Distribution of Ascorbic Acid Analogs and Associated Glycosides in Mushrooms

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Summary Mushrooms contain reducing substances with chemical properties similar to ascorbic acid (AsA). In this study, the four types of reducing substances contained in Flammulina velutipes (Enokitake), Hypsizigus mamoreus (Bunashimeji), Pholiota nameko (Nameko), and Grifola frondosa (Maitake) were respectively purified, and the structure of each was analyzed using nuclear magnetic resonance (NMR) and other methods. The results confirmed that those substances were AsA analogs and associated glycosides (6-deoxy-AsA, 6-deoxy-5-O-(α-D-xylopyranosyl)-AsA, 6-deoxy-5-O-(α-D-glucopyranosyl)-AsA, and 5-O-α-D-glucopyranosyl-erythro-AsA). These substances were characteristic in that saccharide was bonded with the C-5 of the AsA analogs. Osazones were formed from the reducing substances in 19 kinds of edible mushrooms. Using thin-layer chromatography (TLC), they were developed to examine the distribution of the above reducing substances and AsA. The results showed that at least one of the above compounds was certain to be present in any mushroom; that AsA was present in very small quantities if at all; and that several substances similar to the above compounds were present. Key Words ascorbic acid, ascorbic acid analog, mushroom, glycoside, 6-deoxyascorbic acid, erythroascorbic acid, 6-deoxy-5-O-(α-D-xylopyranosyl)-ascorbic acid, 6-deoxy-5-O-(α-D-glucopyranosyl)-ascorbic acid, 5-O-α-D-glucopyranosyl-erythroascorbic acid

Mushrooms have long been known to contain substances that have strong reducing power even in acidic environments. These substances were formerly thought to be ascorbic acid (AsA), when AsA was determined by the 2,6-dichlorophenolindophenol (indophenol) method (1). The advanced analytic methods that are currently available have shown that these substances are not AsA (2), but they have not been clearly identified. Therefore, the reducing substances contained in Enokitake were partially refined and examined. Based on the ultraviolet (UV) spectrum and the formation of osazones from the reaction of the oxidized form with 2,4-dinitrophenylhydrazine (hydrazine), the preliminary exper-
iment indicated the substances had a structure similar to that of AsA. The presence of such substances had not been reported, so I purified them and examined their chemical structure.

METHODS

1. Purification of reducing substances. Five hundred ml of water, 1 liter of methanol, and 30 mg of dithiothreitol (DTT) as an antioxidant were added to 500 g of fresh mushrooms. The mixture was ground in a mixer, and filtered. In cases where a large quantity of extract was needed, the procedure was repeated until the desired quantity of extract was obtained.

Ten liters of mushroom extract were poured into a glass column (30 x 10 cm I. D.) packed with 1 liter of Dowex 1-X8: acetic acid form (Dow Chemical Co.) to make the target substance bond to the resin. After being rinsed with water, the target substance was eluted with 0.2 N HCl. The eluate was taken out 200 ml at a time, 250 g of active charcoal (Norit SX-3, Norit Co.) was then added by totalling the fractions which contained reducing substances (about 3 liters in total), and the mixture was filtered. The Norit remaining on the funnel was transferred to a beaker, mixed with 800 ml of methanol, and filtered. The filtrate was then passed through a Dowex 1-X8 (acetic acid form) column. Since the target substance was oxidized, it passed through the column, but the other acids bonded with the resin and were removed. After adding 10 ml of 3% DTT to the filtrate, the pH was adjusted to about 8, using 10 N NaOH, to return the substance to the reduced form, and concentrated to about one-fifth its former volume in vacuo. After adjusting the pH 1.5–2.0 with 2 N HCl, the solution was further concentrated. After removing NaCl, the precipitate, the resulting syrupy solution was dissolved in 5 ml of methanol, and gel filtration was performed using a 100 x 2.5 cm I.D. glass column packed with Sephadex LH20 (Pharmacia Fine Chem. Co.); eluent, 90% methanol. The fractions containing a large quantity of reducing substance were concentrated and used for experiments. The yield was about 25%.

