Preparation and Physicochemical Analysis of 5-Methyl-3,4-Dihydroxytetrone Produced by Oxidative Degradation of Dehydro-L-Ascorbic Acid

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Abstract

5-Methyl-3,4-dihydroxytetrone was purified by Sephadex G-10 gel chromatography and DEAE-Sepharose ion exchange chromatography from degradation products of dehydro-L-ascorbic acid. 5-Methyl-3,4-dihydroxytetrone was soluble in water and several organic solvents such as ethyl acetate and diethyl ether. Nitroxide radicals dissolved in an aqueous solution and organic solvents were reduced by 5-methyl-3,4-dihydroxytetrone. Vitamin E radicals generated by the reaction of vitamin E with peroxy radical formed from 2,2'-azobis(2,4-dimethylvaleronitrile) were also reduced by 5-methyl-3,4-dihydroxytetrone. These results suggest that 5-methyl-3,4-dihydroxytetrone is a useful amphiphilic radical scavenger.

Introduction

L-Ascorbic acid (AsA) is recognized as the most effective water-soluble antioxidant in human blood plasma.[1,2,3] Although AsA is a useful antioxidant in the biological system, it is easily oxidized to dehydro-L-ascorbic acid (DHA) in a neutral aqueous solution. DHA is quite unstable in an aqueous solution and is easily delactonized to yield 2,3-diketo-L-gulonic acid (DKG), which further undergoes degradation, oxidation, and decarboxylation leading uncontrolled variables.[4,5] The production of degradative compounds of DHA is dependent on pH and temperature.[6,7]

5-Methyl-3,4-dihydroxytetrone (MDT) is one of the reductant generated from the oxidative degradation of DHA.[7] MDT was identified as *aci*-Reductone III derived from DHA heated in sulfuric acid.[8,9] MDT was also prepared from the mixture of DHA and organic acid.[10] Since the degradation product derived from the oxidation of DHA contains several brown intermediates, it is difficult to purify these intermediates.

The present study deals with the preparation and purification of MDT from acidic DHA solution by column chromatography. The physicochemical analysis of MDT suggests that this compound acts as an efficient radical scavenger in an aqueous phase and as an effective reductant of vitamin E(VE) radical.

Materials and Methods

Reagents

L-Ascorbic acid, L-ascorbyl stearate, and

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2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) were purchased from Wako Chemicals (Osaka). Vitamin E (VE) was obtained from Nacalai Tesque Inc. (Kyoto). 3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy was from Aldrich Chem. Comp. Inc. (Milwaukee). All other reagents were used without further purification.

Preparation of 5-methyl-3,4-dihydroxytetrone

Preparation and extraction of MDT were carried out according to the method of Euler and Hasselquest[8] as follows. DHA, prepared by the method of Doner and Hicks,[11] was dissolved in 1 M H_2SO_4 and then heated at 90℃ for 1 h using a water bath. After extraction with ethyl acetate, the extract was evaporated under vacuum. The residue containing MDT was dissolved in 1 mM HCl. The absorption spectra of the solution were recorded on an Ubest-35 UV/VIS Spectrometer (JASCO, Tokyo).

HPLC Analysis

The HPLC analysis was carried out according to our previous report.[7] The system was consisted of JASCO 880-PU pump, Radial PAK cartridge Resolve C_{18} column (0.8×10 cm, Waters Associates) and JASCO 330 multichannel UV detector to obtain three-dimensional (retention time : wavelength : absorbance) chromatographic data. The mobile phase was 50 mM potassium phosphate buffer (pH 6.0) containing 1 mM EDTA, 2.5 mM tetrabutylammonium hydrogensulfate, and 3% methanol. The eluent was pumped at a flow rate of 1 ml min^{-1}.

Purification of 5-methyl-3,4-dihydroxytetrone

A MDT solution was put on a column of Sephadex G-10 (2.6 × 90 cm, Pharmacia) equilibrated with 10 mM HCl. Elution was carried out with 10 mM HCl and 3 ml fractions were collected. The eluates were examined by measuring the absorbance at 245 nm for MDT. The MDT fraction was immediately passed through DEAE-Sepharose Fast Flow column (2.6×15 cm, Pharmacia). DEAE-Sepharose was first washed with 0.2 M HCl, 0.2 M NaOH, and finally distilled water before use. The samples were eluted with 100 ml of distilled water and then with 10 mM HCl, monitoring at 245 nm and 290 nm. MDT-containing fractions were concentrated and extracted by diethyl ether. The white needle crystals were obtained from the solution stood for some days at -20℃. The melting point of the crystal was at 177-178 ℃, which agreed with the value of Euler and Hasselquest.[9] ^{13}C NMR spectra were recorded on a JEOL JNM-GSX 400 spectrometer.

