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Structure of a Precursor to the Blue Components Produced in the Blue Discoloration in Japanese Radish (*Raphanus sativus*) Roots

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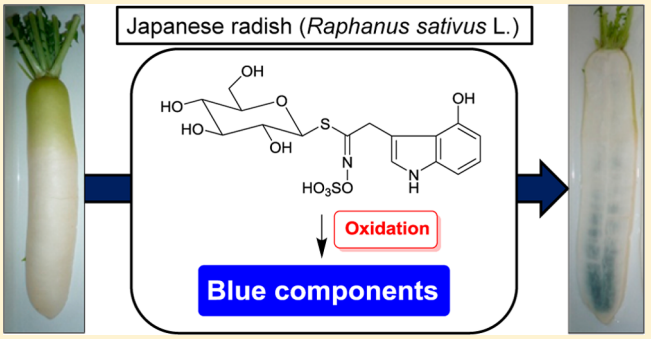
Supporting Information

ABSTRACT: The internal blue discoloration in Japanese radish (*Raphanus sativus* L.) roots has been reported to be a physiological phenomenon after harvest and poses a significant problem for farmers. To avoid this discoloration, the fundamental development of new radish cultivars that do not undergo discoloration and/or improved cultivation methods is required. Elucidating the chemical mechanism leading to this discoloration could help overcome these difficulties. To determine the mechanism underlying this discoloration, this study was designed to probe the structure of a precursor to the blue components generated during the discoloration process. Soaking fresh roots in aqueous H_2O_2 resulted in rapid blue discoloration, similar to the natural discoloration. Using a H_2O_2-based blue discoloration assay, the precursor was extracted and isolated from the fresh roots and identified as the glucosinolate, 4-hydroxyglucobrassicin, via spectroscopy and chemical synthesis.



The internal blue discoloration in Japanese radish (*Raphanus sativus* L.) roots is a physiological phenomenon that occurs when harvested radish roots are stored at approximately 20 °C for several days (Figure 1). Blue components are generated in the roots, and the onset of this discoloration in some radish cultivars has been frequently reported. Because the abnormal blue color decreases the commercial value of the affected radish, this discoloration is a serious problem for shopkeepers and farmers.

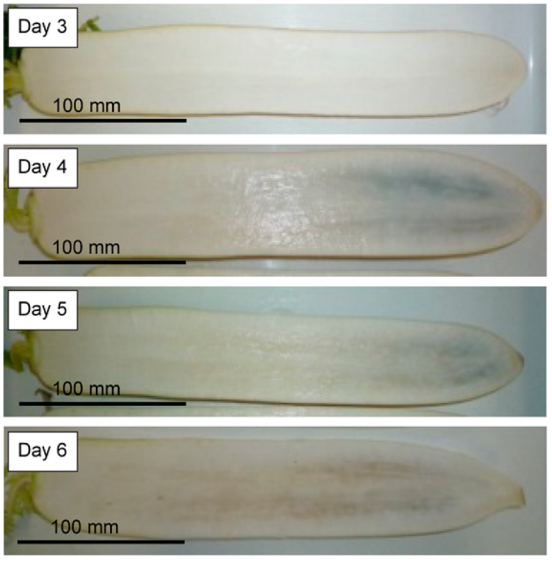


Figure 1. Time course for the discoloration of freshly harvested Hukuhomare roots during storage at 20 °C.

A substantial volume of research on the physiological phenomenon of the internal brown discoloration of radish roots has been reported.[1−4] However, few studies have investigated blue discoloration, and the mechanism underlying this phenomenon remains unknown. One reason for the lack of research on blue discoloration is the belief that the blue components comprise anthocyanins. In our preliminary research, the blue components did not show the characteristics of anthocyanins, including pH-dependent color changes and resistance to reduction by ascorbic acid. It has been reported that the incidence of blue discoloration depends on the radish cultivar and the cultivation conditions, similar to brown discoloration.[5]

To avoid blue discoloration after harvest, the fundamental development of new radish cultivars that do not experience discoloration and/or improved cultivation methods is needed. Elucidating the chemical mechanism of the discoloration can contribute to overcoming these difficulties. Additionally, because this is the first time this discoloration has been observed in white root vegetables, determining the mechanism is chemically and biologically important. This study reports the

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development of a rapid and simple discoloration assay and the isolation and chemical structural determination of the precursors of blue components in Hukuhomare radish roots.

RESULTS AND DISCUSSION

Artificial Blue Discoloration. Rapid and simple methods to artificially induce the discoloration of fresh Hukuhomare root sections were investigated and showed that soaking Hukuhomare root sections in aqueous H_2O_2 induced blue discoloration. The discoloration increased with time as the root sections were soaked in 0.29 M aqueous H_2O_2 at 20 °C, and the degree of discoloration reached a steady state within 5 to 60 min (Figure 2).

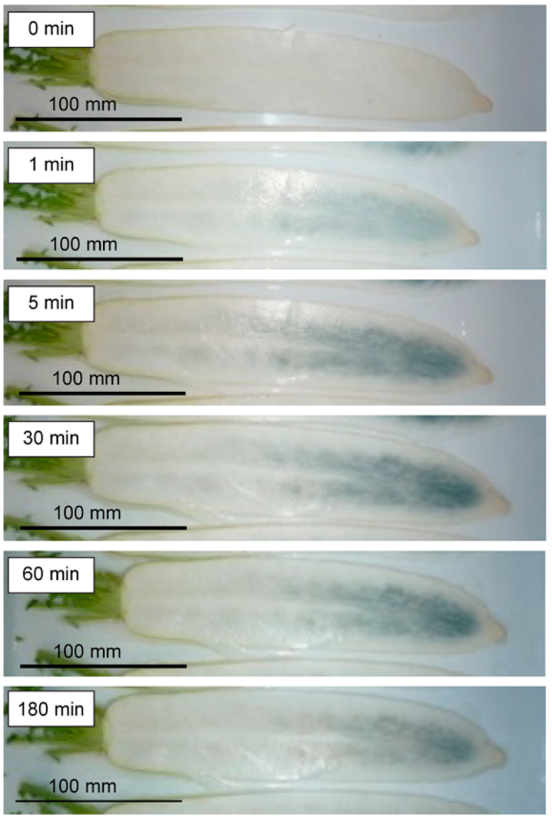

Figure 2. Time course for the discoloration of freshly harvested Hukuhomare root sections by soaking in 0.29 M aqueous H_2O_2 at 20 °C.