2. Hydrolysis method for reducing substances. To the aqueous solution containing reducing substances, an equal amount of 5 N HCl was added, and the resulting solution was heated in a boiling water bath for 15 min. After drying the solution in vacuo, the residue was dissolved in water and used in the experiments.

3. Preparation method for osazone. The hydrazine method (3) for AsA determination was used. Since osazones were formed and precipitated by heating the reducing substances with hydrazine, the untreated hydrazine and sulfuric acid were removed after centrifugal precipitation. The precipitates (mainly osazones) were washed with water, dissolved in acetone and used for TLC.

4. Structural analysis of reducing substances. The structure of the purified samples was analyzed by UV spectra, infrared (IR) spectra, mass (MS) spectra, and NMR spectra. The instruments used and the determination conditions are described in the RESULTS section.
5. **Determination of reducing substances and saccharides.** Reducing substances were determined by the \( \alpha,\alpha' \)-dipyridyl (DP) method (4), xylose by the Bial method (5), and glucose by the mutarotase-glucose oxidase method (6).

**RESULTS**

1. **Structural analysis of reducing substances contained in Enokitake**

1) **Reactions of the sample with DP reagent and hydrazine.** The DP reagent was added to an aqueous solution of the reducing substance of Enokitake (Eno-R) and made to react at 37°C. Absorption at 525 nm was measured in a time series. The color reaction of the sample ended after 30 min, and its color development curve was identical to that of AsA.

Using an aqueous solution of Eno-R, both the reduced form and oxidized form (oxidized by indophenol) were reacted with hydrazine. An orange-colored compound was formed only in the oxidized form. This compound (thought to be osazone) was then developed with TLC using Wakogel FM Plate (Wako Pure Chemicals Co.) with toluene-acetone-acetic acid (2:1:1, \( v/v \)) as the developing solvent. Though AsA-osazone has an \( Rf \) value of 0.56, the value for Eno-R was 0.51. The visible absorption spectrum of the Eno-R osazone (dissolved in methanol) almost agreed with that of AsA: maximum absorption was at 365 and 520 nm.

2) **UV, IR, and MS spectra.** In the UV spectrum, the reduced form showed absorption maxima at 267 nm (pH 5.6, in 0.1 M phosphate buffer), and 243 nm (pH 1.0 in 0.1 M HCl), but almost no absorption was identified in the oxidized form. Both results were the same as the UV spectra results produced by AsA. In the IR spectrum, characteristic absorption was seen at 1763 and 1680 cm\(^{-1} \) (Fig. 1). In AsA lactone carbonyl-based (\( \nu_{C=O} \)) absorption occurs at 1760 cm\(^{-1} \), and conjugate double-bond-based (\( \nu_{C=C} \)) absorption occurs at 1665 cm\(^{-1} \) (7). Values for Eno-R were almost identical. In the MS spectrum, ions thought to be protonized molecules ((\( M+H \))\(^+ \)) were observed at \( m/z=293 \), and ions thought to be (\( M+ \) glycerol+H)\(^+ \) were observed at \( m/z=385 \) (Fig. 2).

3) **NMR spectra.** Nine signals (\( \text{\( \delta_1 \)} \)–\( \text{\( \delta_9 \)} \)) in the \( ^1\text{H} \) NMR spectrum (cf. Fig. 4), and eleven signals (\( \text{\( \delta_1 \)} \)–\( \text{\( \delta_{11} \)} \)) in the \( ^1\text{C} \) NMR spectrum (Fig. 3) were observed. As for the distortionless enhancement by polarization transfer (DEPT) spectrum,

![Graph](image)

Fig. 1. IR spectrum of Eno-R in KBr disk. Instrument, JEOL JIR-100.

