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Role of Caffeic Glucoside Esters in Defense-Repair Processing of Trees I.

Synthesis of the partial structure of acteoside, 2-(3,4-dihydroxyphenyl)-ethyl β-D-glucopyranoside, and peroxidase-catalyzed oxidation*1

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樹木の修復・防御機能発現におけるカフェー酸糖エステル類の役割（第1報）

アクテオシッドの部分構造，2-(3,4-ジハイドロキシフェニル)-エチル β-D-グルコピラノシッドの合成およびペルオキシダーゼによる酵素的酸化反応*1

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カフェー酸糖エステル類は樹体内における生理的役割として障害時の防御-修復機能の発現に関与している可能性を検証するために，カフェー酸糖エステル類の3種類の化合物および部分構造の一つとして合成した2-(3,4-ジヒドロキシフェニル)-エチル β-D-グルコピラノシド（化合物 **9**）の酵素的酸化反応を行った。差スペクトルの検討により，*o*-キノン構造が生成し，縮合物に変化することが示された。アクテオシドのペルオキダーゼによる酵素的酸化反応で褐色の生成物の沈殿が認められ，その収率は種々の反応条件で22～89%であった。この褐色生成物は種々の極性溶媒に難溶であった。化合物 **9** では沈殿が認められなかった。生成物の沈殿には，カフェオイル部分とラムノース部分の両部分構造あるいはそれらのいずれかが必要であることが示唆された。アクテオシッドの縮合物の化学構造について，その構造中に糖部分が存在し，構成成分の縮合にラジカルカップリングが関与していると考察した。合成した化合物 **9** は天然物と同一のものであった。

Caffeic glucoside esters in trees can play a physiological role in the defense-repair processing of wounded tissues. To provide *in vitro* evidence for this explanation, enzymatic oxidation of three caffeic glucoside esters with a synthetic component of them, 2-(3,4-dihydroxyphenyl)-ethyl β-D-glucopyranoside (Compound **9**) was examined. The difference spectra showed that *o*-quinones were formed, followed by a conversion to condensed substances. Acteoside resulted in a brown substance precipitated in the peroxidase-catalyzed oxidation, yielding 22- to 89-% under different incubation conditions. It hardly was soluble in various polar solvents. For Compound **9**, observable precipitation did not occur. It was inferred to have sugar moieties in its condensed structure. Both or one

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of the caffeoyl moieties and the rhamnosyl one likely may be required to cause precipitable substances. An intervention of radical couplings among constitutive moieties of it is discussed. Compound **9** was identical with the natural specimen.

Keywords: caffeic glucoside esters, defense-repair processing, enzymatic oxidation, synthesis, 2-(3,4-dihydroxyphenyl)-ethyl β-D-glucopyranoside.

1. INTRODUCTION

The caffeic glucoside esters (often termed phenylpropanoid glucosides) are found widely distributed in plants.[1] However, their physiological function is unknown.

In our work on the discoloration of kiri (*Paulownia tomentosa* Steud.) wood, caffeic glucoside esters (acteoside and its analogous compounds shown in Fig. 1) were found to be distributed 1.5- to 3.6-mg/g (fresh weight) in the xylem of a tree. Also, the peroxidase activity in the outermost xylem was much greater than that in other parts of the xylem.[2-4] When acteoside was subjected to peroxidase-catalyzed oxidation, the brown substance precipitated was found to be insoluble in neither acetone, methanol, and acetone/water (9/1, v/v), nor in dioxane/water (1/1, v/v).

It is therefore conceivable that this is a greatly condensed substance that would be formed by oxidative coupling. Such a coupling may be formed between caffeoyl- and caffeoyl moieties or between caffeoyl- and 2-(3,4-dihydroxyphenyl)-ethyl moieties, resulting in an intermolecular coupling, and between 2-(3,4-dihydroxyphenyl)-ethyl- and caffeoyl moieties, resulting in an intramolecular coupling. Besides, there may be couplings between sugar moieties and aromatic ones via quinonemethide intermediates. This would cause reducing of their solubilities.