To spectroscopically characterize the resulting blue components, attempts were made to extract the blue components at 0 °C from roots treated with aqueous H_2O_2 and stored at 20 °C. However, the extraction failed because the blue color dissipated immediately after extraction. For the roots soaked in aqueous H_2O_2, the blue color turned brown, and for the roots stored at 20 °C, the blue color disappeared. The instability of the blue components is not surprising because the components resulting from both treatment with H_2O_2 and storage at 20 °C disappeared. Two different partial extraction methods for the blue components generated by soaking in aqueous H_2O_2 and storage at 20 °C were investigated. For the first method, it was hypothesized that the decomposition of the blue components could be promoted by enzymatic oxidation, such as peroxidase and excess H_2O_2. Therefore, 2 mL of MeOH, which generally inactivates enzymes, was added to 4 g of root during homogenization to partially extract the blue components. For the latter method, it was hypothesized that ascorbic acid could lead to discoloration of the blue components. Therefore, ascorbate oxidase was added during homogenization to remove the ascorbic acid by mild oxidation and thus suppress the color fading. Figure 3 compares the visible absorption spectra of the

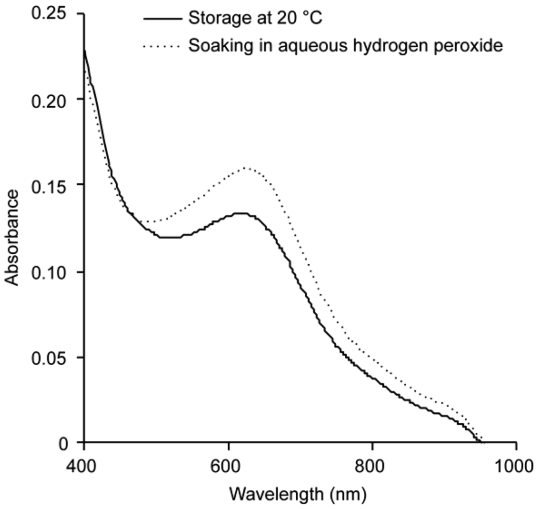

Figure 3. Visible absorption spectra of the blue components. The extracts were prepared from Hukuhomare root soaked in 0.29 M aqueous H_2O_2 or stored at 20 °C for 4 days.

extract prepared from Hukuhomare root treated with 0.29 M aqueous H_2O_2 according to the procedure described in the Experimental Section and the extract prepared from Hukuhomare root stored at 20 °C according to the procedure reported in the Experimental Section. The maximum absorptions in these spectra were observed at ca. 628 nm, and HPLC with PDA detection revealed that the extracts contain many blue components and that their HPLC−PDA characteristics are similar (Figure 4). In addition, the location of the discoloration resulting from the treatment with H_2O_2 was the same as that resulting from storage at 20 °C, as shown in Figures 1 and 2.

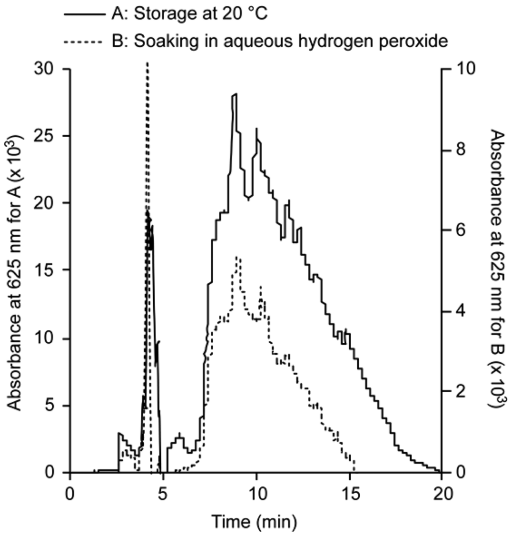

Figure 4. HPLC chromatograms of the extracted components prepared from Hukuhomare root stored at 20 °C for 4 days or soaked in 0.29 M aqueous H_2O_2.

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These results indicate that the blue discoloration can be mimicked by H_2O_2 treatment.

Freshly harvested Hukuhomare roots were vertically cut down the middle, and the xylem sections (10 × 10 × 10 mm) at 50 mm from the root tip were extracted. The addition of 0.29 M aqueous H_2O_2 to the homogenate and filtrate of the sections resulted in immediate discoloration (Figure 5), whereas the

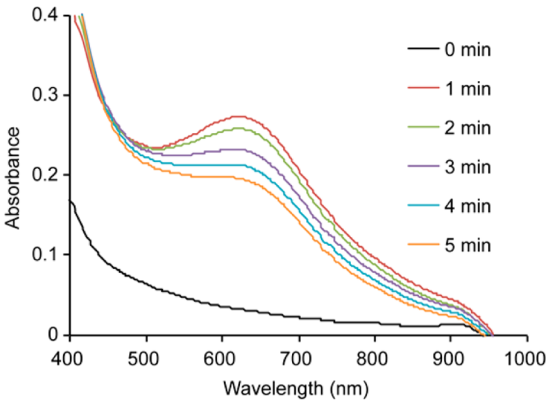

Figure 5. Time course for the discoloration of Hukuhomare root filtrate (0.15 mL) by the addition of 0.29 M aqueous H_2O_2 (7.5 μL) at 20 °C.

addition of aqueous H_2O_2 to the precipitate caused no discoloration, indicating that the component(s) involved in the generation of the blue components exist in the filtrate.