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positive signals were observed at (1) (CH₃) and (3)-(8) (CH): the signal for (2) (CH₂) was negative. Table 1 summarizes the δC values, δH values, J values, and combination of ¹³C and ¹H based on ¹³C-¹H correlated spectroscopy (COSY) spectrum. From the δC values, signal (1) was carbonyl carbon, and signals (9) and (10) were double bond carbon found specifically in α,β-unsaturated cyclic ketone. These values almost agree with the chemical shifts for the lactone ring of AsA (C-1 170.8, C-2 118.3, C-3 152.6 ppm (8)). ¹H-¹H COSY spectrum (Fig. 4) indicates the presence of the partial structure CH₃-CH=CH-O-. The long-range ¹³C-¹H COSY spectrum showed that proton (3) has linked to carbon (1) by a notably firm spin coupling (J_{H-C-C}), and to carbonyl carbon (1) by a weak coupling (J_{H-C-O-C}).
Table 1. Chemical shifts for $^{13}$C and $^1$H, combination of $^{13}$C and $^1$H, and spin coupling constants of Eno-R.

| $\delta_C$ (ppm) | $\delta_H$ (proton numbers) (ppm) | $J$ value (Hz) |
|------------------|-----------------------------------|----------------|
| ① 15.7 (CH$_3$)  | ⑧ 1.33 (3)                        | $J_{\delta,\delta}=6.35$ |
| ② 63.5 (CH$_2$)  | ⑦ 3.50 (1)  ① 3.53 (1)           | $J_{\delta,\delta}=10.5$ |
| ③ 71.6 (CH)      | ⑥ 3.43 (1)                        | $J_{\delta,\delta}=10.5$ |
| ④ 71.6 (CH)      | ⑤ 4.07 (1)  ⑥ 3.32 (1)           | $J_{\delta,\delta}=5.4$  |
| ⑤ 73.6 (CH)      | ⑤ 3.47 (1)                        | $J_{\delta,\delta}=9.3$  |
| ⑥ 75.0 (CH)      | ⑤ 4.68 (1)  ⑥ 3.47 (1)           | $J_{\delta,\delta}=9.3$  |
| ⑦ 79.8 (CH)      | ⑤ 4.82 (1)  ⑥ 3.47 (1)           | $J_{\delta,\delta}=9.3$  |
| ⑧ 98.1 (CH)      | ⑨ 119.9 (\text{=C<})             | $J_{\delta,\delta}=2.68$ |
| ⑩ 155.6 (\text{=C<}) | ⑪ 173.4 (\text{=C=O})  | $J_{\delta,\delta}=9.3$  |

The combinations of $^{13}$C and $^1$H are based on $^{13}$C–$^1$H COSY spectrum.

Fig. 4. $^1$H–$^1$H COSY spectrum of Eno-R. Measurement conditions: instrument, JEOL JNH-GSX500; observation frequency, 500.2 MHz; solvent, methanol-d$_4$; internal standard, TMS.
These results indicate that Eno-R contains 6-deoxy-AsA as a partial structure.

The $^1$H–$^1$H COSY spectrum also indicates the presence of two spin systems (C1–D–C and G–H–C). Since the spin system of C1–D–C was complex, the 1-dimensional homonuclear Hartmann-Hahn (HOHAHA) spectra were measured. By spin locking proton C1 and using variable locking periods of 20, 40, 60, 80, and 100 ms, an $^1$H magnetization shift occurred, and the presence of the following part linked by the spin system was revealed:

\[
\text{CH–CH–CH–CH–CH}_2\text{–O.}
\]

The $^1$H–$^1$H COSY spectrum revealed that proton D2 had a long-range link to anomeric carbon D1. From these results, Eno-R was deduced to be a glycoside in which α-D-xylose was linked to the C-5 of 6-deoxy-AsA (Fig. 5). This structure (C11H16O9, M.W. 292) does not contradict the MS spectral measurement results. However, the absolute configuration of saccharide and aglycone was not elucidated.

