# Protease-Catalyzed Monoacylation of 2-*O*-α-D-Glucopyranosyl-L-ascorbic Acid in Pyridine

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**2-*O*-α-D-Glucopyranosyl-6-*O*-octanoyl-L-ascorbic acid was enzymatically synthesized from 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) and vinyl octanoate with a protease from *Bacillus subtilis* in pyridine. Furthermore, with various linear saturated fatty acid vinylesters as acyl donors, AA-2G was also converted to their corresponding 6-*O*-acyl AA-2G in the same manner. The reactivities of transacylation decreased with increasing length of the acyl groups. Thus, short chain acyl groups were transferred to AA-2G by this protease more efficiently than were long chain acyl groups. This enzymatic method is recommended for the synthesis of 6-Acyl-AA-2G with short or medium length chain acyl groups.**

**Key words** protease; transacylation; 6-Acyl-AA-2G; lipophilic ascorbate; ascorbic acid

We have developed a stable ascorbate derivative, 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G), from ascorbic acid (AA) and maltose or other α-glucans by a regioselective transglucosylation with α-glucosidases from digestive organs of mammals and rice seed, and with cyclomaltodextrin glucanotransferase from *Bacillus stearothermophilus*.[1] This stable hydrophilic vitamin C derivative, which exhibits vitamin C activity *in vivo* after enzymatic hydrolysis to AA by α-glucosidase,[2] has been accepted as a skin care main ingredient by the Japanese Government and already is available as an extra-medicine in commercial cosmetics. Subsequently, we have been successful in the chemical synthesis of a series of monoacylated AA-2G with an efficient transdermal activity in relatively good yields.[3] The monoacyl AA-2G derivatives were identified as 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-Acyl-AA-2G). Some of them with an appropriate length acyl chain group exhibited satisfactory thermal stability[3] and radical scavenging activity.[4] 6-Acyl-AA-2G was susceptible to enzymatic hydrolysis by mammalian tissue esterase and/or α-glucosidase to produce AA-2G and AA.

Regioselective acylation of carbohydrates has been carried out by lipase or protease in hydrophilic organic solvents such as pyridine and dimethylformamide.[5] Lipase-catalyzed transesterification of ascorbic acid in 2-methyl-2-butanol has also been reported.[6]

In this paper, we describe a new enzymatic method for synthesizing 6-Acyl-AA-2G by protease-catalyzed regioselective acylation in pyridine.

## Results and Discussion

In order to develop stable and biodegradable lipophilic ascorbate derivatives, we attempted to prepare the monoacylated derivatives of 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) by use of a variety of enzymes in organic solvents. From these experiments, Bioprase, an alkaline protease from *Bacillus subtilis*, was found to exhibit the highest transacylation activity of all lipases and proteases tested in this study.

AA-2G was dissolved in warm anhydrous pyridine, followed by addition of vinyl octanoate and an enzyme, dried Bioprase. The mixture was stirred at 45 °C; periodically aliquots were withdrawn and analyzed by TLC. The AA-2G spot gradually decreased, while a new major spot appeared. When the spot of AA-2G almost disappeared, the enzyme was removed by filtration and the product isolated and purified by column chromatography and recrystallization. The crystalline product was characterized by $^{1}$H- and $^{13}$C-NMR and UV spectra, optical rotation, and melting point data (see Experimental). Only the signal at C-6 in the $^{1}$H- and $^{13}$C-NMR of the product, compared with the signals of AA-2G, was drastically shifted to downfield. These data agreed well with those chemically synthesized.[3] Therefore, the protease-catalyzed monoacylated compound was identified as 2-*O*-α-D-glucopyranosyl-6-*O*-octanoyl-L-ascorbic acid (6-Octa-AA-2G, Chart 1). In the transacylation reaction between AA-2G and vinyl octanoate, the protease exhibited an overwhelming preference toward acylation of the primary hydroxyl group of the ascorbyl moiety rather than that of the glucose moiety. It is worth mentioning that in the case of chemical monoacylation of AA-2G reported previously,[3] the most reactive site of the molecule was also at the C-6 hydroxyl group.