Assay Method. The isolation of the precursor(s) from the filtrate of freshly harvested Hukuhomare roots was next attempted. First, the filtrate was adsorbed on a reversed-phase column with ODS gel and then eluted by a stepwise increase (10% steps) of the MeOH concentration from 0 to 100%. The fractions were evaporated to the same volume as the filtrate. The blue discoloration activities in the fractions (0.135 mL) were assayed by adding 0.29 M aqueous H_2O_2 (7.5 μL), but no fraction showed activity. However, a mixture reconstructed from all of the fractions and the filtrate exhibited activity. Combination experiments using each fraction were subsequently performed, and the addition of a small amount of the 0% MeOH fraction to the 10 and 20% MeOH fractions was found to show activity. These results indicated that several components in the 0, 10, and 20% MeOH fractions are necessary to produce the blue discoloration using H_2O_2 and that the 0% MeOH fraction must contain component(s) that catalyze the discoloration. Adding EtOH, which generally decreases enzyme activity, inhibited the discoloration in a dose-dependent manner, as shown in Figure S1 (Supporting Information). Thus, the component in the 0% MeOH fraction must be an oxidation enzyme, such as peroxidase. Based on these findings, commercially available horseradish peroxidase (HRP) (15 and 150 units/mL, 0.015 mL) was added to the assay solutions of the 10 and 20% MeOH fractions (0.135 mL) with 0.29 M aqueous H_2O_2 (7.5 μL), and blue discoloration was observed.

Various different experiments were subsequently devised to optimize the amounts of H_2O_2 and HRP added to the discoloration assay reaction solution using a microplate absorbance reader. Finally, addition of 0.029 M aqueous H_2O_2 (7.5 μL) and 150 units/mL HRP (15 μL) to the sample solution (0.15 mL) was found to be optimum to explore the precursor(s).

Extraction and Isolation of the Precursor to the Blue Components. To extract the precursor from a large amount of root sections, the available techniques were severely limited because of the lengthy time required, the instability of the precursor, and the difficulty of isolating the root precipitate. Thus, a method for large-scale extraction was devised. Homogenization in the presence of acetone in a 2:1 ratio relative to the root sections (v/w) prevented the enzymatic decomposition of the precursor, decreased the viscosity of the homogenate, and excluded components that were insoluble in aqueous acetone. When equal amounts of acetone and root sections (v/w) were used, the extract exhibited more gradual blue discoloration in aqueous H_2O_2 in the absence of HRP (Figure S2a, Supporting Information) than in the case of extraction without acetone. Thus, using equivalent amounts was less effective at inactivating the peroxidases. The addition of aqueous H_2O_2 to the extract prepared using the 2:1 (v/w) ratio generated no blue components (Figure S2b, Supporting Information), and the extract exhibited discoloration of the blue color in the presence of HRP and H_2O_2 (Figure S2c, Supporting Information). These results indicated that the peroxidases were inactivated and/or not contained in the extract, and the precursor(s) were extracted as expected. Therefore, twice the amount of acetone was employed for the homogenization. In order to effectively perform the subsequent column chromatography of the extract, unnecessary components were removed as EtOH-insoluble precipitates by the addition of excess EtOH after washing with EtOAc.

The isolation of the final extract solution was achieved using HPLC with a reversed-phase column (Figure S3a, Supporting Information). The blue discoloration activities of the fractions were measured using the method outlined in the Experimental Section (Figure S4, Supporting Information). HPLC analysis of the fraction that exhibited the blue discoloration showed one peak (Figure S3b, Supporting Information). The final yield of purified precursor from 2 kg of root sections was approximately 40 mg, corresponding to approximately 95% of the precursor in the final extract solution based on the HPLC analysis.

Chemical Structure of the Isolated Precursor to the Blue Components. The isolated precursor to the blue components was colorless and soluble in H_2O, MeOH, and EtOH but not in EtOAc. The UV−vis absorption spectrum in H_2O showed a peak at 268 nm (Figure S5, Supporting Information). ^{13}C NMR data revealed the presence of 16 different carbon atoms in the precursor molecule (Figure S6 in the Supporting Information and Table 1). The HRMS data revealed the molecular formula to be $C_{16}H_{19}N_2O_{10}S_2$ (calcd m/z 463.04866, obs m/z 463.04861 $[M - H]^-$) (Figure S7a, Supporting Information), and the HRMS/MS data indicated the presence of a SO_3H group (Figure S7b, Supporting Information). COSY NMR data revealed the presence of one glucose residue (Table 1 and Figure S8 in the Supporting Information). The $^3J_{G\text{-}1,G\text{-}2}$ value (9.8 Hz) of the glucosyl moiety indicated the presence of a β-glucoside bond. The ^{13}C NMR chemical shift of the G-1 carbon was 22 ppm lower than that of methyl β-D-glucopyranoside (Table S1, Supporting Information), suggesting the existence of a thio- or amino-β-glucoside bond. In addition, the ^1H NMR chemical shift of the G-1 proton was 0.66 ppm higher than that of methyl β-D-glucopyranoside. This result indicated that an sp^2 or sp atom should be bound to the sulfur or nitrogen atom in the above β-glucoside bond. The UV spectrum and the HMBC (Figure S9, Supporting Information) and NOESY (Figure S9, Supporting