Radical scavenging activity

The radical scavenging activity of MDT was assayed as follows. The reaction mixture containing 0.1 mM 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy and 1 mM reductant in phosphate buffered saline (PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.4) was incubated for 0-60 min at 37℃. The radical scavenging activity of MDT was quantified by the ESR signal intensity on JES RE1X ESR spectrometer (JEOL Co., LTD. Tokyo). The ESR spectrum of nitroxide radical was recorded under the following conditions; center magnetic field, 336 mT; scanning range, ±10 mT; microwave power, 5 mW; modulation width, 0.1 mT; response, 1 sec.

Reduction of the chromanoxyl radical of VE by MDT

The mixture of 10 mM VE and 10 mM AMVN in chloroform was incubated at 50℃ for 20 min. After the appearance of VE radical was recognized by ESR measurement, 10 mM MDT dissolved in ethanol was added to the solution.

The ESR measurements were carried out under same conditions mentioned above, expect for 10 mW microwave power.

Results

The chemical structures of AsA and MDT are shown in Fig. 1. The reducing activity of MDT is owing to an enediol group in 5-membered γ-lactone ring similar to AsA. As a methyl group is attached to γ-lactone ring, MDT is soluble not only in water but also in organic solvents such as ethyl acetate and ether.

The absorption spectrum of DHA heated at 90℃ for 1 h in 0.5 M H_2SO_4 showed absorption maxima at 245 nm and 290 nm (Fig. 2). Three-dimensional HPLC of the heated sample has showed that AsA, MDT, 3-hydroxy-2-pyrone (3OH2P) and furoic acid are present in the solution (Fig. 3A). Interestingly, MDT was not obtained from DHA heated in H_2O (Fig. 3B).

The preparation and purification procedures of MDT are summarized in Fig. 4. 3OH2P and furoic acid could be removed by the extraction with benzene. The water-soluble substances, such as AsA and erythroascorbic acid, were removed by the extraction with ethyl acetate.

MDT was purified by Sephadex G-10 gel filtration (Fig. 5). MDT-containing fraction was further subjected to an anion-exchange chromatography on a DEAE-Sepharose column, giving two peaks (Fig. 6). MDT was eluted as a sharp single peak with 10 mM HCl and crystallized in diethyl ether.

Fig. 7 shows the ^{13}C NMR spectra of MDT at pH 2, 4, and 6. Each signal was assigned making reference to the data of Berger.[12] Chemical shifts for C-1 and C-3 were shifted to low magnetic field at pH 2 as similar to AsA.

To investigate whether MDT can reduce

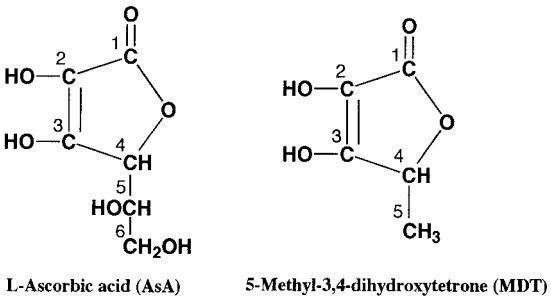

Fig. 1 Chemical structures of AsA and MDT.

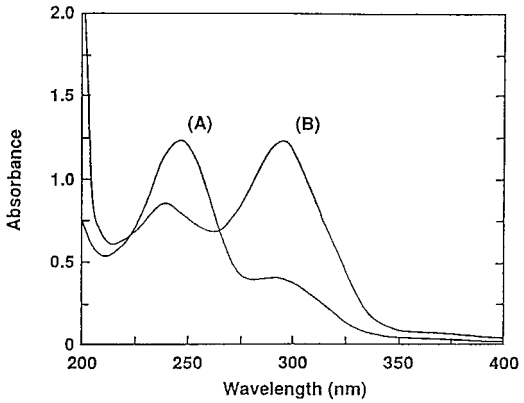

Fig. 2 Absorption spectra of DHA solution heated at 90℃ for 1 h in 0.5 M H_2SO_4 (A) and in H_2O (B).