Furthermore, we determined the reactivities in protease-catalyzed acylation of AA-2G with different linear saturated fatty acid vinylesters as acyl donors. A time course of these reactions is shown in Fig. 1. AA-2G was regioselectively converted to its corresponding 6-*O*-acyl AA-2G in the same manner. In the case of vinyl butyrate and vinyl hexanoate, the yields of 6-Buty- and 6-Hexa-AA-2G rapidly increased at the beginning and reached a maximum (67 and 82%, respectively) after 6 h. However, the yields were gradually decreased by subsequent reaction, while AA-2G as the acyl acceptor was consumed. HPLC analyses also showed that by-products increased with the passage of time. These results suggested that acyl groups were further transferred to the primary hydroxyl group of the glucose moiety and to the secondary hydroxyl groups. In the case of vinyl octanoate and vinyl decanoate, the yields of 6-Octa- and 6-Deca-AA-2G gradually increased, and 78 and 32% conversion was achieved after 24 h, respectively. In these reactions, by-products such as di- and tri-substituted derivatives were detected in trace amounts.

In contrast, when vinyl laurate and vinyl palmitate were used as acyl donors, the yields of 6-Dode- and 6-Palm-AA-2G were much lower than those of 6-Octa- and 6-Deca-AA-2G, because of their low reactivities. These results indicated

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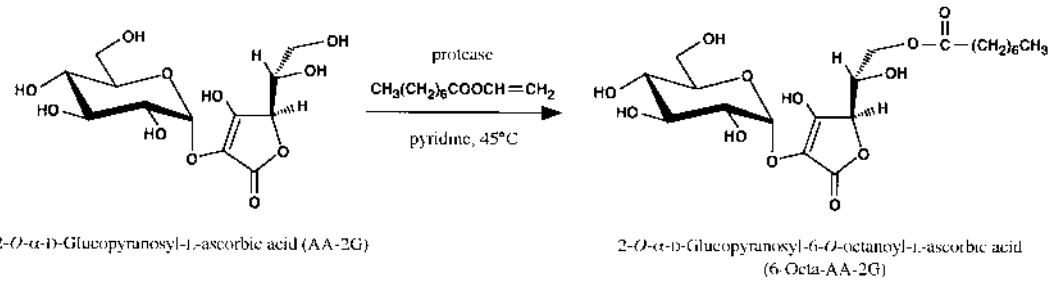


2-*O*-α-D-Glucopyranosyl-L-ascorbic acid (AA-2G)

2-*O*-α-D-Glucopyranosyl-6-*O*-octanoyl-L-ascorbic acid (6-Octa-AA-2G)