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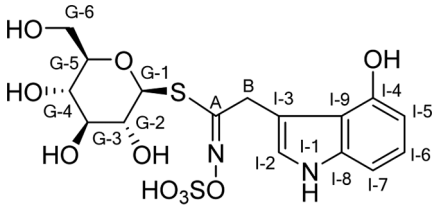
Table 1. ^{1}H and ^{13}C NMR Data of the Isolated Precursor to the Blue Components[a]

position[b]	δ_C, type	δ_H (J in Hz)
G-1	82.1, CH	4.67, d (9.8)
G-2	72.6, CH	3.06, dd (8.5, 9.8)
G-3	78.0, CH	2.99, dd (8.5, 8.5)
G-4	68.8, CH	3.26, dd (8.5, 9.8)
G-5	80.4, CH	3.12, m
G-6	60.1, CH_2	3.56, dd (4.3, 12.0), 3.67, d (12.0)
A	156.6, C	
B	29.3, CH_2	4.03, d (16.5), 4.50, d (16.5)
I-1-NH		10.76, br s
I-2	121.2, CH	6.96, br s
I-3	109.8, C	
I-4	151.8, C	
I-5	103.06 or 103.10, CH	6.39, d (7.3)
I-6	121.9, CH	6.87, dd (7.3, 7.9)
I-7	103.06 or 103.1, CH	6.83, d (7.9)
I-8	138.3, C	
I-9	115.9, C	
I-4-OH		9.4, br s

[a]Assignments are based on 2D NMR methods. The ^{1}H and ^{13}C NMR data of the isolated precursor to the blue components were obtained in DMSO-d_6 at 30 °C. [b]The positions of the atoms corresponding to the ^{1}H and ^{13}C NMR peaks are presented in the structure of **1**.

Information) NMR spectra suggested that the precursor might contain an indole moiety. Based on these spectroscopic data, it was speculated that the precursor might be 4-hydroxyglucobrassicin (**1**), which is a natural glucosinolate found in plants of the Brassicaceae family.[6,7] However, the ^{1}H and ^{13}C NMR data of **1** have not been reported thus far, and therefore, we used the data of desulfo-4-hydroxyglucobrassicin for comparison (Table S1, Supporting Information).[6] The ^{1}H and ^{13}C NMR data of the isolated precursor to the blue components and those of desulfo 4-hydroxyglucobrassicin were similar, strongly supporting the speculation regarding the structure of the precursor to the blue components. The UV spectrum was also similar to that of desulfo-4-hydroxyglucobrassicin.[8]



4-Hydroxyglucobrassicin (**1**)

Glucosinolates, including **1**, are found in plants of the Brassicaceae family, and in 2011, the total number of documented natural glucosinolates exceeded 130.[8] Compound **1**, isolated from cabbage seed and rapeseed, was identified using desulfonylation and subsequent GC/MS spectrometry and ^{1}H NMR data in 1982.[9] Although many glucosinolates have been synthesized,[10] the synthesis of **1** has not been reported. Gardrat et al. described the preparation of 4-benzyloxydesulfoglucobrassicin from 4-benzyloxyindole for the synthesis of **1** in 1999,[11] but since then, a synthesis of **1** has not been reported. In the present study, the synthesis of **1** was achieved from 4-hydroxyindole and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose using methods adapted from the procedures described by Cassel et al.[12] (Scheme 1). The ^{1}H and ^{13}C NMR data, HPLC−PDA−MS analyses, $[\alpha]_D^{25}$ value, and UV spectrum of the synthesized **1** were consistent with those of the isolated precursor to the blue components, confirming that the isolated precursor to the blue components is definitely **1**.

Blue Discoloration Reaction of Compound 1. As seen in Figure 6A, the blue discoloration of **1** occurred in the reaction with H_2O_2 and HRP, and the generated blue components were stable under the reaction conditions used. However, the blue color afforded by soaking the root sections in aqueous H_2O_2 or storing them at 20 °C turned brown over time. Thus, it is assumed that the components in the roots would affect the stability of the blue components. The HPLC−PDA analyses of the discoloration over time revealed that many types of blue components were produced during the initial reaction period and after 18 h (Figure 6B). Given the complicated root system, it seems reasonable to assume that many types of blue components could be produced by soaking the roots in aqueous H_2O_2 or storing them at 20 °C.

Compound **1** is known to be susceptible to oxidation and is readily converted to blue compound(s).[9,13] The oxidation mechanism was investigated by Truscott and Manthey using 4-hydroxyindole as a model.[14] They reported that oxidation proceeds via the formation of indol-(4,7)-*p*-quinone. Subsequently, Latxague and Gardrat found evidence supporting Truscott's results.[15] The UV−vis absorption spectrum of the oxidation products reported in their study was similar to that of the crude blue components obtained here (Figures 3 and 6A). Therefore, glucosinolates and their derivatives possessing an indol-(4,7)-*p*-quinone skeleton were likely produced via the blue discoloration reaction in the roots.

Using the Hukuhomare radish, which is susceptible to blue discoloration, an assay employing H_2O_2 and HRP was developed to observe the discoloration precursor and identified it as compound **1** by spectroscopy and chemical synthesis. This is the first report indicating that **1** is the precursor to the blue components in plants. These results should contribute to elucidating the molecular physiological mechanism underlying the blue discoloration.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined using an ASONE ATM-01 apparatus and are uncorrected. Optical rotation data were obtained using a P-2200 instrument (JASCO Corp. Tokyo, Japan). UV−vis spectra were obtained with a V-530 DS spectrometer (JASCO Corp.). IR data were obtained using an FT/IR 410 spectrometer (JASCO Corp.). ^{1}H, ^{13}C, $^{1}H-^{1}H$ COSY, $^{13}C-^{1}H$ COSY, HMBC, and NOESY NMR spectra were measured on a JNM-A500 spectrometer (JEOL, Tokyo, Japan) operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. TMS and acetone were used as the internal standards for DMSO-d_6 and D_2O, respectively. Chemical shift values and coupling constants are reported as δ (ppm) and J (Hz), respectively. HRMS analysis was performed using an LTQ Orbitrap Velos EDT Ultimate 3000 system and Xcalibur version 2.2 SPI (Thermo Fisher Scientific Inc., MA., USA) in ESI mode. HPLC−PDA−MS analyses were performed at 25 °C on a JASCO Gulliver HPLC system equipped with an MD-910 detector and a ZQ 4000 mass spectrometer (Waters Corporation, Milford, MA, USA). Mass analyses were carried out in positive ESI mode. Thin-layer chromatography was performed on Merck Kieselgel 60 F254 plates (layer thickness, 0.25 mm; Merck KGaA, Darmstadt, Germany), and the spots were visualized with a UV lamp (254 nm). Preparative chromatography was performed on BW-200 silica gel columns (Fuji Silysia, Aichi, Japan). Elemental analyses were achieved with a Yanaco CHNCORDER MT-3 instrument. HRP and 2,3,4,6-tetra-*O*-acetyl-1-