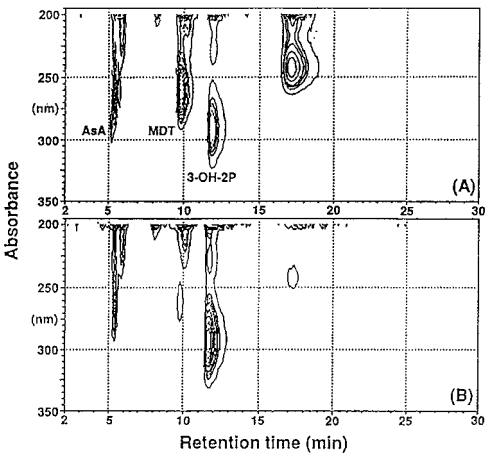

Fig. 3 Ion-pairing reversed phase HPLC of DHA solution heated at 90℃ for 1 h in 0.5 M H_2SO_4 (A) and in H_2O (B).

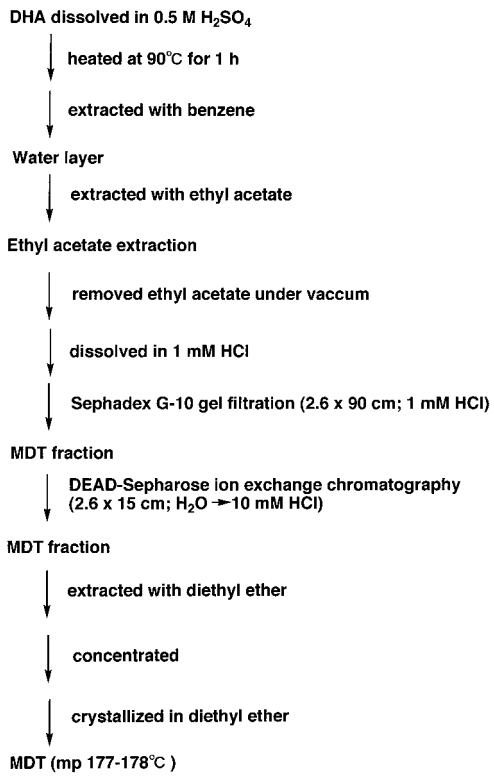

Fig. 4 Summary of preparation of MDT.

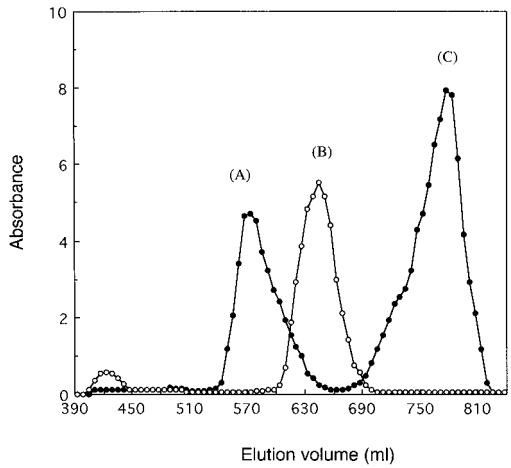

Fig. 5 Gel filtration of ethyl acetate extract of DHA solution heated at 90℃ for 1 h on a Sephadex G-10 column. Elution was carried out with 10 mM HCl and 3 ml fractions were collected. Closed and open circles indicate the absorbance at 245 and 290 nm, respectively. (A)MDT, (B)3OH2P, (C)Furoic acid.

free radical, MDT was mixed with the nitroxide radical, 3-carbamoyl-2,2,5,5-tetramethyl-3-yloxy, in PBS and ethanol (Fig. 8). The reducing activity of MDT was estimated from the reduction of ESR signal intensity of the nitroxide radical. The ESR signal intensity of the nitroxide radical in PBS decreased im-