The idea of oxidative phenolic coupling has been proposed in the initiation of lignification,[5-7] the formation of phenol-carbohydrate coupling,[8-17] the induction of a defense against fungal attack,[18,19] the lessening of cell wall extensibility,[20] and the browning of food.[21] Our interest in oxidative phenolic coupling, especially the peroxidase-catalyzed coupling centers around a role involved in the defense-repair processing of trees. When trees are injured, their cell walls and cell membranes are ruptured, and thus the integrities of cellular compartments change, leading to the mixing of substrates and enzymes and concomitantly to the efflux of vacuolar contents. In this context, the above condensed substance could play a possible role as a repairing material, a constitutive material, or an infusing substance in the ligno-suberized boundary zone or other structural barriers induced in the wounded tissues.[22-24]

The present work is aimed at realizing physiological functions of caffeic glucoside esters in trees through the characterizations of linkage types of oxidative couplings among constitutive groups in the above condensed substances. Its chemical structure would be expected to be of a greatly complex polymer because acteoside has two *o*-diphenol moieties and two sugar ones in its structure. To develop the problem; synthesis of acteoside and its partial structures would be required, which would help labelling studies of the enzymatic oxidation of it *in vitro* and *in vivo*.

In this study, one of the partial structural compounds of acteoside, Compound **9**, was synthesized and enzymatic oxidations of three caffeic glucoside esters with it by a crude soluble enzyme solution (PTP) of kiri and the commercial horseradish peroxidase solution (HRP) were spectroscopically and visually examined.

2. RESULT AND DISCUSSION

2.1 Synthesis of 2-(3,4-dihydroxyphenyl)-ethyl β-D-glucopyranoside (**9**)

The structure of the intermediates and final compound was confirmed by spectroscopic methods or spectral comparisons with the natural specimen.

2-(3,4-dihydroxyphenyl)-ethyl β-D-glucopyranoside (**9**) was a known compound previously reported as a constituent of the fruits of *Ligustrum obtusifolium* Sieb. et Zucc., and the bark of *Prunus grayana* Maxim.[25,26] Its aglycone, 3,4-dihydroxyphenethyl alcohol, was recognized first as a component of echinacoside, a glucoside from *Echinacea angustifolia*[27] and unambiguously was prepared from

Compound **2**.[28]

Compound **9** was synthesized according to the synthetic scheme shown in Fig. 1.

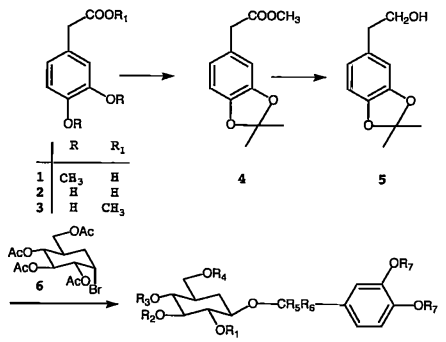
3,4-Dihydroxyphenylacetic acid **2** was prepared from homoveratric acid **1** according to the procedure of Shaw et al. in a yield of 85%.[29]

Methyl 3,4-dihydroxyphenylacetate **3** was prepared from Compound **2** by the conventional acid-catalyzed esterification with methanol in a yield of 93.6%.

Methyl 3,4-isopropylidenedioxyphenylacetate **4** was prepared from Compound **3** by the conventional acetalation with acetone, and *p*-toluenesulfonic acid monohydrate as a catalyst in a yield of 50%.[30]

3,4-Isopropylidenedioxyphenethyl alcohol **5** was prepared from Compound **4** by the conventional sodium borohydride reduction in a yield of 71%.[31]

3,4-Isopropylidenedioxyphenethyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranoside **7** was prepared from Compounds **5** and **6** by the Koenigs-Knorr method in a yield of 23%. The yield was not satisfactory, but it could be improved by a much larger addition of Compound **6**, because 20% of starting Compound **5** was recovered. Compound **7** was deacetylated with sodium methoxide in methanol, followed by deprotection of the isopropylidene group with 99% trifluoroacetic acid to give the target Compound **9** in a yield of 83.5% *via* Compound **8**.



