Food Chemistry 155 (2014) 221–226

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Novel oxidation products of cyanidin 3-*O*-glucoside with 2,2′-azobis-(2,4-dimethyl)valeronitrile and evaluation of anthocyanin content and its oxidation in black rice

Haruna Kamiya, Emiko Yanase *, Shin-ichi Nakatsuka

Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

ARTICLE INFO

Article history:
Available online 31 January 2014

Keywords:
Anthocyanin
Cyanidin 3-*O*-glucoside
Radical oxidation
2,2′-Azobis-(2,4-dimethyl)valeronitrile (AMVN)
Black rice

ABSTRACT

The radical oxidation mechanism of anthocyanin derivatives was investigated by the reaction of cyanidin 3-*O*-glucoside in the presence of radical initiator 2,2′-azobis-(2,4-dimethyl)valeronitrile (AMVN) in EtOH and aqueous CH_3CN. Six different oxidation products were isolated, depending on the solvent employed. These products were identified using NMR spectroscopy and multistep derivatisation reactions. Of the products obtained, two novel oxidised anthocyanin derivatives were isolated from black rice under prolonged storage. A radical reaction mechanism is proposed on the basis of these reaction products. Quantification of oxidised anthocyanins in black rice is demonstrated as a method to verify freshness of the rice.

1. Introduction

The pigment anthocyanin is widely distributed in the plant kingdom and is well known to be responsible for the red, blue, and violet colours in flowers and fruits of some plants (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; Harborne, 1994; Harborne & Williams, 1995). Anthocyanins, members of the flavonoid group of phytochemicals, are recognised to play an important role in the enhancement of an array of bioactivities in animals, including improving vision (Jang, Zhou, Nakanishi, & Sparrow, 2005; Matsumoto & Iida, 2007), antioxidative activities (Castañeda-Ovando et al., 2009; Tsuda et al., 1994), and anticancer activities (Hou, 2003; Katsube, Iwashita, Tsushida, Yamaki, & Kobori, 2003; Lule & Xia, 2005; Nichenametla, Taruscio, Barney, & Exon, 2006). Structurally, anthocyanins are the sugar (glucose)-containing analogues of anthocyanidin aglycone, which are composed of a characteristic flavylium (2-phenylchromenium) cation (Fig. 1). Numerous anthocyanin derivatives are known with varying phenolic substitution about the chromenylium (2-benzopyrylium) core and the 2-phenyl moiety, in addition to differences in sugar combinations (Harborne, 1994; Harborne & Williams, 1995).

Several authors have reported the oxidation reactions of anthocyanins (Es-Safi et al., 2008; Jurd, 1964, 1972; Karrer & Widmer, 1927; Karrer et al., 1927; Tsuda, Ohshima, Kawakishi, & Osawa, 1996). Tsuda et al. first reported the radical oxidation of **1** in 1996; they investigated the reaction of **1** (Fig. 1) with an alkylperoxyl radical species and reported the chemical structures of the resultant products. In our continuing studies directed at natural product isolation and characterisation, we noted the appearance of a new peak that had been initially absent when a solution of cyanidin 3-*O*-glucoside **1** in MeOH was kept under daylight conditions for three days; in contrast, this peak was not observed when the solution was kept in the dark. Accordingly, we believed this peak to be the product of radical oxidation of **1**. To investigate this hypothesis, we performed the oxidation of **1**, with a radical initiator in EtOH at 60 °C and analysed the reaction mixture using HPLC. Three new peaks appeared in the chromatogram with the concomitant decrease of **1**; one of the peaks was observed at the same retention time as the peak noted under daylight conditions, indicating that the compound produced under daylight conditions was formed by radical oxidation.

In this study, we investigated the reaction of **1** with the alkylperoxyl radical 2,2′-azobis-(2,4-dimethyl)valeronitrile (AMVN) in detail and report the isolation of two novel oxidative products. We propose an oxidation mechanism based on the identity of the obtained products. Furthermore, we report the isolation of these oxidative products from black rice that had been stored.

2. Materials and methods

2.1. Instruments

NMR spectra were recorded on a JEOL ECA-500 or a JEOL ECA-600 instrument (Tokyo, Japan), using tetramethylsilane (TMS) as

* Corresponding author. Tel./fax: +81 58 293 2914.
E-mail address: e-yanase@gifu-u.ac.jp (E. Yanase).

http://dx.doi.org/10.1016/j.foodchem.2014.01.077
0308-8146/

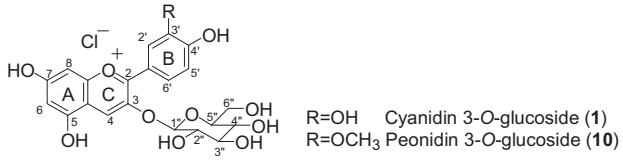

Fig. 1. Representative anthocyanins; cyanidin 3-*O*-glucoside **1** and peonidin 3-*O*-glucoside **10**.

an internal standard. Multiplicities are reported using the following abbreviations: singlet (s), doublet (d), triplet (t), doublet of doublets (dd), multiplet (m), broad (br). Coupling constants (*J*) are reported in hertz (Hz). IR spectra were recorded on a Perkin Elmer 2000 FT-IR spectrometer (Perkin Elmer Japan Co., Ltd., Kanagawa, Japan). UV–Vis spectra were recorded on a Hitachi 4000U UV–Vis spectrometer (Tokyo, Japan) using methanol as the solvent. High-resolution electron impact mass spectrometry (HREIMS) and high-resolution electrospray ionisation mass spectrometry (HRESIMS) data were obtained using a JEOL JMS-700/GI mass spectrometer (Tokyo, Japan). High-performance liquid chromatography (HPLC) analyses were carried out with a JASCO PU-2089 intelligent pump equipped with a JASCO MD-2010 PDA detector, and JASCO CO-2065 column oven (Tokyo, Japan). The HPLC columns used for analytical and preparative HPLC were COSMOSIL 5C18-MS-II (4.6 mm ID × 150 mm; Nacalai Tesque Inc., Kyoto, Japan) and NB-ODS-9 (10 mm ID × 250 mm; Nagara Science Co., Ltd., Gifu, Japan), respectively.