4) Sample hydrolysis and xylose detection. Eno-R was hydrolyzed and developed with TLC using Wakogel FM Plate with ethylacetate-acetic acid-H2O (6:3:2, v/v) as the developing solvent. In a saccharide detection reaction (p-anisidine spray and heat at 120°C), coloring was observed at Rf 0.53, which agreed with the value for pure xylose. The substance corresponding to the spot at Rf 0.53 was reacted with the Bial reagent (5), and its visible absorption spectrum agreed completely with those of pure xylose.

2. Distribution of AsA analogs in mushrooms

1) Reducing substance preparation and osazone formation. Nineteen kinds of edible mushrooms belonging to Agaricales (14 kinds) and Aphyllophorales (5 kinds) were examined in order to determine whether an Eno-R type glycoside was contained in any of them and whether other analogs were present. For each kind of mushroom, half of the reducing substances obtained were hydrolyzed, and used to form osazone in the same manner as the unhydrolyzed sample.

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2) **TLC of osazones from unhydrolyzed samples.** As Fig. 6 shows, at least one kind of osazone was generated from any unhydrolyzed mushroom sample. *Pleurotus salmoneostramineus* (5) and *Naematoloma sublateritium* (13) produced 4 kinds of osazones. Eight kinds of osazones ((a)–(h)) were found among the 19 kinds of mushrooms. Content values varied widely, however. Four kinds of osazones—(b), (c), (e), and (f)—were present in large quantities. Osazone (c) is an Eno-R type osazone, which is produced only by *Pleurotus ostreatus* (2), *P. salmoneostramineus* (5), and *Naematoloma sublateritium* (13). Osazones (e) and (f) were generated in most mushrooms, and the reducing substances that form them were widely distributed among the mushrooms. Osazone (b) in *Nameko* (14) and *Naematoloma sublateritium* (13) formed the same osazone with hydrolyzed Eno-R.

3) **TLC of osazones from hydrolyzed samples.** A mixture of chloroform, ethylacetate and acetic acid (60:35:5, v/v) was used as the developing solvent for the osazones of hydrolyzed samples, because the solvent used for unhydrolyzed samples produced excessive Rf values in the osazones. As Fig. 7 shows, five kinds

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**Fig. 6.** Thin-layer chromatogram of osazones derived from reducing substances of 19 mushrooms and AsA. Media: Wakogel FM. Developing solvent: toluene/acetone/acetic acid (2:1:1, v/v). (A) AsA, (1) Flammulina velutipes (Enokitake) (♀, osazone of hydrolysis product), (2) Pleurotus ostreatus (Hiratake), (3) *P. cystidiosus* (Oohiratake), (4) *P. cornucopiae* (Tamogitate), (5) *P. salmoneostramineus* (Tokiirohiratake), (6) *Lentinus edodes* (Shiitake), (7) *Hygrophorus russula* (Sakurashimeji), (8) *Armillariella mellea* (Naratake), (9) *Hypsizigus marmoreus* (Bunashimeji), (10) *Tricholoma matsutake* (Matsutake), (11) *Pleurocybella porrigens* (Sugihiratake), (12) *Phaeolepiota aurea* (Koganetake), (13) *Naematoloma sublateritium* (Kuritake), (14) *Pholiota nameko* (Nameko), (15) *Myceloptodonoides aitchisonii* (Bunaharitake), (16) *Boletopsis leucoloma* (Kurokawa), (17) *Sarcodon aspratus* (Koutake), (18) *Grifola frondosa* (Maitake), (19) *Laetiporus sulphureus* (Masutake). ♀, main spot; ○, neutral spot; _, minor spot.

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of osazones ((A)–(E)) were observed; (B) and (D) were the most common. Osazone (A) was found only in Naematoloma sublateritium (13). Osazone (C) was found in 4 kinds of mushrooms and osazone (E) in 7 kinds, though both were present in very small quantities. The Rf values, and UV and visible spectra (solvent: methanol) of osazone (E) agreed completely with those of AsA osazone.