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
7	Ac	Ac	Ac	Ac	H	H	$\succ^{CH_3}_{CH_3}$
8	H	H	H	H	H	H	$\succ^{CH_3}_{CH_3}$
9	H	H	H	H	H	H	H
Acteoside	H	rhamnosyl	caffeoyl	H	H	H	H
Isoacteoside	H	rhamnosyl	H	caffeoyl	H	H	H
Tomentoside A	H	rhamnosyl	caffeoyl	caffeoyl	O	-	H
Campneoside I	H	rhamnosyl	caffeoyl	caffeoyl	H	OCH_3	H

Fig. 1. Synthetic scheme of Compound **9** and structures of caffeic glucoside esters.

Our Compound **9** was synthesized first, proving to be identical to the corresponding natural specimen mentioned above on the basis of spectral comparisons.[25,26]

2.2 Enzymic oxidation

As Fig. 2 shows, all four compounds resulted in fast increases of absorptions at 380–430 nm in 1 or 3 min upon the enzymatic oxidations by the HRP, and after that time the absorptions decreased slowly for acteoside or fast for isoacteoside and Compound **9**, but tomentoside A increased slowly. As Fig. 3 shows, isoacteoside and tomentoside A resulted in fast increases of absorption at 370 nm in 20- and 10 min, respectively, upon the enzymatic oxidation by the PTP, and after that time the absorption decreased fast for isoacteoside or increased slowly through 30 min and then decreased slowly for tomentoside A. Acteoside resulted in relatively fast and small increases of it, at 410 nm in 10 min and after that time in the very slow increase of it. Compound **9** resulted

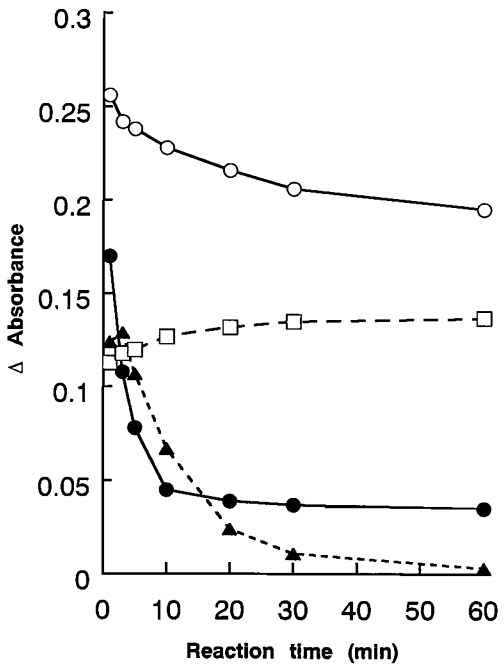


Fig. 2. Difference visible-UV spectra of the HRP-catalyzed oxidation of caffeic glucoside esters at specified wave lengths.
Legend: ○: acteoside at 430 nm, ●: isoacteoside at 400 nm, □: tomentoside A at 380 nm, ▲: Compound **9** at 400 nm.

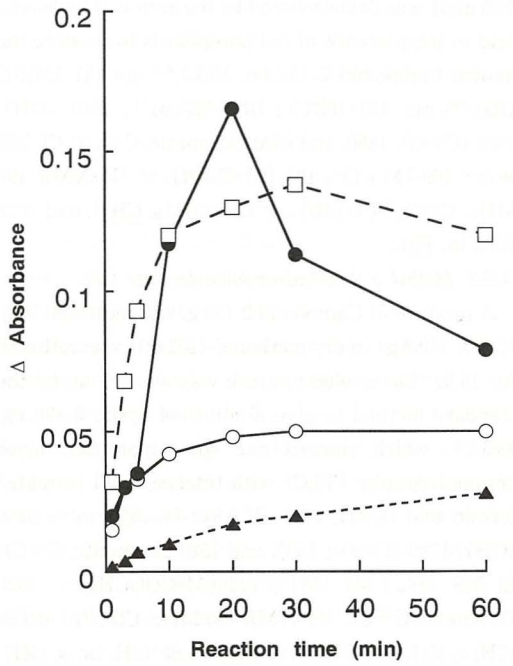


Fig. 3. Difference visible-UV spectra of the PTP-catalyzed oxidation of caffeic glucoside esters at specified wave lengths.
Legend: ○: acteoside at 410 nm, ●: isoacteoside at 370 nm, □: tomentoside A at 370 nm, ▲: Compound **9** at 410 nm.

