhibited negligible scavenging activity. On the other hand, 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) and a series of 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acids (6-Acyl-AA-2G) themselves exhibited the radical scavenging activity of EC_{50}: 6.1×10^{-5} M and 4.4×10^{-5}—5.9×10^{-5} M, respectively, although their activities were lower than that of AA. Among 6-Acyl-AA-2G derivatives, the EC_{50} values tended to decrease with increasing length of their acyl carbon group. Increasing pH of the buffer resulted in decrease in the scavenging activity of all compounds tested as expected. We speculate that the difference in the radical scavenging activity of derivatives *O*-substituted at the C-2 position of AA may be ascribed to the linkage type of the substituent group to the endiol-lactone resonance system and the degree of dissociation of the C-3 proton.

Key words ascorbic acid 2-glucoside; 6-acyl ascorbic acid 2-glucoside; radical scavenge; lipophilic ascorbate; stable ascorbate

It is now generally accepted that oxygen radicals play a vital role *in vivo* not only as a mediator of signal transduction but also as causative species for oxidative damage of membranes and tissues, eventually resulting in a variety of diseases, cancer, aging *etc.*[1,2] Vitamin C acts as a potent water-soluble antioxidant in biological fluids by scavenging the reactive oxygen species.

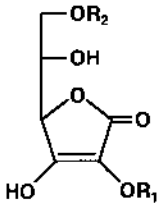
The well-known susceptibility of vitamin C to thermal and oxidative degradation has led to interest in derivatives with increased stability *in vitro.* In particular, the chemical modification of hydroxyl group at the C-2 position of vitamin C is of interest. 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (ascorbic acid 2-glucoside, AA-2G)[3,4] and a series of 6-acyl ascorbic acid 2-glucoside (6-Acyl-AA-2G), and ascorbic acid 2-phosphate (AA-2P),[5,6] ascorbic acid 2-sulfate (AA-2S),[7,8] 2-*O*-octadecylascorbic acid (CV-3611),[9] and ascorbyl 2,6-dipalmitate (2,6-Palm-AA) have been developed as stable derivatives (Fig. 1) and it is found that they themselves have no two-electron reductivity.

The stable ascorbate derivatives *O*-substituted at the C-2 position are roughly divided into two groups. The first group is composed of the derivatives which can exhibit the inherent vitamin C activity of radical scavenging activity as well as anti-scorbutic activity and collagen synthesis *in vivo* after enzymatic degradation. The second group is composed of the derivatives which are not capable of exhibiting these vitamin C activities, because they are not enzymatically metabolized *in vivo.*[10] AA-2G, 6-Acyl-AA-2G, AA-2P and 2,6-Palm-AA, which themselves have no two-electron reductivity, belong to the former group, but they are demonstrated to exhibit vitamin C activity *in vivo* after enzymatic hydrolysis by α-glucosidase, α-glucosidase and esterase (unpublished data), phosphatase, and esterase, respectively, as suggested by their biological effects and examinations with given enzymes.

In the present article, we examined whether the derivatives *O*-substituted at the C-2 position of AA scavenge the free-radicals under non-enzymatic conditions, and compared their ability with ascorbate and α-tocopherol.

The radical scavenging activity of AA-2G and other AA derivatives was measured using a relatively stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). The results are summarized in Table 1. At 0.1 mM which corresponds to an equal molar amount of DPPH, AA-2G and 6-Acyl-AA-2G exhibited the radical scavenging activity (68.9—77.5%), although their activities were lower than that of AA (94.3%), a conventional lipophilic ascorbate derivative, 6-Palm-AA (95.7%), and a typical lipophilic antioxidant, vitamin E (90.9%). It was also found that the activities of all 6-Acyl-AA-2G tested were slightly superior to AA-2G, and the scavenging activity gradually rose with increasing length of their acyl group. In contrast, 2,6-Palm-AA, AA-2P and AA-2S, which are stable 2-*O*-substituted ascorbate derivatives like AA-2G and 6-Acyl-AA-2G, exhibited only limited activity.