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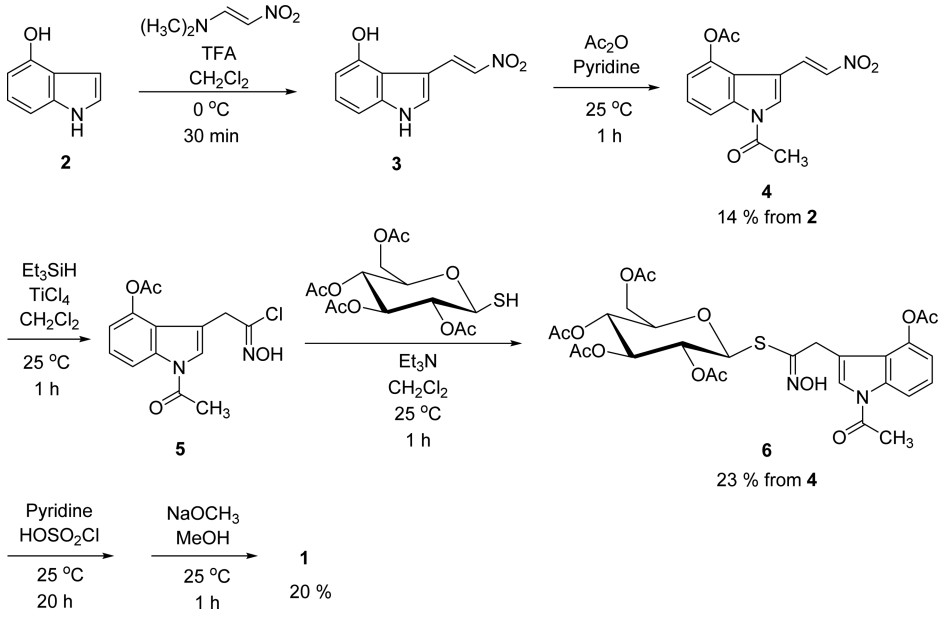
Scheme 1. Chemical Synthesis of 1



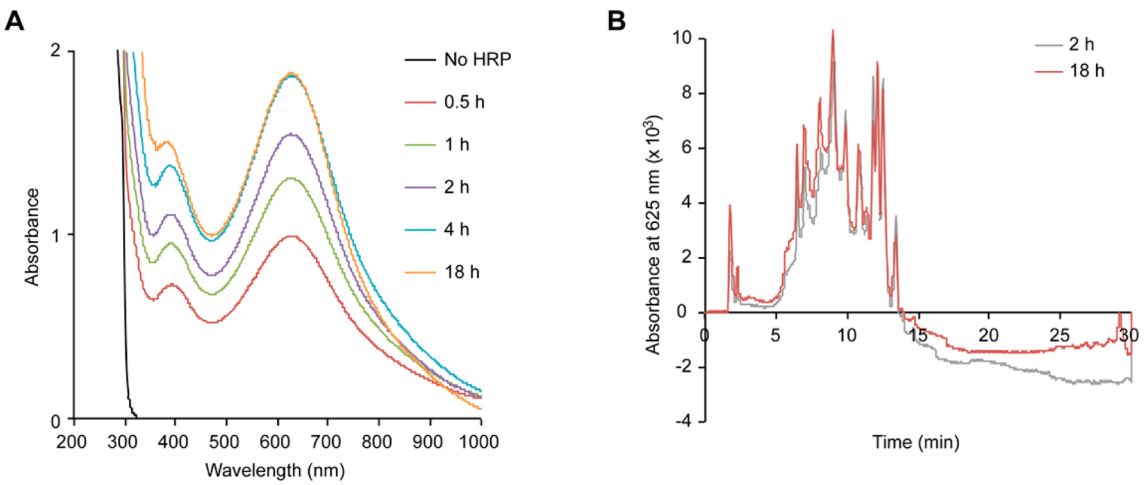


Figure 6. Monitoring the reaction of **1** with H_2O_2 and HRP. (A) UV−vis absorption spectra and (B) HPLC chromatograms at 2 and 18 h after the addition of H_2O_2 and HRP to the solution of **1**. The reaction and HPLC conditions are described in the Experimental Section.

thio-β-D-glucopyranose were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan.

Plant Material and Cultivation Conditions. *R. sativus* L. cv. Hukuhomare radish seeds were purchased from the Mikado Kyowa Seed Co., Ltd., in Japan. The radish cultivar was grown in the field of the Ishikawa Sand Dune Agricultural Research Center in 2013 using conventional methods. The sowing date was the September 5, and the roots were harvested approximately 60 days after sowing.