in a slow increase of it at 410 nm through 60 min.

The increase in absorption at that range of absorption wavelengths indicates the formation of *o*-quinones and hence, its decrease means that *o*-quinones would be converted mostly into condensed substances that are insoluble and precipitable or soluble in the measuring solution.

Actually, as Fig. 4 shows, for the HRP-catalyzed oxidation of acteoside, the turbidity of the solution occurred in 1 min after the addition of the hydrogen peroxide, and the observable precipitation of the brown substance occurred in 30 min. For the PTP-catalyzed oxidation of it, slight turbidity occurred in 3 min, but the observable precipitation of the brown substance occurred in 60 min. Furthermore, for Compound **9**, any observable precipitation of the brown substance did not occur in 24 h by either the HRP-catalyzed oxidation or the PTP-catalyzed one.

The yield of the brown substance was 89- and 65-% in 6 months at 4°C for the HRP- and PTP-catalyzed oxidations of acteoside, respectively, and 22- and 59

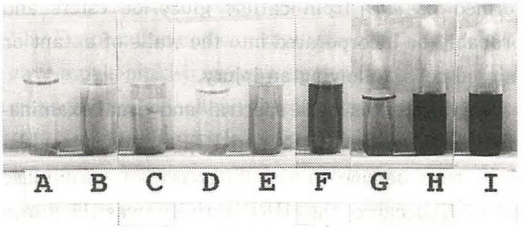


Fig. 4. Visual observations of the enzymic oxidation. **A**: reference with acteoside at time=1 min; **B**: acteoside, with HRP at time=1 min; **C**: acteoside, with HRP at time=30 min; **D**: reference with acteoside at time=3 min; **E**: acteoside, with PTP at time 3 min; **F**: acteoside, with PTP at time=1 h; **G**: reference with Compound **9** at time=24 h; **H**: Compound **9**, with HRP at time=24 h; **I**: Compound **9**, with PTP at time=24 h.

-% (cumulative values) in 24 h and four months at room temperatures, respectively, for the HRP-catalyzed oxidations of it, as described under "Experiment". Note that the yields obtained do not always result solely after such lengthy reaction times because the present time-yield experiments were not enough to lead to conclusiveness.

These brown substances hardly were soluble in acetone, methanol, and acetone/water (9/1, v/v), nor dioxane/water (1/1, v/v). They had sugar moieties in their condensed structures, because not any the glucose and rhamnose was detected in the centrifugal supernatant of the reaction mixture.

From these results, the following consideration can be made for the structural formation of the brown substances: they are greatly condensed substances that would be formed by oxidative coupling. Radical couplings intervene among the constitutive moieties so as to produce polymeric substances insoluble in aqueous, organic, and aqueous organic media. Such couplings may be formed between caffeoyl- and caffeoyl moieties[32,33] or between caffeoyl- and 2-(3,4-dihydroxyphenyl)-ethyl moieties for an intermolecular coupling, and also between 2-(3,4-dihydroxyphenyl)-ethyl- and caffeoyl moieties for an intramolecular coupling. Besides, there are potential couplings between sugar moieties and aromatic ones for an intermolecular coupling and an intramolecular one *via* quinonemethide intermediates.[8] Considering all of the results and discussions, we believe it reasonable to predict that polymeric substances will be

formed *in vivo* from caffeic glucoside esters and probably be incorporated into the walls of extant or injured cells, following an injury.