The EC_{50} value was also determined as the concentration of each sample required to give 50% of the absorbance shown by a blank test. The EC_{50} values of all of 6-Acyl-AA-2G (4.1—5.9×10^{-5} M) were lower than that of AA-2G (6.1×10^{-5} M), indicating that 6-Acyl-AA-2G are more efficient than AA-2G in terms of the radical scavenging potency. As mentioned above, the EC_{50} values also suggested that 6-



Compound	R_1	R_2
Ascorbic acid	H	H
Ascorbyl 6-palmitate	H	$COC_{15}H_{31}$
Ascorbyl 2,6-palmitate	$COC_{15}H_{31}$	$COC_{15}H_{31}$
Ascorbic acid 2-phosphate	PO_3^{2-}	H
Ascorbic acid 2-sulfate	SO_3^-	H
CV-3611	$C_{18}H_{37}$	H
Ascorbic acid 2-glucoside	Glc	H
6-Acyl-AA-2G	Glc	COR

Fig. 1. Chemical Structures of AA and Its Derivatives

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Acyl-AA-2G tended to increase in scavenging activity with increasing length of their acyl groups. The EC_{50} values of 6-Palm-AA (2.9×10^{-5} M) and α-tocopherol (1.9×10^{-5} M) were shown to be almost the same as that of AA (2.2×10^{-5} M). In contrast, the EC_{50} of 2,6-Palm-AA, AA-2P and AA-2S was not determined, indicating that the radical scavenging activities are extremely weak.

The effects of pH and polarity on the EC_{50} value of AA-2G and 6-Acyl-AA-2G were investigated in four different solvents, namely, 60% EtOH/40% water, 60% EtOH/40% pH 5.0 or pH 7.4 buffer and EtOH. The EC_{50} of AA, AA-2G and 6-Acyl-AA-2G was found to decrease sharply with increase in pH (Table 2). In 60% EtOH/40% pH 5.0 buffer, the scavenging activity of AA-2G and 6-Acyl-AA-2G was about one eighth to one ninth of that observed in 60% EtOH/40% water, although AA and α-tocopherol retained high activity. In 60% EtOH/40% pH 7.4 buffer, the activity disappeared in all AA derivatives and in α-tocopherol, while slight activity of AA was still observed. In EtOH, the radical scavenging activity of AA was reduced to one-half the value in 60% EtOH, whereas the activities of AA-2G and 6-Acyl-AA-2G were not changed.

One molecule ascorbate can scavenge two DPPH radicals by releasing two hydrogen radicals, while in the case of 2-*O*-substituted AA derivatives, the one molecule can release only one hydrogen radical. Thus, the decreased radical scavenging activity of 2-*O*-substituted AA derivatives could, in principle, be explained by the inherent electron donating potency. Our results show the diversity of the radical scavenging activity among 2-*O*-substituted ascorbate derivatives. This could be attributed to the linkage type of the substituent group to the enediol–lactone resonant system. AA-2G and 6-Acyl-AA-2G have ether linkages, while 2,6-Palm-AA, AA-2P and AA-2S possess ester linkages. Thus, introduction of electron-attracting groups such as phosphate and sulfate into the enediol–lactone resonant system may hinder dehydrogenation at the C-3 of AA, resulting in the reduced radical scavenging activity.

The radical scavenging activity of AA and its derivatives was found to decrease sharply with increase in pH. The pH dependent activity of a-tocopherol was similar to that of AA derivatives, the degree of dissociation at the reactive sites. So far, the reason there is only a small difference in the activity between AA and AA derivatives in low polar regions is unclear. In any event, 6-Acyl-AA-2G may function as an efficient radical scavenging agent in the biomembrane and corneum *etc*.

Our present findings, and the high stability and susceptibility[10] to enzymatic hydrolysis of AA-2G and 6-Acyl-AA-2G suggest that both glucosides are promising ascorbate derivatives as an AA source for medical and skin care applications.