Radish Root Internal Blue Discoloration at 20 °C and Using H_2O_2. Freshly harvested radish roots were stored at 20 °C and approximately 80% relative moisture content for 3−6 days in the dark. Freshly harvested radish roots were vertically cut down the middle and soaked in 0.29 M aqueous H_2O_2 at approximately 20 °C, and the resulting discoloration was visually observed for 180 min.

Extraction of the Blue Components from Radish Roots Treated with H_2O_2. Freshly harvested Hukuhomare roots were vertically cut down the middle, and the sections were soaked in 0.29 M aqueous H_2O_2 at 20 °C for 5 min. The roots were lightly rinsed with distilled H_2O. The blue portion (4 g) of the root was homogenized with MeOH (2 mL) in an ice bath, and the homogenate was filtered through absorbent cotton. The resulting filtrate was centrifuged at 10 000*g* and 0 °C for 5 min. The supernatant was filtered through a 0.45 μm membrane filter (Millipore Millex-LH 13NL, Merck Millipore, and Darmstadt, Germany). UV−vis absorption spectra of the filtrate were obtained using a V-530 DS spectrometer, and HPLC−PDA analysis of the filtrate was performed immediately.

Extraction of the Blue Components from Radish Roots Stored at 20 °C. Freshly harvested Hukuhomare roots were stored at 20 °C and approximately 80% relative moisture content for 4 days in the dark. The roots were vertically cut down the middle, and the blue portion (4 g) was homogenized with a mixture of 0.4 mL of 100 units/mL ascorbate oxidase and MeOH (0.8 mL) in an ice bath. The homogenate was filtered through absorbent cotton, and the resultant filtrate was centrifuged at 10 000*g* and 0 °C for 5 min. The supernatant was filtered through a 0.45 μm membrane filter. UV−vis absorption spectra of the filtrate were obtained using a UV−vis spectrometer, and HPLC−PDA analysis of the filtrate was performed immediately, as outlined in the next section.

HPLC-PDA Analysis of Root Extracts. The HPLC−PDA instrument was equipped with a Cosmosil 5C18-PAQ column. The mobile phase was a mixture of aqueous 10 mM phosphate buffer (pH 7.0) (A) and MeOH (B). The flow rate was 0.8 mL/min in a linear gradient starting with 0% B and reaching 100% B at 30 min.

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Blue Discoloration Using the Filtrate. Freshly harvested Hukuhomare roots were vertically cut down the middle, and xylem sections (10 × 10 × 10 mm) at 50 mm from the root tip were picked out. Each section was homogenized with 0.1 mL of MeOH in an ice bath, and the homogenate was filtered through absorbent cotton. The resulting filtrate was centrifuged at 10 000*g* and 0 °C for 10 min. The supernatant was filtered through a 0.45 μm membrane filter. The filtrate (0.15 mL) was added to a microquartz cell (Hellma Ultramicro Cell, Hellema Analytics, Müllheim, Germany), and the absorbance at 628 nm was measured at 20 °C using a UV−vis spectrometer, with the absorbance at 628 nm calibrated to 0. Aqueous H_2O_2 (0.29 M, 7.5 μL) was added to the quartz cell at 20 °C, and the absorbance at 628 nm was immediately recorded every 1 s for 5 min.

Measuring the Blue Discoloration Activity. Sample solutions (0.15 mL) were placed in the wells of a 96-well plate, and 0.029 M aqueous H_2O_2 (7.5 μL) and 150 units/mL HRP (15 μL) in 0.1 M phosphate buffer (pH 7.0) were added. The absorbance at 620 nm was measured at 20 °C every 2 min for 2 h using a microplate absorbance reader (Sunrise Rainbow, Tecan Japan Co., Ltd., Kanagawa, Japan).

Extraction and Isolation of the Precursor to the Blue Components. The precursor to the blue components was extracted and isolated using the following sequence.

Xylem sections (150 g, at 50 to 150 mm from the root tip) of freshly harvested Hukuhomare radish roots were homogenized with 300 mL of acetone in an ice bath using a Hsiangtai HG-200 homogenizer and then filtered through a glass filter. The filtrate was evaporated under reduced pressure to approximately 100 mL. The resultant concentrate was washed with EtOAc (2 × 150 mL). The water extract was evaporated under reduced pressure to approximately 70 mL, and the concentrate was filtered through a 0.45 μm membrane filter. The filtrate was evaporated to approximately 15 mL, to which 90 mL of EtOH was added with stirring at room temperature for 2 h. The resulting supernatant was separated from the precipitate and rinsed with EtOH. The EtOH solutions were combined and evaporated to near dryness, and the residue was dissolved in H_2O (30 mL). This aqueous solution was designated as the "extract solution". This extraction process was also performed for 2 kg of radish root sections.

Isolation of the extract solution (0.4 mL) was achieved using a JASCO Gulliver HPLC system with an MD-910 detector and a Cosmosil 5C18-PAQ column (20 mm × 250 mm; Nacalai Tesque, Inc., Kyoto, Japan). The mobile phase was a mixture of aqueous 10 mM phosphate buffer (pH 7.0) (A) and MeOH (B). A linear gradient was used, starting with 5% B and reaching 30% B in 60 min at room temperature with a flow rate of 5 mL/min. The precursor to the blue components eluted between 30 and 35 min as a single peak. The fraction containing the precursor to the blue components was evaporated under reduced pressure to near dryness. HPLC purification of the 2 kg of radish root sections was conducted. The residue containing the precursor to the blue components was dissolved in H_2O (25 mL), and the resulting solution (1 mL) was purified with HPLC using the aforementioned conditions. The fractions of precursor to the blue components were pooled, evaporated under reduced pressure to near dryness, and then dissolved in H_2O (1 mL). MeOH (15 mL) and EtOH (15 mL) were added to the solution. The resulting suspension was centrifuged at 10 000*g* and 4 °C for 10 min, and the supernatant was separated, which was evaporated to complete dryness under reduced pressure to afford the pure precursor (40 mg) to the blue components containing a small amount of phosphate solid. The purity of the precursor was confirmed using HPLC−PDA−MS at 25 °C on a JASCO Gulliver HPLC system equipped with an MD-910 detector and a ZQ 4000 mass spectrometer. The HPLC elution was conducted on a Cosmosil 5C18-PAQ column (4.6 mm × 250 mm) at an elution rate of 0.8 mL/min with a linear gradient system of aqueous 10 mM phosphate buffer (pH 7.0) and MeOH (changing the ratio from 100:0 to 0:100 in 30 min). Mass analysis was carried out in negative ESI mode.