2.2. Materials

Cyanidin 3-*O*-glucoside **1** was isolated from a commercial black bean extract and recrystallised using a HCl–MeOH mixture (Lee et al., 2009; Valls, Millán, Martí, Borràs, & Arola, 2009). 2,2′-Azobis-(2,4-dimethyl)valeronitrile (AMVN) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Black rice samples were obtained from the Gifu Field Science Center at Gifu University.

2.3. Reaction of Cyanidin 3-O-glucoside *1* with AMVN in EtOH

Compound **1** (50.0 mg) and AMVN (25.0 mg, 1 equiv.) were dissolved in 40 mL of EtOH, and the mixture was stirred at 60 °C for 4 h. The reaction mixture was purified by HPLC (column: NB-ODS-9) with CH_3CN/H_2O (20:80 v/v) containing 0.5% trifluoroacetic acid (TFA) to obtain compound **2** (13.5 mg, 28.2%), protocatechuic acid ethyl ester (**3b**; 6.2 mg, 33.0%), and compound **2a** (5.0 mg, 9.6%). 2-(3,4-Dihydroxyphenyl)-4,6-dihydroxybenzofuran-3-carboxylic acid glucose ester **2**: ^{1}H NMR (600 MHz, CD_3OD): δ 3.26 (1H, t, J = 8.9 Hz, H-2″), 3.41 (1H, t, J = 8.9 Hz, H-4″), 3.41–3.45 (1H, m, H-5″), 3.45 (1H, t, J = 8.9 Hz, H-3″), 3.75 (1H, dd, J = 4.5, 12.0 Hz, H-6″), 3.87 (1H, dd, J = 2.4, 12.0 Hz, H-6″), 5.81 (1H, d, J = 8.2 Hz, H-1″), 6.25 (1H, d, J = 2.1 Hz, H-5), 6.46 (1H, d, J = 2.1 Hz, H-7), 6.87 (1H, d, J = 8.3 Hz, H-5′), 7.27 (1H, dd, J = 2.0, 8.3 Hz, H-6′), 7.33 (1H, d, J = 2.0 Hz, H-2′); ^{13}C NMR (150 MHz, CD_3OD): δ 61.9 (C6″), 70.4 (C4″), 73.4 (C2″), 78.0 (C3″), 78.5 (C5″), 90.4 (C7), 96.5 (C1″), 100.0 (C5), 107.1 (C3), 108.1 (C9), 115.6 (C5′), 118.4 (C2′), 122.1 (C1′), 123.7 (C6′), 145.3 (C3′), 148.8 (C4′), 152.4 (C4), 157.1 (C8), 158.7 (C6), 162.4 (C2), 167.8 (C10); UV λ_{max} (MeOH) nm (ε): 253 (15,100), 347 (9400).

2.4. Acid hydrolysis of *2*

A solution of **2** (5.6 mg) in 2% TFA aq. (1 mL) was stirred at 80 °C for 1 h. After cooling, the solution was concentrated under reduced pressure and purified by HPLC (column: NB-ODS-9) using CH_3CN/H_2O (22:78 v/v) containing 0.5% TFA. Compound **2a** (3.2 mg, 87.8%) was obtained: ^{1}H NMR (600 MHz, CD_3OD): δ 6.22 (1H, d, J = 2.0 Hz, H-5), 6.43 (1H, d, J = 2.0 Hz, H-7), 6.84 (1H, d, J = 8.2 Hz, H-5′), 7.20 (1H, dd, J = 2.0, 8.2 Hz, H-6′), 7.27 (1H, d, J = 2.0 Hz, H-2′); ^{13}C NMR (150 MHz, CD_3OD): δ 90.1 (C7), 99.6 (C5), 108.2 (C3), 108.7 (C9), 115.5 (C5′), 118.0 (C2′), 122.5 (C1′), 123.4 (C6′), 145.4 (C3′), 148.3 (C4′), 152.7 (C4), 157.1 (C8), 158.5 (C6), 161.1 (C2), 170.5 (C10); UV λ_{max} (MeOH) nm (ε): 247 (14,400), 334 (12,600).

2.5. Methylation of *2a*

A solution of diazomethane (excess) in Et_2O was added to a solution of **2a** (10 mg) in MeOH (1 mL) and kept at room temperature for 1.5 h. The reaction mixture was concentrated under reduced pressure and then purified by preparative thin-layer chromatography (p-TLC) with 1% MeOH in CH_2Cl_2 to give **2b** (8.0 mg, 65.0%). EIMS: m/z 372 $[M]^+$; ^{1}H NMR (600 MHz, CD_3OD): δ 3.86 (3H, s, OMe), 3.88 (3H, s, OMe), 3.90 (6H, s, OMe), 3.92 (3H, s, OMe), 6.42 (1H, d, J = 1.7 Hz, H-5), 6.75 (1H, d, J = 1.7 Hz, H-7), 7.03 (1H, d, J = 8.6 Hz, H-5′), 7.37 (1H, dd, J = 2.1, 8.6 Hz, H-6′), 7.39 (1H, d, J = 2.1 Hz, H-2′); IR ν_{max} (CH_2Cl_2) cm^{-1}: 1512, 1606, 1727, 3054, 3058, 3686.