The differences in the spectral- and visual examinations between the HRP- and PTP-catalyzed oxidations may be due to the difference of peroxidase activity, because the HRP-activity was 11 times greater than the PTP-activity. However, it is worthwhile to note that the PTP may contain other polyphenol oxidases, like tyrosinase, and they can affect in various ways such differences.

The apparent spectral differences observed among three analogous caffeic glucoside esters and the effects of their structural features on the specified precipitancy remain to be explained, based on chemical structures of their condensed substance. Nevertheless, the overall results may suggest that both or one of the caffeoyl moieties, and the rhamnosyl moiety are required to cause precipitable substances at least under the present conditions of enzymatic oxidations.

In conclusion, this work established first a synthetic route to Glucoside **9** and consequently provided useful information concerning the effects of the structual features on the specified precipitancy and solubility of the condensed substance of this type of glucoside.

3. EXPERIMENT

3.1 Instrument and chromatography

Melting points (mps) are uncorrected. The following instruments were used: UV spectra, Shimadzu UV-300; IR spectra, JASCO IRA-1; NMR spectra, Hitachi R-22 (^{1}H: 90 MHz), BRUKER AM 500 (^{1}H: 500 MHz) with TMS (tetramethylsilane) as an internal standard; MS spectra, FD-MS (JEOL JMS-OISG -2), EI-MS and CI-MS (Hitachi, M-2000); HPLC, RI detector (Shodex RI-72, SHOWA DENKO) and pump (MP-312, Lab-Quatec). Chemical shifts (δ) and coupling constants (J) are given in ppm and Hz, respectively. Silica gel TLC and silica gel column chromatography employed silica gel 60 G (Merck) and Wakogel C-300 (Wako), respectively; HPLC, TSK gel Sugar AXI (TOSOH, 4.6 mm I.D.×15 cm).

3.2 Synthesis of 2-(3,4-dihydroxyphenyl)-ethyl β-D-glucopyranoside (**9**)

3.2.1 3,4-Dihydroxyphenylacetic acid (**2**)

Commercially available homoveratric acid **1** (50 g, 0.26 mol) was demethylated by the action of hydriodic acid in the presence of red phosphorus to produce the known Compound **2** (36.4 g, 85%).[29] mp 131-131.5°C (Ref.[29] mp 131-132°C); IR ν_{Max}^{KBr} cm^{-1}: 3440 (OH), 1690 (C=O), 1600, and 1520 (aromatic. C=C). CI-MS m/z: 169 $[M+1]^+$, 123 $[M-COOH]^+$. ^{1}H-NMR (90 MHz, $CDCl_3+CD_3OD$): δ 3.47 (2H, s, $\underline{CH_2}$), 6.61-6.78 (3H, m, $\underline{Ph}$).

3.2.2 Methyl 3,4-dihydroxyphenylacetate (**3**)

A mixture of Compound **2** (30 g, 0.18 mol) and 97% H_2SO_4 (10.2 g) in dry methanol (182 ml) was refluxed for 15 h. The cooled mixture was worked up by the standard method to give a colorless Syrup **3** (30.4 g, 93.6%), which showed one spot upon thin layer chromatography (TLC) with toluene/ethyl formate/formic acid (5/4/1, v/v), Rf 0.54; IR ν_{Max}^{KBr} cm^{-1}: 3400 (OH), 1720 (C=O), 1600, and 1520 (aromatic. C=C). EI-MS m/z: 182 $[M]^+$, 123 $[M-COOCH_3]^+$. 1520 (aromatic. C=C). ^{1}H-NMR (90 MHz, $CDCl^3$): δ 3.50 (2H, s, $\underline{CH_2}$), 3.70 (3H, s, $\underline{CH_3}$), 5.85 (2H, br. s, $\underline{OH}$), 6.58-6.80 (3H, m, Ph).

This was employed in the next step without further purification.