Isolated Precursor to the Blue Components: White, amorphous solid; $[\alpha]_D^{25}$ −7 (*c* 0.4, H_2O); UV (H_2O) λ_{max} 268 nm; IR (KBr) ν_{max} 3410, 1638, 1252, 1060 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 463.04861 $[M - H]^-$ (calcd for $C_{16}H_{19}N_2O_{10}S_2$, 463.04866).

*(E)-3-(2-Nitroethenyl)-1H-indole-4-ol (**3**).* To a mixture of 4-hydroxyindole (3 g, 0.023 mol), 1-(dimethylamino)-2-nitroethylene (2.5 g, 0.022 mol), and CH_2Cl_2 (60 mL) was added TFA (3.4 mL, 0.046 mol) at 0 °C with stirring for 30 min. EtOAc (50 mL) was added and the solution neutralized by addition of aqueous saturated $NaHCO_3$ (90 mL). The mixture was extracted with EtOAc (2 × 100 mL), and the combined EtOAc layers were dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The resulting residue was adsorbed on a silica gel column in EtOAc/*n*-hexane (1:1 v/v) and eluted with the same solvent. The eluent was evaporated under reduced pressure to afford crude compound **3** (3.6 g), which was used in the next step without further purification. ESI-MS *m*/*z* 205.13 $[M + H]^+$ (calcd for $C_{10}H_8N_2O_3$ + H, 205.06).

*(E)-1-Acetyl-3-(2-nitroethynyl)-1H-indol-4-yl acetate (**4**).* A mixture of crude compound **3** (3.6 g), pyridine (30 mL), and Ac_2O (30 mL) was stirred at room temperature for 1 h and the solvent evaporated under reduced pressure. MeOH (5 mL) was added to the residue, and the mixture was evaporated to dryness under reduced pressure. The resulting solid was dissolved in CH_2Cl_2 and purified by silica gel column chromatography using CH_2Cl_2 as the eluent. The fraction containing compound **4** was evaporated under reduced pressure and crystallized from a mixture of EtOAc and Et_2O to give yellow prisms of **4** (0.9 g, 14% yield from 4-hydroxyindole): mp 210−212 °C; λ_{max}/nm (log ε) (MeOH) 208 (4.25), 226 (4.24), 266 (4.06), 360 (4.11); IR (KBr) ν_{max} 1742, 1632, 1544, 1509, 1432, 1375, 1336, 1301, 1234, 1197 cm^{-1}; ^1H NMR (DMSO-d_6, 27 °C) δ/ppm 2.41 (3H, s, CH_3), 2.69 (3H, s, CH_3), 7.19 (2H, d, *J* = 7.9 Hz, indol-H5 or 7), 7.42 (1H, t, *J* = 7.9 Hz, indol-H6), 8.18 (1H, d, *J* = 13.4 Hz, C═CH), 8.23 (1H, d, *J* = 13.4 Hz, C═CH), 8.25 (1H, d, *J* = 7.9 Hz, indol-H5 or 7), 8.79 (1H, s, indol-H2) (see Figure S10, Supporting Information); ^{13}C NMR (DMSO-d_6, 27 °C) δ/ppm 20.72 (CH_3), 23.86 (CH_3), 110.31 (C), 114.07 (CH, indol-C5 or 7), 118.16 (CH, indol-C5 or 7), 120.07 (C), 126.25 (CH, indol-C6), 130.85 (CH, C═C), 131.32 (CH, indol-C2), 136.93 (CH, C═C), 137.01 (C), 143.24 (C), 168.72 (C, C═O), 169.80 (C, C═O) (see Figure S11, Supporting Information). Anal. Calcd for $C_{14}H_{12}N_2O_5$: C, 58.33; H, 4.20; N, 9.72%. Found: C, 58.39; H, 4.16; N, 9.68%.