2.6. Catalytic reduction of *2b*

A solution of compound **2b** (8.0 mg) in EtOH (2 mL) was hydrogenated over Pd/C catalyst (10 wt% loading) under hydrogen at atmospheric pressure and under reflux for 1 h. After removal of the catalyst by filtration, the filtrate was concentrated under reduced pressure, and the residue was purified by p-TLC (1% MeOH in CH_2Cl_2) to give **4** (3.8 mg, 47.2%). HREIMS: m/z 374.1360 $[M]^+$ (calcd. for $C_{20}H_{22}O_7$, 374.1366); ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.24 (3H, s, OMe), 3.77 (3H, s, OMe), 3.81 (3H, s, OMe), 3.87 (3H, s, OMe), 3.88 (3H, s, OMe), 4.41 (1H, d, J = 9.7 Hz, H-3), 5.93 (1H, d, J = 9.7 Hz, H-2), 6.07 (1H, d, J = 2.3 Hz H-5), 6.20 (1H, d, J = 2.3 Hz, H-7), 6.84 (1H, d, J = 9.2 Hz, H-5′), 6.92–6.93 (2H, m, H-2′and H-6′); ^{13}C NMR (125 MHz, $CDCl_3$): δ 51.7 (C3 & OMe), 55.5 (OMe), 55.6 (OMe), 55.9 (OMe), 56.0 (OMe), 87.0 (C2), 88.5 (C7), 91.8 (C5), 105.0 (C9), 109.5 (C2′), 110.6 (C5′), 119.1 (C6′), 129.2 (C1′), 148.7 (C3′ or 4′), 148.9 (C3′ or 4′), 157.0 (C4), 162.3 (C8), 162.8 (C6), 170.7 (C10); IR ν_{max} (CH_2Cl_2) cm^{-1}: 1608, 1737, 3055, 3061, 3686.

2.7. $LiAlH_4$ reduction of *4*

A solution of compound **4** (3.8 mg) in Et_2O (anhydrous, 1.5 mL) was added to a solution of $LiAlH_4$ (6.1 mg) in Et_2O (anhydrous, 0.5 mL), and the mixture was stirred at room temperature for 10 min. The reaction was then quenched by the dropwise addition of EtOAc and the mixture was extracted with 1 M HCl aq./CH_2Cl_2. The organic layer was collected and concentrated under vacuum. The residue was purified by p-TLC (40% EtOAc in hexane) to give compound **5**: HREIMS: m/z 346.1430 $[M]^+$ (calcd. for $C_{19}H_{22}O_6$, 346.1416); ^{1}H NMR (600 MHz, CD_3OD): δ 3.37 (1H, dd, J = 5.5, 11.0 Hz, H-10), 3.49 (1H, dd, J = 5.5, 11.0 Hz, H-10), 3.65–3.72 (1H, m, H-3), 3.78 (3H, s, OMe), 3.81 (3H, s, OMe), 3.83 (3H, s, OMe), 3.85 (3H, s, OMe), 5.77 (1H, d, J = 8.2 Hz, H-2), 6.12 (1H, d, J = 1.8 Hz, H-5), 6.15 (1H, d, J = 1.8 Hz, H-7), 6.93 (1H, d, J = 8.3 Hz, H-5′), 6.96 (1H, dd, J = 1.4, 8.3 Hz, H-6′), 7.03 (1H, brs, H-2′); ^{13}C NMR (150 MHz, $CDCl_3$): δ 48.4 (C3), 55.8 (OMe), 56.0 (OMe), 56.4 (OMe), 61.9 (C10), 88.7 (C2), 89.7 (C7), 92.3 (C5), 108.1 (C9), 111.5 (C2′), 112.3 (C5′), 120.2 (C6′), 131.3 (C1′), 149.8 (C3′ or 4′), 149.9 (C3′ or 4′), 158.5 (C4), 162.9 (C8), 163.4 (C6); IR ν_{max} (CH_2Cl_2) cm^{-1}: 1501, 1519, 1602, 3054, 3686.

2.8. Reaction of **1** with AMVN in aqueous CH_3CN

Compound **1** (50.0 mg) and AMVN (25.0 mg, 1 equiv.) were dissolved in 20% CH_3CN aq. (40 mL), and the mixture was stirred at 60 °C for 6 h. The reaction mixture was purified by HPLC (column: NB-ODS-9) using CH_3CN/H_2O (15:85 v/v) containing 0.5% TFA. Compound **6** (5.4 mg, 15%), protocatechuic acid (**3a**; 5.3 mg, 34.0%), and **7** (6.8 mg, 13.6%) were obtained. 2-*O*-(3,4-Dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid glucose ester **7**: ^{1}H NMR (600 MHz, CD_3OD): δ 3.27–3.40 (4H, m, H-2″ ∼ 5″), 3.55 (1H, d, J = 16.3 Hz, H-7), 3.61 (1H, d, J = 16.3 Hz, H-7), 3.64 (1H, brd, H-6″), 3.81 (1H, brd, J = 12.3 Hz, H-6″), 5.42 (1H, d, J = 8.3 Hz, H-1″), 6.17 (1H, d, J = 2.0 Hz, H-5), 6.27 (1H, d, J = 2.0 Hz, H-3), 6.87 (1H, d, J = 8.3 Hz, H-5′), 7.56–7.58 (2H, m, H-2′, 6′); ^{13}C NMR (150 MHz, Acetone-d_6): δ 29.6 (C7), 62.2 (C6″), 70.8 (C4″), 73.5 (C2″), 77.4 (C3″), 78.0 (C5″), 95.5 (C1″), 100.8 (C3), 102.2 (C5), 106.2 (C1), 115.9 (C5′), 117.5 (C2′), 121.5 (C1′), 124.3 (C6′), 145.5 (C3′), 151.5 (C4′), 152.2 (C6), 157.6 (C2), 158.1 (C4), 165.1 (C7′), 170.7 (C8).