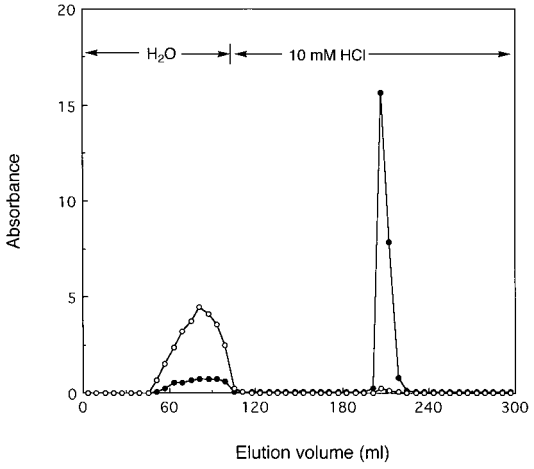

Fig. 6 Anion-exchange chromatography of Sephadex G-10 fraction on a DEAE-Sepharese Fast Flow column. Elution was carried out with 100 ml of distilled water and then with 10 mM HCl. Three ml fractions were collected. Closed and open circles indicate the absorbance at 245 and 290 nm, respectively.

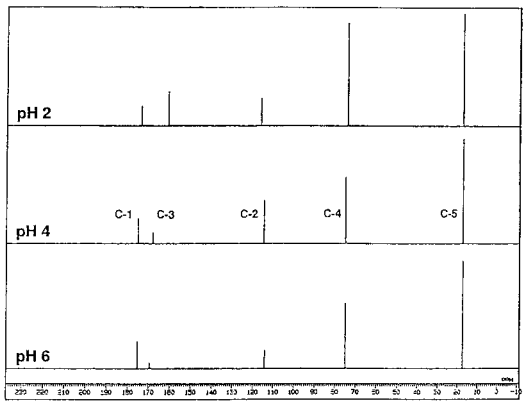

Fig. 7 ^{13}C-NMR spectra of MDT at various pHs.

mediately after the addition of MDT. Furthermore, the ESR intensity of the nitroxide radical in ethanol was more rapidly decreased by the addition of MDT than that of ascorbyl stearate, a lipophilic radical scavenging antioxidant in membrane. However, there was little change in ESR intensity when VE was added to the nitroxide radical in ethanol. VE, a strong chain-breaking antioxidant, could not reduce the nitroxide radical in ethanol.

The VE radical was detected by the ESR measurement when the mixture of VE and AMVN is heated at 50℃ in chloroform (Fig. 9A). VE radical was immediately reduced by the addition of MDT dissolved in ethanol (Fig. 9B).

Discussion

DHA changes to a variety of degradation products under different conditions such as pH and temperature.[7] MDT is generated by the oxidative degradation of DHA in a strong acid solution (Fig. 3). A possible mechanism on the formation of MDT from DHA is shown in Fig. 10. DHA is very unstable in an aqueous solution and easily delactonized to DKG. DKG is decarboxylated to L-xylosone (XLS).[13] XLS is converted to 2-keto-5-hydroxy-3-pentenoic acid (KHP). Under weak acid condition, KHP undergoes ring closure to 3OH2P.

As an alternative pathway, KHP is converted to the enediol form and leads to rear-

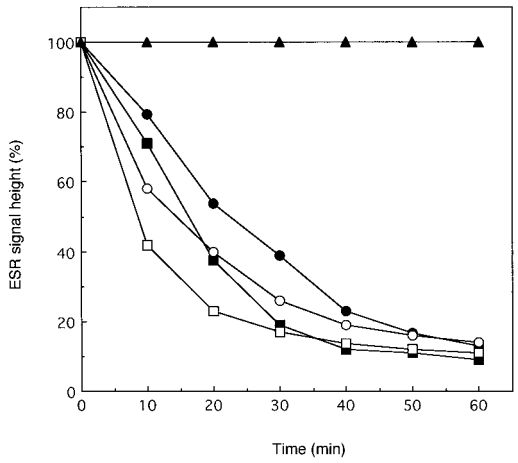

Fig. 8 Time course of radical scavenging reaction with various reductants. The reaction mixtures contained 0.1 mM 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy and 1 mM reductant were incubated at 37℃. Changes in ESR signal intensity due to scavenging of the nitroxide radical were measured at indicated time.
●; Ascorbyl stearate in ethanol,
■; MDT in ethanol,
▲;VE in ethanol,
○; AsA in PBS, □; MDT in PBS.