*2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl-[(4-acetoxy-1-acetyl-1H-indol-3-yl)methyl]thiohydroxymate (**6**).* Compound **4** (0.5 g, 1.7 mmol) was dissolved in dry CH_2Cl_2 (25 mL). To the solution, were added Et_3SiH (0.36 mL, 2.3 mmol) and 0.91 M $TiCl_4$ in dry CH_2Cl_2 (2.9 mL, 2.6 mmol) at 0 °C. After being stirred at 25 °C for 1 h, cold H_2O (50 mL) was added and the solution was extracted with CH_2Cl_2 (2 × 25 mL). The combined CH_2Cl_2 layers were dried with anhydrous Na_2SO_4 and evaporated under reduced pressure to give crude (4-acetoxy-1-acetyl-1*H*-indol-3-yl)ethanimidoyl chloride (**5**), which was used in the next step without purification. To crude compound **5** in CH_2Cl_2 (25 mL) were added 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (0.32 g, 0.88 mmol) and Et_3N (0.72 mL, 5.2 mmol) at 25 °C. After being stirred at 25 °C for 1 h, cold H_2O (25 mL) and aqueous 1 M HCl (5 mL) were added and the solution was extracted with CH_2Cl_2 (2 × 25 mL). The CH_2Cl_2 layer was dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 and purified by silica gel column chromatography using EtOAc/CH_2Cl_2 (1:4, subsequently 1:2 v/v). The fraction containing compound **6** was evaporated under reduced pressure, and compound **6** was crystallized from MeOH to afford pure **6** (0.25 g, 23% yield from compound **4**): mp 235−237 °C; $[\alpha]_D^{25}$ −25 (*c* 0.5, $CHCl_3$); λ_{max}/nm (log ε) (MeOH) 208 (4.24), 236 (4.30), 302 (3.85); IR (KBr) ν_{max} 1756, 1433, 1370, 1219, 1047 cm^{-1}; ^1H NMR (DMSO-d_6, 27 °C) δ/ppm 1.92 (3H, s, CH_3), 1.97 (3H, s, CH_3), 2.00 (3H, s, CH_3), 2.01 (3H, s, CH_3), 2.28 (3H, s, CH_3), 2.61 (3H, s, CH_3), 3.97 (1H, d, *J* = 17.1 Hz, CH_2), 4.07 (1H, d, *J* = 10.4 Hz, glucose-H6), 4.10 (1H, d, *J* = 17.1 Hz, CH_2), 4.13 (1H, m, glucose-H6), 4.23 (1H, m, glucose-H5), 4.93 (1H, t, *J* = 9.8 Hz, glucose-H2), 4.97 (1H, t, *J* = 9.8 Hz, glucose-H4), 5.45 (1H, t, *J* = 9.8 Hz, glucose-H3), 5.76 (1H, d, *J* = 9.8 Hz, glucose-H1), 7.01 (1H, d, *J* = 7.9 Hz, indol-H5 or 7), 7.29 (1H, t, *J* = 7.9 Hz, indol-H6), 7.73 (1H, s, indol-

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H2), 8.18 (1H, d, J = 7.9 Hz, indol-H5 or 7), 11.27 (1H, s, OH) (see Figure S12, Supporting Information); ^{13}C NMR (DMSO-d_6, 27 °C) δ/ppm 20.27 (CH_3), 20.29 (CH_3), 20.33 (CH_3), 20.35 (CH_3), 20.84 (CH_3), 23.79 (CH_3), 28.65 (CH_2), 62.20 (CH_2, glucose-C6), 68.04 (CH, glucose-C4), 69.73 (CH, glucose-C2), 72.85 (CH, glucose-C3), 74.27 (CH, glucose-C5), 77.85 (CH, glucose-C1), 113.27 (CH, indol-C5 or 7), 114.72 (C), 117.00 (CH, indol-C5 or 7), 122.62 (C), 124.88 (CH, indol-C6), 125.84 (CH, indol-C2), 136.97 (C), 143.41(C), 147.69 (C), 168.77 (C, C═O), 169.07 (C, C═O), 169.10 (C, C═O), 169.31 (C, C═O), 169.58 (C, C═O), 170.07 (C, C═O) (see Figure S13, Supporting Information). Anal. Calcd for $C_{28}H_{32}N_2O_{13}S$: C, 52.83; H, 5.07; N, 4.40%. Found: C, 52.87; H, 5.01; N, 4.35%.

*4-Hydroxyglucobrassicin (**1**).* Compound **6** (0.1 g, 0.16 mmol) was dissolved in dry pyridine (3 mL), and chlorosulfonic acid (0.21 mL, 3.1 mmol) was added to the solution at −50 °C. After being stirred at 25 °C for 20 h, aqueous saturated $NaHCO_3$ (0.12 mL) was added and the solution was extracted with CH_2Cl_2 (2 × 25 mL). The CH_2Cl_2 layer was dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was dissolved in MeOH (3 mL), to which 0.5 M NaOMe in MeOH (0.56 mL, 0.28 mmol) was added at 25 °C. After 1 h, HOAc (0.032 mL) and 0.1 M phosphate buffer (pH 7.6) (1 mL) were added to neutralize the solution, which was then evaporated under reduced pressure. The residue was dissolved in H_2O and filtered through a 0.45 μm membrane filter. The filtrate was purified using a JASCO Gulliver HPLC system with an MD-910 detector and a Cosmosil 5C18-PAQ column (20 mm × 250 mm) using the elution conditions described above. The fractions containing compound **1** were pooled, evaporated under reduced pressure to near dryness, and dissolved in H_2O (1 mL). MeOH (10 mL) was added, and the resulting suspension was centrifuged at 10 000g and 4 °C for 10 min. The supernatant was separated and evaporated under reduced pressure to complete dryness to afford pure **1** (15 mg, 20% yield from **6**) containing a small amount of phosphate solid. ^{1}H NMR, ^{13}C NMR, UV−vis, IR, and HPLC−PDA−MS analyses were performed, and the data were found to be consistent with those of the isolated precursor to the blue components: $[\alpha]_D^{25}$ −7 (c 0.4, H_2O).

Blue Discoloration Reaction of Compound 1. An aqueous solution (0.5 mL) of 1 mM compound **1** was added to a microquartz cell, and the absorption spectrum was measured at 20 °C using the V-530 DS spectrometer. Aqueous H_2O_2 (0.29 M, 7 μL) and 1500 units/mL HRP solution (10 μL) were added to the solution at 20 °C, and UV−vis absorption spectra were collected. Simultaneously, a sample (20 μL) of the solution was removed from the quartz cell for HPLC−PDA analysis. HPLC−PDA analysis was performed using a JASCO Gulliver HPLC system with the MD-910 detector. The HPLC was equipped with a Cosmosil 5C18-PAQ column (4.6 mm × 250 mm), and the mobile phase was a mixture of aqueous 10 mM phosphate buffer (pH 7.0) (A) and MeOH (B). The flow rate was 0.8 mL/min in a linear gradient starting with 0% B and reaching 100% B in 30 min.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00121.

Figures S1−S13 and Table S1 (PDF)

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Notes

The authors declare no competing financial interest.

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