2.9. Acid hydrolysis of **7**

A solution of **7** (6.8 mg) in 2% TFA aq. (1 mL) was stirred at 80 °C for 1 h. After cooling, the solution was concentrated under reduced pressure and purified by HPLC (column: NB-ODS-9) using CH_3CN/H_2O (15:85 v/v) containing 0.5% TFA to obtain **7a** (4.4 mg, 98%). The ^{1}H NMR data of **7a** were identical to that described in the literature (Ryu, Park, & Ho, 1998).

2.10. Methylation of **7a**

A solution of diazomethane (excess) in Et_2O was added to a solution of **7a** (3.5 mg) in MeOH (0.5 mL) and kept at room temperature for 1.5 h. The reaction mixture was concentrated under reduced pressure and then purified by p-TLC (2% MeOH in CH_2Cl_2) to give **7b** (2.6 mg, 61.4%). HRESIMS: m/z 391.1383 $[M+H]^+$ (calcd. for $C_{20}H_{23}O_8$, 391.1393); ^{1}H NMR (600 MHz, CD_3OD): δ 3.54 (2H, s, H-7), 3.56 (3H, s, COOMe), 3.80 (3H, s, OMe), 3.84 (3H, s, OMe), 3.90 (3H, s, OMe), 3.94 (3H, s, OMe), 6.42 (1H, d, J = 2.4 Hz, H-5), 6.49 (1H, d, J = 2.4 Hz, H-3), 7.08 (1H, d, J = 8.9 Hz, H-5′), 7.64 (1H, d, J = 2.0 Hz, H-2′), 7.80 (1H, dd, J = 2.0, 8.9 Hz, H-6′); ^{13}C NMR (150 MHz, CD_3OD): δ 29.8 (C7), 52.4 (COO*Me*), 56.0 (OMe), 56.5 (OMe), 56.6 (OMe), 97.2 (C5), 100.8 (C3), 109.6 (C1), 112.0 (C5′), 113.6 (C2′), 122.5 (C1′), 125.6 (C6′), 150.2 (C3′), 152.0 (C2), 155.4 (C4′), 160.3 (C6), 161.5 (C4), 165.8 (C7′), 173.7 (C8).

2.11. $LiAlH_4$ reduction of **7b**

A solution of **7b** (1.8 mg) in Et_2O (anhydrous, 0.1 mL) was added to a solution of $LiAlH_4$ (2 mg) in Et_2O (anhydrous, 0.1 mL), and the mixture was stirred at room temperature for 20 min. The reaction was then quenched with EtOAc, and the mixture was extracted with EtOAc/1 M HCl aq. The organic layer was collected and concentrated under vacuum. The residue was purified by p-TLC (55% EtOAc in hexane) to give **8** (0.9 mg) and **9** (0.9 mg). The chemical structures of **8** and **9** were identified using ^{1}H NMR.

2.12. Isolation of **1**, **2**, **7**, and **10** from black rice

Samples of black rice (10 g) were extracted overnight using 20 mL of 50% MeOH aq. with 1% TFA and then filtered. The sample was extracted twice. The filtrate was washed with chloroform after concentration under reduced pressure. The aqueous layer was concentrated under reduced pressure and loaded onto a Diaion HP20SS (Mitsubishi Chemical Corporation) column packed with 0.5% TFA aq. The column was eluted with the following solvent systems in the presented order: 0.5% TFA/H_2O; 10% CH_3CN/0.5% TFA/H_2O; 15% CH_3CN/0.5% TFA/H_2O; 20% CH_3CN/0.5% TFA/H_2O; 30% CH_3CN/0.5% TFA/H_2O; 0.5% TFA/MeOH. Fraction 2 (38.7 mg) was further purified by reversed-phase HPLC (15% CH_3CN/0.5%TFA/H_2O) to yield **1**. Fraction 3 (10.1 mg) was further purified by reverse-phase HPLC twice (18% CH_3CN/0.5% TFA/H_2O and 11.5% CH_3CN/0.5% TFA/H_2O) to yield **7** and **10**. Fraction 4 (22.3 mg) was further purified by reversed-phase HPLC (15% CH_3CN/0.5% TFA/H_2O) to yield **2**. The ^{1}H NMR spectra obtained for the isolated compounds were in agreement with previously reported data. As a result of quantitative analysis by HPLC, the amount of each compound in black rice per 10 g sample was as follows: **1**, 14 mg; **2**, 0.07 mg; **7**, 0.1 mg; **10**, 0.9 mg.

2.13. Quantitative analysis

Black rice samples (20 grain, approx. 500 mg) were extracted overnight with 2 mL of 50% MeOH aq. with 1% TFA and then filtered. The residue and filtrate were collected. The residue was dried at 105 °C for 5 h and weighed. The filtrate was concentrated under reduced pressure and weighed. This extract was dissolved in 0.2 mL of 50% MeOH aq. in 1% TFA and was used for the quantitative analysis of **1** and **10** by HPLC (15% CH_3CN/0.5% TFA/H_2O). After the quantitative analysis of **1** and **10**, this sample solution was redissolved in 1 mL of 2% TFA aq. after concentration and extracted three times with EtOAc. The organic layer was concentrated and dissolved in 0.2 mL of 50% MeOH aq. with 1% TFA. This solution was used for the quantitative analysis of **2** and **7** by HPLC (15% CH_3CN/0.5% TFA/H_2O).

3. Results and discussion

3.1. Radical oxidation of **1** in EtOH

Tsuda et al. previously described the radical oxidation of **1** with the radical initiator AMVN in 1996 (Tsuda et al., 1996) and reported the isolation of a 2,3-benzofuran derivative after reaction in aqueous CH_3CN; the derivative is formed by elimination of the B-ring from **1**, yielding protocatechuic acid (3,4-dihydroxybenzoic acid). We conducted the same reaction under the reported conditions and analysed the reaction mixture by HPLC. A similar HPLC chromatographic pattern was observed; however, the peaks differed from those observed from the analogous radical oxidation in EtOH (Fig. 2).