Figures 6 and 7 illustrate the following relationships between the osazones of the unhydrolyzed reducing substances and those of hydrolyzed reducing substances: c → (B) (example: Enokitake), e → (B) (example: Maitake), and f → (D) (example: Bunashimeji). Since (B) is thought to be an osazone of 6-deoxy-AsA, the reducing substance that produces (e) is thought to consist of 6-deoxy-AsA. Since osazone (D) differs from the osazone of 6-deoxy-AsA in mobility, it is thought to be the osazone of an unknown AsA analog. The reducing substances in Nameko formed the same osazone whether the sample was hydrolyzed or not.

3. Structural analysis of reducing substances contained in Bunashimeji, Nameko, and Maitake

1) Saccharides generated by hydrolysis. As Fig. 6 shows, the reducing substances that formed osazones (e) and (f) were present in many mushrooms. Therefore, the reducing substance in Maitake (converted into osazone (e)) and Bunashimeji (converted into osazone (f)) were purified, and their respective structures were analyzed. The reducing substances in Nameko (Name-R) was also examined since Name-R was thought to be 6-deoxy-AsA. The substances were used in the form of Na salt to make them easier to handle.
Samples of the reducing substances in Bunashimeji (Buna-R) and Maitake (Mai-R) were hydrolyzed, and developed with TLC by the same method used for Eno-R. Both in Buna-R and Mai-R, coloring was consistent with that of glucose (RF 0.66). The above substance reduced a Benedict reagent, and responded to the mutarotase-glucose oxidase determination method (6). The amount of glucose in Buna-R and Mai-R showed mol equivalent to that of the reducing substance. Accordingly, Mai-R is a glycoside in which glucose is linked to 6-deoxy-AsA, and Buna-R is a glycoside in which glucose is linked to an unknown AsA analog.

2) UV, IR, and MS spectra. Table 2 summarizes the measurement results for these spectra. In the 3 samples, the maximum absorption of the reduced forms in the UV region was around 265 nm at pH 5.6, and 245 nm at pH 1.0. Little absorption was detected in the oxidized forms. Characteristic IR absorption was seen around 1726 and 1597 cm\(^{-1}\). These wavenumbers agreed well with those of AsA: Na (1719 and 1597 cm\(^{-1}\)) (7). As for the MS spectra measurement results in Buna-R: Na, a suspected protonized molecule peak was observed at \(m/z = 331\), and suspected Na-added ions were observed at \(m/z = 353\) and 375. Measuring the positive ions in Name-R: Na and Mai-R: Na was difficult because impurities were present and Na salt samples were used. In the negative ion measurements, quasi-molecular ions \((M - Na)^{-}\) were observed at \(m/z = 159\) in Name-R: Na and \(m/z = 321\) in Mai-R: Na.

3) NMR spectra. a) NMR spectra of the Buna-R: Table 3 summarizes the \(\delta_c\) values, \(\delta_h\) values, combinations of \(^{13}\)C and \(^1\)H, and J values. The \(^1\)H NMR spectrum showed 10 signals (2–11). The \(^{13}\)C NMR spectrum showed 11 signals (1–11), but no signal corresponding to CH\(_3\) at 15.7 ppm in the Eno-R was seen. From the \(\delta_c\) values, signal 1 was carbonyl carbon, signals 9 and 10 were double-bond carbon, and 1–8 were CH\(_2\) and CH. A DEPT spectrum revealed that 1 and 2 were CH\(_2\), and 3–8 were CH. Based on NMR and MS spectra, the molecular weight of this substance is 330, and the molecular formula is \(C_{11}H_{15}D_{10}Na\). The \(\delta_c\) values of 9, 10, and 11, and the UV and IR information suggest that this substance has a lactone ring structure similar to AsA.