3.2.3 Methyl 3,4-isopropylidenedioxyphenylacetate (**4**)

A mixture of Compound **3** (8.20 g, 45.1 mmol), and *p*-toluenesulfonic acid monohydrate (89 mg, 0.47 mmol) in dry acetone (5.7 ml, 78 mmol) and dry benzene (100 ml) was refluxed for 24 h, removing water produced.[30] The cooled mixture was worked up by the standard method to give a colorless Syrup **4** (5.0 g, 50%), which showed one spot upon the TLC with hexane/ethyl acetate (36/1, v/v), Rf 0.28; IR ν_{Max}^{KBr} cm^{-1}: 1740 (C=O), 1490 (aromatic. C=C), 1380, and 1370 (>C$(CH_3)_2$). ^{1}H-NMR (90 MHz, $CDCl_3$): δ 1.68 (6H, s, $\underline{>C(CH_3)_2}$, 3.52 (2H, s, $\underline{CH_2}$), 3.67 (3H, s, $\underline{CH_3}$), 6.68 (3H, br. s, Ph).

This was employed in the next step without further purification.

3.2.4 3,4-Isopropylidenedioxyphenethyl alcohol (**5**)

Compound **4** (9.1 g, 41 mmol) was dissolved in a peroxide-free dioxane/water (1/1, v/v) mixture (300 ml), and sodium borohydride (17.4 g, 460 mmol) was added portion-wise with stirring for 20 min.[31] The mixture was stirred for 6 h at room temperature and worked up by the standard method to give a crude syrup (7.1 g), which was purified on a silica gel column (214 g). Elution with *n*-hexane/ethyl acetate

(9/2, v/v) gave a colorless syrup **5** (5.7 g, 71%) ; IR ν_{Max}^{KBr} cm^{-1} : 3420 (OH), 1490 (aromatic. C=C), 1380, and 1370 (>$C(CH_3)_2$). ^{1}H-NMR (90 MHz, $CDCl_3$) : δ 1.65 (6H, s, >$\underline{C(CH_3)_2}$, 2.76 (2H, t, J=6.0, $\underline{\beta\text{-}CH_2}$), 3.80 (2H, t, J=6.0, $\underline{\alpha\text{-}CH_2}$), 6.63 (3H, br. s, Ph).

3.2.5 3,4-Isopropylidenedioxyphenethyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**7**)

Compound **5** (1.2 g, 6 mmol) was dried by co-evaporation with dry benzene three times and then over phosphorus pentoxide *in vacuo*. To a mixture of it and mercury(II) cyanide (0.8 g, 3 mmol) in dry acetonitrile (30 ml), 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **6** (2.5 g, 6.2 mmol, prepared by the method of Bárczai-Martos and Körösy[34]) was added. The mixture was stirred for 6 h at room temperature and worked up by the standard method to give the crude product (3.6 g) which was subjected to column chromatography on silica gel (217 g). Elution with *n*-hexane/ethyl acetate (10/0.2 to 10/5, v/v, stepwise elution) gave an amorphous Powder **7** (0.7 g, 23%) ; IR ν_{Max}^{KBr} cm^{-1} : 1740 (C=O), 1490 (aromatic. C=C), 1380, and 1370 (>$C(CH_3)_2$). FD-MS m/z : 524 $[M]^+$, 525 $[M+1]^+$. ^{1}H-NMR (500 MHz, $CDCl_3$) : for an aglycone moiety, δ 1.65 (6H, s, >$\underline{C(CH_3)_2}$, 2.78 (2H, m, $\underline{\beta\text{-}CH_2}$), 3.61 (1H, m, $\underline{\alpha\text{-}CH_2}$), 4.07 (1H, m, $\underline{\alpha\text{-}CH_2}$), 6.57-6.63 (3H, m, Ph), and for an glucose moiety, δ 4.49 (1H, d, J=8.0, $\underline{H\text{-}1}$), 5.0 (1H, t, J=9.0, $\underline{H\text{-}2}$), 5.19 (1H, t, J=9.3, $\underline{H\text{-}3}$), 5.09 (1H, t, J=10.0, $\underline{H\text{-}4}$), 3.68 (1H, ddd, J=2.0, 5.0, 10.0, $\underline{H\text{-}5}$), 4.14 (1H, dd, J=2.0, 12.0, $\underline{H_{ax}\text{-}6}$), 4.27 (1H, dd, J=5.0, 12.0, $\underline{H_{eq}\text{-}6}$), 1.97, 2.00, 2.02, and 2.09 (each 3H, s, $\underline{OAc}$).