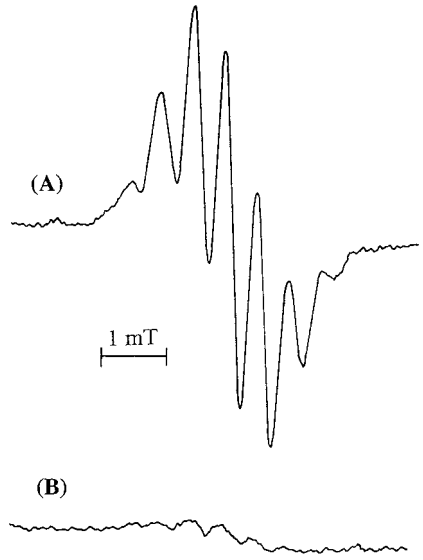

Fig. 9 Reduction of chromanoxyl radical of VE in chloroform.
The mixture of 10 mM VE and 10 mM AMVN in chloroform was incubated at 50℃ for 20 min. After the appearance of VE radical was recognized by ESR measurement, 10 mM MDT dissolved in ethanol was added to the solution.
The ESR spectra of VE radical were recorded before (A) and immediately after (B) the addition of MDT.

rangement from the aldehydic to the carboxylic group and from the hydroxylmethyl at the C-5 position to the methyl group, thus producing 2,3,4-trihydoxy pentenoic acid (THP) under strong acid condition. THP undergoes ring closure to give MDT.

The free nitroxide radical, 3-carbamoyl-2,2,5,5-tetramethyl-3-yloxy, was reduced by AsA and MDT at nearly equal rate in PBS (Fig. 8). In ethanol, the nitroxide radical was reduced more rapidly by MDT than by the ascorbyl stearate, which is known to act as a lipophilic and radical-scavenging antioxidant in membrane. Takahashi et al.[14,15] reported that fatty acid esters of AsA function as an antioxidant not only in homogeneous solution of methyl linolate but also in soybean phosphatidylcholine liposomal membrane. These results suggest the possibility that MDT may act as a radical-scavenger both in hydrophilic and in lipophilic environments.

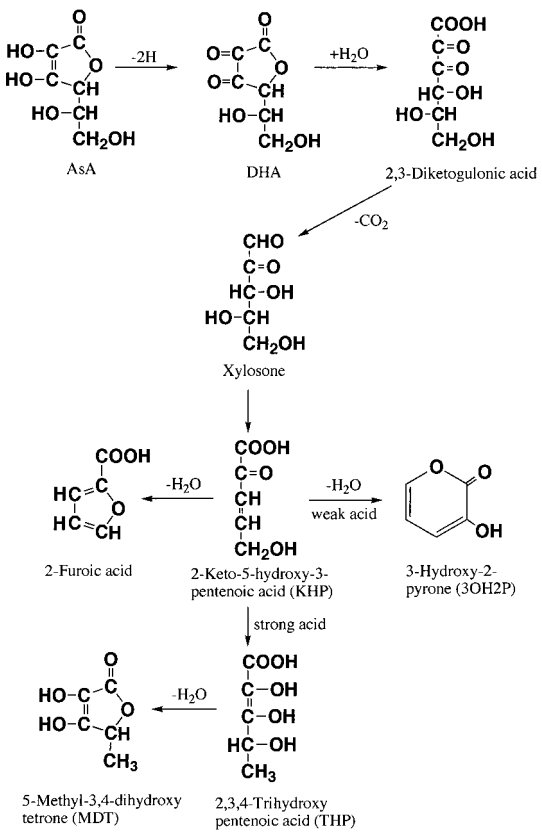

Fig. 10 A possible mechanism of MDT formation.

The VE radical was induced by the reaction of VE with peroxyl radicals formed from AMVN in chloroform at 50℃ (Fig. 9). The ESR signal of VE radical is rapidly disappeared by the addition of MDT to the solution. This result shows that MDT is accessible to VE radical in lipid region. It is well known that AsA can reduce the nitroxide radical in external monolayer of the vesicle membrane, judging from the disappearance of the ESR signal due to nitroxide radical by the addition of AsA.[16] It has been considered that the synergistic interaction between AsA and VE prevents lipid peroxidation in membrane,[16] though the mechanism of the reduction and the location of such an interaction in membrane have not yet definitely elucidated.[17,18] The results in Figs. 8 and 9 show that MDT can suppress lipid peroxidation both by the reducing of VE radical and by the scavenging of peroxyl radicals in membrane.

As mentioned above, MDT can reduce nitroxide radical and VE radical. This study show some possibilities that MDT may act as an efficient radical scavenger both in membranes and in aqueous solutions, and that many reductants generated from the oxidative degradation of AsA may also scavenge active oxygen species.

Acknowledgement

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