The \(^1\)H–\(^1\)H COSY spectrum indicated the presence of the following two

| Table 2. Properties of UV absorption spectra, IR spectra, and MS spectra of Buna-R: Na, Name-R: Na, and Mai-R: Na. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| UV spectra (nm) | Reduced form | Oxidized form | IR spectra | MS spectra |
|                 | \(\lambda_{\text{max}}^{\text{pH 5.6}}\) | \(\lambda_{\text{max}}^{\text{pH 1.0}}\) | Characteristic absorption (cm\(^{-1}\)) | Molecular ion peak, \(m/z\) |
| Buna-R: Na      | 267            | 243            | None        | 1726, 1597     | 331\(^1\) |
| Name-R: Na      | 266            | 243            | None        | 1722, 1593     | 159\(^2\) |
| Mai-R: Na       | 267            | 244            | None        | 1726, 1593     | 321\(^2\) |

\(^1\)Positive ion (\((M + H)^+\) detection, \(^2\)negative ion ((M – Na)\(^-\)) detection.
Table 3. Chemical shifts for $^{13}$C and $^1$H, combination of $^{13}$C and $^1$H, and spin coupling constants of Buna-R:Na.

| $\delta_{C}$ (ppm) | $\delta_{H}$ (proton numbers) (ppm) | $J$ value (Hz) |
|--------------------|-----------------------------------|----------------|
| 61.3 (CH$_2$)      | 3.77 (1)                          |                |
| 66.8 (CH$_2$)      | 3.85 (1)                          | $J_{\delta, \gamma}$=12.45 |
| 70.4 (CH)          | 3.92 (1)                          | $J_{\delta, \gamma}$=11.5  |
| 72.2 (CH)          | 3.89 (1)                          | $J_{\delta, \gamma}$=10.1   |
| 72.4 (CH)          | 3.90 (1)                          | $J_{\delta, \gamma}$=9.8    |
| 73.8 (CH)          | 3.69 (1)                          | $J_{\delta, \gamma}$=4.65   |
| 78.9 (CH)          | 3.68 (1)                          | $J_{\delta, \gamma}$=2.45   |
| 98.8 (CH)          | 4.93 (1)                          | $J_{\delta, \gamma}$=3.65   |
| 9 (C<)             |                                   |                |
| 10 (C<)            |                                   |                |
| 11 (C=)            |                                   |                |

The combinations of $^{13}$C and $^1$H are based on $^{13}$C-$^1$H COSY spectrum.

b) NMR spectra of the Name-R:Na: Table 4 gives measurement results of the $^1$H NMR and $^{13}$C NMR spectra. Three signals (8-10) were observed in the $^1$H NMR spectrum, and the $\delta_{H}$ values reveal the presence of a partial CH$_3$-CH$_2$-CH$_2$-structure. Six signals (1-6) were observed in the $^{13}$C NMR spectrum, and the $\delta_{C}$ values of 4, 5, 6 agreed with those of the lactone ring in Buna-R:Na. The UV, IR, and NMR data suggests that Name-R:Na is 6-deoxy-AsA:Na (C$_{11}$H$_{15}$O$_{10}$Na, M. W. 330) (Fig. 8-(1)). This structure (C$_{11}$H$_{15}$O$_{10}$Na, M. W. 330) does not contradict the MS spectral measurement results. From the cross peak with 1 and 2, signal 10 was assigned to C-3 of erythro-AsA. The $\delta_{C}$ values showed a magnetic field shift of 20.7 ppm, which was low compared to that of Eno-R. This downfield shift endorses the conclusion that C-3 is $-\text{ONa}$ (10).
Fig. 8. Proposed structures of the reducing substances obtained from Bunashimeji, Nameko, and Maitake. (1) Buna-R:Na, (2) Name-R:Na, (3) Mai-R:Na. 1–12 and a–j indicate the assignment of the signals observed in $^{13}$C NMR spectrum and $^1$H NMR spectrum respectively (see Tables 3–5).