The reaction products were purified using preparative HPLC (Fig. 2A, **P1**–**P3**), and the structures were determined using NMR techniques and mass spectrometry (MS). We found that the peak labelled **P2** was due to ethyl protocatechuate **3b**, which originated from the cleavage of the B-ring of **1**. The ^{1}H NMR spectrum of the compound corresponding to **P1** indicated that the C-ring moiety of the unknown anthocyanin was altered by the reaction and that the A- and B-rings, as well as the glucoside unit remained intact.

To confirm the identity of **P1**, a pentamethoxy derivative was produced by permethylation using diazomethane, followed by acid hydrolysis to remove the glucoside unit (Fig. 3). The ^{1}H NMR and IR spectra of the transformed pentamethoxy **P1** derivative indicated the presence of four phenolic hydroxyl groups and an ester moiety. Upon catalytic hydrogenation of the permethylated **P1** derivative, the appearance of two additional proton signals was observed (compound **4**); furthermore, alcohol **5** was generated upon reduction of **4** with $LiAlH_4$. From this information, **P1** was identified as 2-(3,4-dihydroxyphenyl)-4,6-dihydroxybenzofuran-3-carboxylic acid glucose ester (**2**) based on its reactivity as described above (Fig. 3). HRESIMS data showed that the molecular formula of **2** was $C_{21}H_{20}O_{12}$ (calcd. for $C_{21}H_{20}O_{12}Na$, 487.0852; found,

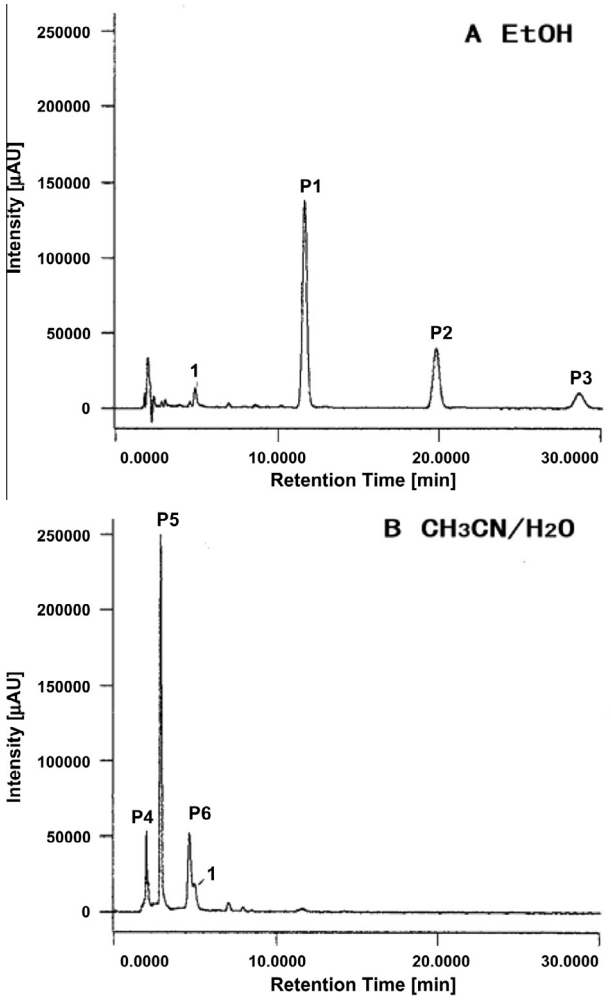

Fig. 2. HPLC chromatogram of products from the reaction of **1** with AMVN conducted in (A) EtOH, and (B) CH_3CN/H_2O. The reversed-phase HPLC system was operated under the following conditions: column, COSMOSIL 5C18-MS-II, 4.6 mm ID × 150 mm; solvent, 20% CH_3CN in a 0.5% aqueous TFA solution; flow rate, 1 mL/min; temp., 35 °C; detector, UV (254 nm).

487.0880), which is in good agreement with the structure obtained from the ^{1}H NMR spectrum. The ^{1}H NMR spectrum of **P3** was very similar to that of **2**, except for the absence of a characteristic glycoside peak at approximately 3–4 ppm, suggesting that **P3** was in fact **2a**, *i.e.*, the aglycone of **2**, which was confirmed through ^{1}H NMR spectral analysis after hydrolysis of **2**.

Compound **2c**, in which the glucoside unit of **2** is substituted with a methyl residue, has been previously isolated from black rice (*Oryza sativa* L.) and bran (Han, Ryu, & Kang, 2004). To provide further confirmation for the structure of **2**, **2a** was methylated in 2% H_2SO_4/MeOH. Compound **2c** was obtained and analysed using ^{1}H NMR, which was identical to that previously published.

The yields of **2**, **2a**, and **3b** from the oxidation of **1** in EtOH were 28%, 10%, and 32%, respectively.

3.2. Radical oxidation of *1* in aqueous CH_3CN

Compound **1** was treated with AMVN in aqueous CH_3CN using conditions modified from a previous report (Tsuda et al., 1996), and the solution was analysed using HPLC. Three new peaks were formed with an accompanying decrease of **1** (Fig. 2B, **P4**–**P6**). The reaction products were purified using preparative HPLC and the structures were determined using NMR and MS techniques. The results showed that the compounds corresponding to the peaks **P4** and **P5** were **6** and protocatechuic acid **3b**, respectively, as previously reported by Tsuda et al. (1996).