Chart 1

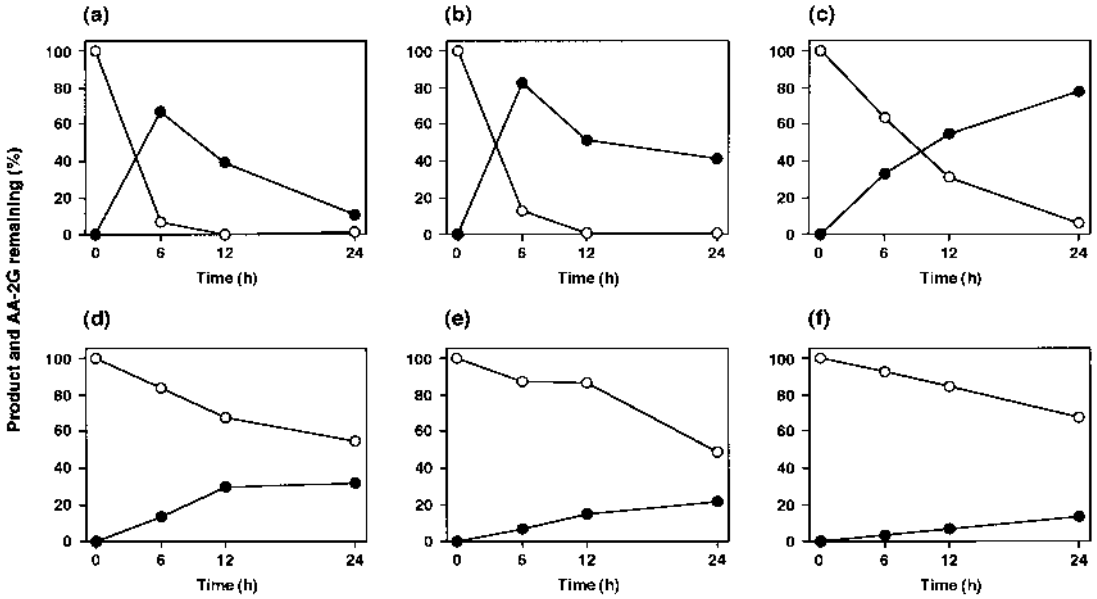


Fig. 1. Formation of 6-Acyl-AA-2G (●) by Protease-Catalyzed Acylation of AA-2G (○) with Different Fatty Acid Vinylesters

The reaction mixture contained AA-2G, each fatty acid vinylester and protease in dry pyridine. The reaction was carried out at 45 °C for 24 h. Aliquots were periodically withdrawn and analyzed by HPLC. When vinyl butyrate, vinyl hexanoate, vinyl octanoate, vinyl decanoate, vinyl laurate and vinyl palmitate were used as acyl donors, their products were 6-Buty- (a), 6-Hexa- (b), 6-Octa- (c), 6-Deca- (d), 6-Dode- (e) and 6-Palm-AA-2G (f), respectively. Each value is the average of duplicate determinations.

that their reactivities of transacylation decreased with increasing length of the acyl groups. Thus, short chain acyl groups were transferred to AA-2G by this protease more efficiently than were long chain acyl groups.

In conclusion, we succeeded in the protease-catalyzed regioselective acylation of AA-2G and here propose a new enzymatic method to prepare 6-Acyl-AA-2G. This enzymatic method is recommended for the synthesis of 6-Acyl-AA-2G with short or medium length chain acyl groups.

## Experimental

**General Experimental Procedure** $^1$H- and $^{13}$C-NMR spectra were recorded on a Varian VXR-500 Instrument with TMS. UV spectra were obtained on a Shimadzu UV-1200 spectrophotometer. Melting points were determined on a Yanagimoto micro-melting point apparatus, and are uncorrected. Optical rotations were obtained at JASCO DIP-1000. 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. Thin layer chromatography was performed on Kieselgel 60$F_{254}$ (Merck) and spots were detected under UV lamp. Column chromatography was carried out on Sephadex LH-20 (Pharmacia Biotech).

**Chemicals** 2-*O*-α-D-Glucopyranosyl-L-ascorbic acid (AA-2G) was a gift from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Bioprase (900000 U/g), an alkaline protease from *Bacillus subtilis*, was a gift from Nagase Biochemicals, Ltd., Osaka. A series of novel monoacylated AA derivatives, 6-*O*-acyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-Acyl-AA-2G) was chemically synthesized as described previously.[3] They were identified as 6-*O*-butyryl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-Buty-AA-2G), 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), 6-*O*-decanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-Deca-AA-2G), 6-*O*-dodecanoyl-2-*O*-α-D-glucopyranosyl-L-ascorbic acid (6-Dode-AA-2G), and 2-*O*-α-D-glucopyranosyl-6-*O*-hexadecanoyl-L-ascorbic acid (6-Palm-AA-2G). Vinyl butyrate, vinyl hexanoate, vinyl octanoate, vinyl decanoate, vinyl laurate and vinyl palmitate were purchased from Wako Pure Chemical Industries, Ltd., Osaka.