Table 4. Chemical shifts for $^{13}$C and $^1$H, combination of $^{13}$C and $^1$H, and spin coupling constants of Name-R:Na.

| $\delta_C$ (ppm) | $\delta_H$ (proton numbers) | $J$ values (Hz) |
|------------------|-----------------------------|-----------------|
| 19.4 (CH$_3$)    | 1.34$^d$ (3)                |                 |
| 66.5 (CH)        | 4.12$^m$ (1)                | $J_{\beta,\beta}$ = 6.6 |
| 82.7 (CH)        | 4.33$^d$ (1)                | $J_{\alpha,\alpha}$ = 2.5 |
| 113.9 (=C<)      |                             |                 |
| 176.8 (=C<)      |                             |                 |
| 178.3 (>C=O)     |                             |                 |

$^d$ Doublet, $^m$ multiplet.

c) NMR spectra of the Mai-R:Na: Ten signals (⑤–①) in the $^1$H NMR spectrum and 12 signals (①–⑬) in the $^{13}$C NMR spectrum were observed (Table 5). A DEPT spectrum indicated that ① was CH$_3$, ② was CH$_2$, and ③–⑪ were CH. The $\delta_C$ values indicated that ⑫ was carbonyl carbon, and ⑩ and ⑪ were double-bond carbon, which conforms to the lactone ring of Buna-R:Na and Name-R:Na. The $^1$H–$^1$H COSY spectrum showed two spin-coupled partial structures: (i) ③–⑩–⑦ (CH$_3$–CH$_2$–CH$_2$–CH$_2$–), (ii) ①–⑤–⑩–⑦–⑫–①, ⑧ (–CH$_3$–CH$_2$–CH$_2$–CH$_2$–CH$_2$–CH$_2$–). Partial structure (i) was thought to be sidechain...
Table 5. Chemical shifts for $^{13}$C and $^1$H, combination of $^{13}$C and $^1$H, and spin coupling constants of Mai-R:Na.

| δ_c  | δ_h (proton numbers) | J value (Hz) |
|------|-----------------------|--------------|
| (ppm) | (ppm)                |              |
| ① 15.8 (CH₃) | ② 1.35 (3)         |              |
| ② 61.1 (CH₂) | ④ 3.76 (1)         | J₉,₀ = 12.2  |
| ③ 70.0 (CH) | ⑥ 3.82 (1)         |              |
| ④ 70.1 (CH) | ⑧ 3.39 (1)         | J₉,₀ = 10.1  |
| ⑤ 72.2 (CH) | ⑧ 4.18 (1)         | J₉,₀ = 6.6   |
| ⑥ 72.4 (CH) | ⑪ 3.48 (1)         | J₉,₀ = 10.0  |
| ⑦ 73.8 (CH) | ④ 3.64 (1)         | J₉,₀ = 4.4   |
| ⑧ 82.1 (CH) | ④ 3.57 (1)         | J₉,₀ = 2.4   |
| ⑨ 96.0 (CH) | ① 4.44 (1)         | J₉,₀ = 9.0   |
| ⑩ 113.5 (= C<) | ① 4.99 (1)       | J₉,₀ = 1.95  |
| ⑪ 176.8 (= C<) | ① 3.7 (1)        | J₉,₀ = 3.7   |
| ⑫ 178.2 (> C=O) | ① 3.57 (1)     |              |

The combinations of $^{13}$C and $^1$H are based on $^{13}$C-$^1$H COSY spectrum.

of 6-deoxy-AsA. The δ_c values of partial structure (ii) agreed with those of methyl-α-D-glucose (9), previously mentioned in the section on Bunashimeji. The δ_c values of ④ and ⑧ indicate a glucosidic linkage of glucose and C-5 present in 6-deoxy-AsA. Thus, Mai-R: Na was deduced to be a glycoside where α-D-glucose was linked to the C-5 of 6-deoxy-AsA:Na (C₁₂H₁₇O₁₀Na, M.W. 344) (Fig. 8-(3)). A quasi-molecular ion ($(M-Na)^-$) supporting this conclusion was observed at m/z = 321.