The NMR data obtained indicated that **P6** was a novel compound containing the A-, B-rings, and glucoside unit from **1**. HRESIMS data showed that the molecular formula of **P6** was $C_{21}H_{22}O_{13}$ ($C_{21}H_{22}O_{13}Na$: calcd. for $C_{21}H_{22}O_{13}Na$, 505.0958; found, 505.0949). The chemical structure of **P6** was determined using derivatisation reactions, *i.e.*, acid hydrolysis, methylation, and reduction (Fig. 3). The results allowed us to identify **P6** as 2-*O*-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid glucose ester **7**, generated by the cleavage of the C2–C3 bond in compound **1**. Compound **7a**, the acid hydrolysate of **7**, was previously isolated from the petals of *Papaver rhoeas* and the fruit of Jaboticaba (*Myrciaria cauliflora*); the spectral data for **7a** were in agreement with published data (Hillenbrand, Zapp, & Becker, 2004; Reynertson et al., 2006).

The yields of **6**, **3a**, and **7** from the oxidation of **1** in aqueous CH_3CN were 15%, 34%, and 14%, respectively.

3.3. Proposed radical oxidation mechanism of *1*

Our experiments indicated that the radical oxidation of **1** with AMVN produced different products in the two solvent systems examined; *i.e.*, **2**, **2a**, and **3b** were produced in EtOH, and **3a**, **6**, and **7** were produced in aqueous CH_3CN (Fig. 4). To explain these results, we propose mechanisms for the formation of these compounds in each solvent, as shown in Fig. 5.

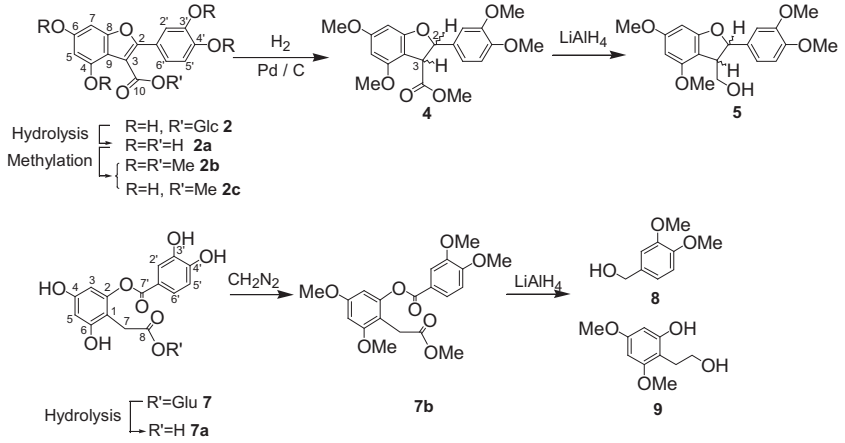

Fig. 3. Transformation of **2** and **7**.

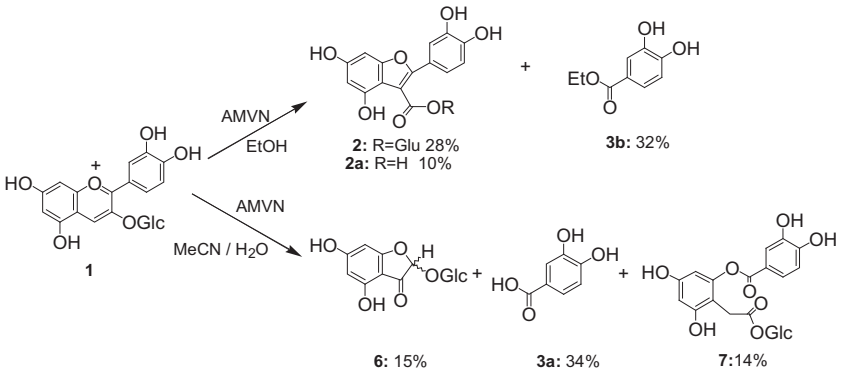

Fig. 4. Chemical structures of the radical oxidation products of **1**.

It is known that anthocyanins exist in different chemical forms in solution (Castañeda-Ovando et al., 2009). An equilibrium exists between the flavylium ion **I**, anhydrobase **II**, and carbinol pseudobase **III** because of reversible hydration at the 2-position; thus, all three anthocyanin forms can coexist in solution. Therefore, we suppose that AMVN oxidation occurs on each of the three forms of anthocyanin **1**.

In the AMVN-mediated oxidation of **III** in aqueous CH_3CN, the species undergoing oxidation is chalcone **IV**, a ring-opened species existing under aqueous conditions. The position in chalcone **IV** corresponding to the 4-position of **1** is oxidised, resulting in formation of **3a** and **6**. In addition, oxidation of tautomeric species **II** occurs at the position corresponding to the 3-position in **1**, resulting in formation of intermediate product **V**. In the presence of water, **V** is subsequently transformed to **7** by hydration at the 2-position and successive ring opening.

In the AMVN-mediated oxidation of **1** in EtOH, **III** can be formed by the nucleophilic attack of EtOH; however, oxidation to a compound analogous to **6** does not proceed because the C-ring opening reaction does not occur. Therefore, because of the equilibrium reaction described above, **III** is converted to **I** or **II**. Oxidation of **II** in EtOH with AMVN generates **V** by oxidation at the 3-position, in a manner similar to that in aqueous CH_3CN, and is then converted to **VI** by the nucleophilic attack of EtOH. Compound **VI** is converted to **2** by a rearrangement reaction accompanied by ring contraction. In EtOH, because the oxidation product derived from **III** is not produced, compound **2** and its aglycone **2a** are the main oxidation products.