**Synthesis of 2-*O*-α-D-Glucopyranosyl-6-*O*-octanoyl-L-ascorbic Acid (6-Octa-AA-2G)** AA-2G (0.30 g, 0.887 mmol) was dissolved in dry pyridine (150 ml). Vinyl octanoate (1.37 ml, 0.887×8 mmol) and dried Bioprase (12.0 g) were added to the solution. The mixture was vigorously stirred under an atmosphere of argon at 45 °C for 18 h. The reaction mixture was filtered and the solvent was removed under reduced pressure. The residue dissolved in MeOH/AcOH/$H_2O$ (40 : 5 : 60) was chromatographed on a Sephadex LH-20 column ($\phi$2.0×21 cm) eluted with MeOH–$H_2O$ (4 : 6, v/v). Recrystallization of the product from EtOH gave 6-Octa-AA-2G (0.153 g, 37.2%). UV $\lambda_{max}$ (MeOH+HCl) nm ($\varepsilon$): 233 (10100); UV $\lambda_{max}$ (MeOH+NaOH) nm ($\varepsilon$): 260 (15300). $^1$H-NMR (500 MHz, $CD_3OD$) $\delta$: 0.90 (3H, t, $J$=7.0 Hz), 1.32 (8H, m), 1.63 (2H, qn, $J$=7.3 Hz), 2.37 (2H, t, $J$=7.3 Hz), 3.40 (1H, t, $J$=9.7 Hz, 4′-H), 3.53 (1H, dd, $J$=3.7, 9.7 Hz, 2′-H), 3.70 (1H, dd, $J$=4.9, 11.6 Hz, 6′-Ha), 3.78 (1H, t, $J$=9.7 Hz, 3′-H), 3.81 (1H, dd, $J$=2.4, 11.6 Hz, 6′-Hb), 4.03 (1H, ddd, $J$=2.4, 4.9, 9.7 Hz, 5′-H), 4.13 (1H, ddd, $J$=1.8, 6.1, 7.0 Hz, 5-H), 4.18 (1H, dd, $J$=6.1, 11.0 Hz, 6-Ha), 4.26 (1H, dd, $J$=7.0, 11.0 Hz, 6-Hb), 4.82 (1H, d, $J$=1.8 Hz, 4-H), 5.38 (1H, d, $J$=3.7 Hz, 1′-H). $^{13}$C-NMR (125 MHz, $CD_3OD$) $\delta$: 14.34, 23.59, 25.95, 30.01, 30.11, 32.80, 34.87, 62.20 (6′-C), 65.62 (6-C), 67.93 (5-C), 71.18

(4′-C), 73.41 (2′-C), 74.51 (3′-C), 74.84 (5′-C), 77.35 (4-C), 101.73 (1′-C), 120.38 (2-C), 162.02 (3-C), 172.10, 175.11 (1-C). $[\alpha]_D^{24}$ +144.2° ($c$=0.50, MeOH). mp 170.5—174.0 °C

**Protease-Catalyzed Monoacylation of AA-2G Using Different Fatty Acid Vinylesters as Acyl Donors** The reaction mixture contained AA-2G (10.0 mg, 29.6 $\mu$mol), each fatty acid vinylester (29.6×8 $\mu$mol) and the enzyme (400 mg) in 5.0 ml of dry pyridine. The reaction was carried out by vigorous stirring under an atmosphere of argon at 45 °C for 24 h. Aliquots of 25 $\mu$l were withdrawn at the indicated times, concentrated to dryness under a stream of argon, and dissolved in 250 $\mu$l of MeOH. Ten microliters of the methanolic solution was subjected to HPLC analysis. Separation for monoacylated AA-2G was achieved by isocratic elution of an Inertsil Ph column ($\phi$ 4.6×250 mm, 5 $\mu$m, GL Sciences Inc., Tokyo) 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. The reaction product was determined quantitatively by the peak area of the sample with reference to calibration with a chemically synthesized 6-Acyl-AA-2G.

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