DISCUSSION

Structural analyses by the UV, IR, MS, and NMR spectra revealed that the reducing substances in mushrooms with chemical properties similar to those of AsA were actually AsA analogs; 6-deoxy-AsA (6-deoxy-threo-hexono-1,4-lactono-2-ene), erythro-AsA (glycero-pentono-1,4-lactono-2-ene), and their glycosides: 6-deoxy-5-O-(α-D-xylopyranosyl)-AsA, 6-deoxy-5-O-(α-D-glucopyranosyl)-AsA, and 5-O-α-D-glucopyranosyl-erythro-AsA. Accordingly, osazone (b) in Fig. 6 is 6-deoxy-AsA, osazone (c) is 6-deoxy-AsA with linked xylose, osazone (e) is 6-deoxy-AsA with linked glucose, and osazone (f) is erythro-AsA with linked glucose. Other reducing substances with a similar structure were also present, such as osazones (a) and (d).

It is noteworthy that the reducing substances contained in mushrooms were AsA analogs and associated glycosides. AsA osazone formations were not found in any unhydrolyzed sample (Fig. 6). Since the TLC developing method for osazones

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lacks precision, it is obviously impossible to insist that AsA is not present in a free state. However, even if the AsA is contained, its amount is present in very small quantity. When the hydrolyzed reducing substances were converted into osazones, some mushrooms formed osazone (E), or AsA osazone (Fig. 7). Therefore, AsA glycosides may exist, but in far smaller quantities than the other osazones; their relationship with the unhydrolyzed reducing substances remains unclear. It is possible that, together with osazone (C), osazone (E) may be artificially produced during osazone formation. To test this possibility, a method of separating these reducing substances (without osazone formation) by high-performance liquid chromatography (HPLC) is currently under investigation.

The significant feature of the reducing substances in mushrooms is that saccharide is linked to the C-5 of 6-deoxy-AsA and erythro-AsA. Ascorbigen (11) and AsA-2-sulfuric acid (12) have been reported as naturally occurring AsA-related compounds, but the binding site of the indol and sulfuric acid groups is the C-2 position. The fatty acid in many synthesized AsA-fatty acid esters also links in the 2, 3, and 6 positions. In order for fatty acid to bond with the 5 position, the 2, 3, and 6 positions need to be closed (13). It is very interesting that the saccharide in mushroom glycosides should preferentially bond with C-5, and the reason for this phenomenon is being studied.

6-Deoxy-AsA was artificially synthesized in order to examine the relationship between AsA structure and vitamin C activity (14). It has been reported that 6-deoxy-L-AsA is one-third as physiologically effective against scorbutus as AsA (15). Since 6-deoxy-AsA is contained in an independent form in Nameko, eating Nameko would provide some of the partial benefits of AsA. If the Eno-R and Mai-R types are hydrolyzed in the digestive tract or elsewhere in the body, a similar effect can be expected. For such an effect to occur, however, the 6-deoxy-AsA involved must be of the L-form; but such a determination was not made in this experiment. On the other hand, L-erythro-AsA is considered ineffective in treating scorbutus (16). Erythro-AsA is present in an independent form in Candida (d-form) (17), Neurospora crassa (18), and Saccharomyces cerevisiae (19). These reports suggest that erythro-AsA may be a widely distributed substance in fungi. However, no other study has reported the presence of the glycosides revealed in this study. The author plans to use HPLC to assess the distribution of the two AsA analogs and the associated glycosides in plants, animals, and other fungi; results will be reported in an upcoming paper. Further research on the functional roles and vitamin C effects of these substances is to be conducted.

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