3.4. Isolation of *1* and its oxidised derivatives from black rice

Black rice (*Oryza Sativa* L. *indica*) is a variety of rice with dark purple colouration primarily cultivated in Asia. Anthocyanins are thought to play an important antioxidative role in black rice during its storage; thus, it is likely that black rice contains an anthocyanin radical oxidant as above. Early studies have shown compound **1** as a major anthocyanin component in black rice (Abdel-Aal, Young, & Rabalski, 2006; Kim et al., 2008; Ryu et al., 1998). Accordingly, we

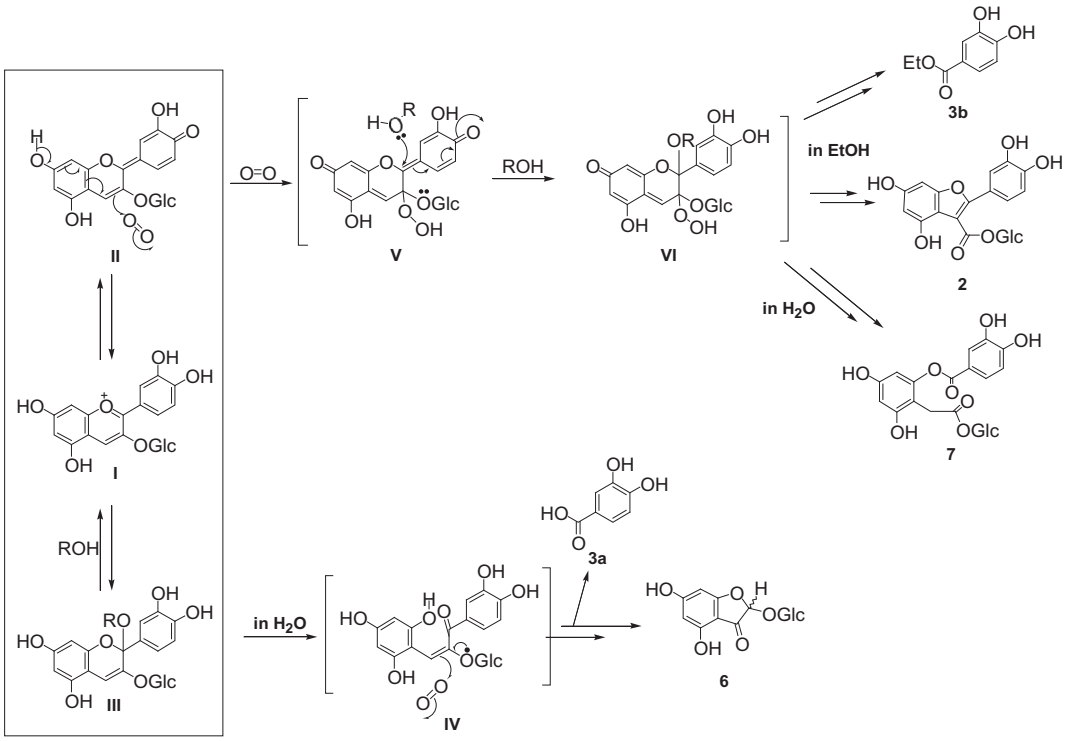

Fig. 5. Hypothetical oxidation mechanism for **1**.

Table 1
The amounts of **1**, **7**, and **10** in black rice at different storage periods.

Storage period	Content (mg/g)[a]			Ratio[b]		
	1	**10**	**7**	**1**	**10**	**7**
20 days[c]	8.25	0.44	–[d]	9.5	0.5	–[d]
0 years	1.70	0.07	0.01	9.4	0.4	0.03
1 years	1.74	0.07	0.03	9.1	0.4	0.2
2 years	0.97	0.03	0.05	8.5	0.3	0.4
3 years	1.29	0.05	0.08	8.4	0.3	0.5

[a] Total weight of samples were calculated from residue and extract weights.
[b] Content ratio of compound **1**, **10**, and **7** in sample.
[c] 20 days before harvest.
[d] Not detected.

investigated the isolation of **1** and its oxidised derivatives from a black rice sample subjected to long-term storage and performed a quantitative analysis of the isolated materials.

A black rice sample that had been stored for one year in the dark at room temperature was extracted with 50% MeOH aq. with 1% TFA overnight, and the extract was purified by a Diaion HP20SS (Mitsubishi Chemical Co.) column and reversed-phase HPLC. Compounds **1** (as the major anthocyanin) and peonidin 3-*O*-glucoside (**10**; minor) were obtained. The amounts of **1** and **10** in black rice were 0.19% and 0.007%, respectively. In addition, HPLC analysis of this extract revealed the presence of two peaks having the same retention time as those of **2** and **7**, which were further purified using preparative HPLC. The ^{1}H NMR spectra of the isolated compounds were in good agreement with the corresponding compounds obtained by the laboratory oxidation of **1**.

Quantitative analyses of **2** and **7** were performed on black rice samples at varying storage periods, *i.e.*, 20 days before harvest, just after harvest and after 1–3 years of storage; the results are shown in Table 1. Because growth conditions and water content differed with each sample, data are shown as the ratio of each component relative to the total dry weight. Compound **2** was not detected for all conditions examined due to its low concentration. In contrast, **7** was detected after harvest, although it could not be detected before harvest. The data suggested that **7** increased with concomitant decrease in anthocyanin compounds (**1**, **10**) with prolonged storage.