Table 1. Radical Scavenging Activity of 6-Acyl-AA-2G and other AA Derivatives against DPPH in 60% EtOH aq

Antioxidant	Scavenging activity (%)	EC_{50} (10^{-5} M)
AA	94.3±1.7	2.2
6-Palmi AA	95.7±0.1	2.9
2,6-Palmi AA	23.7±7.2	—
AA-2P	1.5±0.7	—
AA-2S	0.0±0.8	—
AA-2G	68.9±1.1	6.1
6-Buty-AA-2G	69.2±3.6	5.9
6-Hexa-AA-2G	73.8±1.2*	5.4
6-Octa-AA-2G	76.6±0.7**,†	5.7
6-Deca-AA-2G	73.6±3.7	4.2
6-Dode-AA-2G	76.2±0.8**	4.6
6-Myri-AA-2G	74.1±1.2**	4.4
6-Palm-AA-2G	76.4±1.4**	4.1
6-Stea-AA-2G	77.5±0.1***,†	4.4
α-Tocopherol	90.9±0.7	1.9

After 20 min-reaction at 25 °C, the free-radical scavenging activity of each antioxidant was quantified by the decolorization of DPPH (10^{-4} M) at 516 nm. The fifty percent of effective concentration (EC_{50}) value was determined as the concentration of each sample required to give 50% of the absorbance shown by a blank test. Each value is the average±S.D. of triplicate determinations. The statistical significance of differences in the mean of each data was calculated with Student's *t* test. $*p<0.05$, $**p<0.01$, $***p<0.001$: compared with AA-2G. $\dagger p<0.05$: compared with 6-Buty-AA-2G.

Experimental

Materials 6-Palm-AA, 2,6-Palm-AA and DPPH were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). A and AA-2P magnesium salt *n*-hydrate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). AA-2S barium salt was provided by Sigma Chemical Co. (St. Louis, MO, U.S.A.). α-Tocopherol was purchased from nacalai tesque, Inc. (Kyoto, Japan). AA-2G was obtained from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). A series of novel stable amphipathic vitamin C derivatives, 6-Acyl-AA-2G was synthesized from AA-2G and acid anhydrides in pyridine (unpublished data). They were identified as 6-*O*-butyryl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-Buty-AA-2G), 6-Hexa-AA-2G, 6-Octa-AA-2G, 6-Deca-AA-2G, 6-Dode-AA-2G, 6-Myri-AA-2G, 6-Palm-AA-2G, 6-Stea-AA-2G.

Radical Scavenging Activity Radical scavenging activity of AA derivatives was assayed using a relatively stable free radical, DPPH, according to the method of Blois.[11] An ethanol solution (1.25×10^{-6}, 1.25×10^{-5}, 6.25×10^{-5}, 1.25×10^{-4} and 1.25×10^{-3} M, 4 ml) of AA derivatives dissolved in ethanol or a mixture of ethanol/water, 0.1 M acetate buffer (pH 5.0) or 0.1 M phosphate buffer (pH 7.4), 1/1, was added to 1 ml of 5×10^{-4} M DPPH in ethanol. After mixing for 3 s vigorously, the solution was placed in a shading tube (ϕ 13.0×17.5 mm), and the end of the tube was sealed under a stream of argon. The mixture was incubated for 20 min at 25 °C. The radical scavenging activity (%) of each antioxidant was quantified by the decol-

Table 2. Radical Scavenging Activity against DPPH under Different Conditions

Antioxidant	EC_{50} (10^{-5} M)			
	in 60% EtOHaq	in 60% EtOH/40% pH 5.0 buffer	in 60% EtOH/40% pH 7.4 buffer	in 100% EtOH
AA	2.2	3.3	752	4.8
AA-2G	6.1	47	ND	4.4
6-Buty-AA-2G	5.9	54	ND	4.2
6-Deca-AA-2G	4.2	33	ND	4.0
6-Palm-AA-2G	4.1	31	ND	4.5
α-Tocopherol	1.9	1.7	ND	2.0

After 20 min-reaction at 25 °C, the fifty percent of effective concentration (EC_{50}) value was determined as the concentration of each sample required to give 50% of the absorbance shown by a blank test. Each value is the average of triplicate determinations. ND: not determined.

orization of DPPH at 516 nm on a Shimadzu UV-1200 spectrophotometer. The fifty percent effective concentration (EC_{50}) value was determined as the concentration of each sample required to give 50% of the absorbance shown by a blank test.

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