4. Conclusions

In conclusion, two novel oxidation products, compounds **2** (2-(3,4-dihydroxyphenyl)-4,6-dihydroxybenzofuran-3-carboxylic acid glucose ester) and **7** (2-*O*-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid glucose ester), were isolated from the AMVN-mediated oxidation reaction of **1**, with the discovery that different oxidation products were generated in different solvents. Based on these products, a radical reaction mechanism is proposed (*cf.* Fig. 5). Under the oxidation conditions of the proposed mechanism, **1** was transformed to the corresponding oxidised products with a total yield of around 70%, as shown in Fig. 5. This result indicates that our proposed oxidative mechanism is a preferred route for the radical degradation of anthocyanin. Investigations are currently underway for complete identification of oxidation products of other anthocyanin derivatives (*e.g.*, delphinidin glycoside) under similar oxidative conditions, in order to provide further insights into this radical oxidation route. In addition, the presence of these oxidised compounds in black rice after prolonged periods of storage under dark conditions indicates a high probability of this radical reaction occurring in food products by a similar mechanism. Indeed, the amount of compound **7** increased during prolonged storage periods. This result suggests the possibility that the amount of **7** detected is a suitable criterion for the determination of the freshness of black rice.

Acknowledgments

We thank Dr. Yoshihiro Miwa, professor emeritus at Gifu University, for providing the black rice samples. This work was partially supported by a C19 Kiyomi Yoshizaki Research Grant.

References

Abdel-Aal, E. S. M., Young, J. C., & Rabalski, I. (2006). Anthocyanin composition in black blue, pink, purple, and red cereal grains. *Journal of Agricultural and Food Chemistry, 54*, 4696–4704.
Castañeda-Ovando, A., Pacheco-Hernández, M. L., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry, 113*, 859–871.
Es-Safi, N. E., Meudec, E., Bouchut, C., Fulcrand, H., Ducrot, P. H., Herbette, G., et al. (2008). New compounds obtained by evolution and oxidation of malvidin 3-*O*-glucoside in ethanolic medium. *Journal of Agricultural and Food Chemistry, 56*, 4584–4591.
Han, S. J., Ryu, S. N., & Kang, S. S. (2004). A new 2-arylbenzofuran with antioxidant activity from the black colored rice (*Oryza satuca* L.) bran. *Chemical and Pharmaceutical Bulletin, 52*, 1365–1366.
Harborne, J. B. (1994). The flavonoids: Advances in research since 1986. Chapman & Hall.
Harborne, J. B., & Williams, C. A. (1995). Anthocyanins and other flavonoids. *Natural Product Reports*, 639–657.
Hillenbrand, M., Zapp, J., & Becker, H. (2004). Depsides from the petals of *Papaver rhoeas*. *Planta Medica, 70*, 378–380.
Hou, D.-X. (2003). Potential mechanisms of cancer chemoprevention by anthocyanins. *Current Molecular Medicine, 3*, 149–159.
Jang, Y. P., Zhou, J., Nakanishi, K., & Sparrow, J. R. (2005). Anthocyanins protect against A2E photooxidation and membrane permeabilization in retinal pigment epithelial cells. *Photochemistry and Photobiology, 81*, 529–536.
Jurd, L. (1964). Anthocyanins and related compounds. III. Oxidation of substituted flavylium salts to 2-phenylbenzofurans. *Journal of Organic Chemistry, 29*, 2602–2605.
Jurd, L. (1972). Recent progress in the chemistry of flavylium salts. *Recent Advances in Phytochemistry, 5*, 135–164.
Karrer, P., & Widmer, R. (1927). Plant coloring matters. V. Colors of primrose. *Helvetica Chimica Acta, 10*, 758–763.
Karrer, P., Widmer, R., Helfenstein, A., Hurliman, W., Nievergelt, O., & Monsasrrate-Thomas, P. (1927). Plant coloring matters. IV. Anthocyans and anthocyanidins. *Helvetica Chimica Acta, 10*, 729–757.
Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K., & Kobori, M. (2003). Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. *Journal of Agricultural and Food Chemistry, 51*, 68–75.
Kim, M. K., Kim, H., Koh, K., Kim, H. S., Lee, Y. S., & Kim, Y. H. (2008). Identification and quantification of anthocyanin pigments in colored rice. *Nutrition Research and Practice, 2*, 46–49.
Lee, J. H., Kang, N. S., Shin, S. O., Shin, S. H., Lim, S. G., Suh, D. Y., et al. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry, 112*, 226–231.
Lule, S. U., & Xia, W. (2005). Food phenolics, pros and cons: A review. *Food Reviews International, 21*, 367–388.
Matsumoto, H., & Iida, H. (2007). The improvement of refractive function by oral intake of blackcurrant anthocyanins and its action mechanism. *Japanese Pharmacology & Therapeutics, 35*, 447–455.
Nichenametla, S. N., Taruscio, T. G., Barney, D. L., & Exon, J. H. (2006). A review of the effects and mechanisms of polyphenolics in cancer. *Critical Reviews in Food Science and Nutrition, 46*, 161–183.
Reynertson, K. A., Wallace, A. M., Adachi, S., Gil, R. R., Yang, H., Basil, M. J., et al. (2006). Bioactive depsides and anthocyanins from jaboticaba (*Myrciaria cauliflora*). *Journal of Natural Products, 69*, 1228–1230.
Ryu, S. N., Park, S. Z., & Ho, C. T. (1998). High performance liquid chromatographic determination of anthocyanin pigments in some varieties of black rice. *Journal of Food and Drug Analysis, 6*, 729–736.
Tsuda, T., Ohshima, K., Kawakishi, S., & Osawa, T. (1996). Oxidation products of cyanicin 3-O-β-D-glucoside with a free radical initiator. *Lipids, 31*, 1259–1263.
Tsuda, T., Watanabe, M., Ohshima, K., Norinobu, S., Choi, S. W., Kawakishi, S., et al. (1994). Antioxidative activity of the anthocyanin pigments cyanidin 3-O-β-D-glucoside and cyanidin. *Journal of Agricultural and Food Chemistry, 42*, 2407–2410.
Valls, J., Millán, S., Martí, M. P., Borràs, E., & Arola, L. (2009). Advanced separation methods of food anthocyanins, isoflavones and flavanols. *Journal of Chromatography A, 1216*, 7143–7172.