3.2.6 3,4-Isopropylidenedioxyphenethyl β-D-glucopyranoside (**8**)

To a stirred and ice-cooled solution of Compound **7** (0.68 g, 1.3 mmol) in dry methanol (20 ml), a solution of sodium methoxide (76 mg, 1.4 mmol) in dry methanol (5 ml) was added dropwise. The mixture was stirred for 50 min at 0°C and neutralized with Dowex 50-X4 (1 g), filtered, washed and concentrated in vacuo to give a white amorphous Powder 8 (0.44 g, 96%). This showed one spot on the TLC with chloroform/methanol/water (30/10/1, v/v), Rf 0.73. This was employed in the next step without further purification.

3.2.7 2-(3,4-dihydroxyphenyl)-ethyl β-D-glucopyranoside (**9**)

Compound 8 (0.44 g, 1.2 mmol) was dissolved in 99% trifluoroacetic acid (3 ml) at 0°C, and stirred for 1 h. Evaporation and co-evaporation with dry toluene gave a pale pinkish amorphous powder (0.46 g). This was subjected to column chromatography on silica gel (66 g). Elution with chloroform/methanol/water (40/10/1, v/v) gave an pale pinkish amorphous Powder **9** (0.34 g, 87%) ; IR ν_{Max}^{KBr} cm^{-1} : 3360 (OH), 1600, and 1520 (aromatic. C=C). FD-MS m/z : 316 $[M]^+$, 317 $[M+1]^+$. ^{1}H-NMR (500 MHz, CD_3OD) : for an aglycone moiety, δ 2.77 (2H, m, $\underline{\beta\text{-}CH_2}$), 3.67-3.71 (1H, m, $\underline{\alpha\text{-}CH_2}$), 3.99-4.04 (1H, m, $\underline{\alpha\text{-}CH_2}$), 6.68 (1H, d, J=2.0, $\underline{Ph\text{-}H\text{-}2}$), 6.67 (1H, d, J=7.9, $\underline{Ph\text{-}H\text{-}5}$), 6.55 (1H, dd, J=2.0, 7.9, $\underline{Ph\text{-}H\text{-}6}$), and for a glucose moiety, δ 4.28 (1H, d, J=8.0, $\underline{H\text{-}1}$), 3.18 (1H, dd, J=8.0, 9.0, $\underline{H\text{-}2}$), 3.23-3.37 (3H, m, $\underline{H\text{-}3}$, $\underline{H\text{-}4}$, $\underline{H\text{-}5}$), 3.67 (1H, dd, J=2.0, 12.0, $\underline{H_{ax}\text{-}6}$), 3.86 (1H, dd, J=5.0, 12.0, $\underline{H_{eq}\text{-}6}$).

3.3 Enzymic oxidation

3.3.1 Preparation of enzyme solutions

Two enzyme solutions were used: PTP and HRP. The former one was prepared as follows:[35,36] the outermost sapwood of a three-year-old kiri was scraped off to give 66 g of the tissue, which then was frozen with liquid nitrogen. This was ground for two min with a cold vibrating-ball mill and then extracted in a mortar for 10 min at 4°C with a 0.1 M potassium phosphate buffer solution, pH 7.0, which contained 1.0 g of Polyclar AT (GAF Chem. Co. Ltd.) per gram of the tissue. The extract was separated from the residues and the Polyclar AT with a Büchner funnel and further clarified by centrifugation at 8400 g for 20 min at 4°C. The supernatant was salted out with ammonium sulfate (75% saturation) and then centrifuged at 8400 g for 20 min at 4°C. The precipitate obtained was dissolved in 70 ml of 0.1 M potassium phosphate buffer solution, pH 7.0, and then dialysed with a pure water (1 Lx8) for 24 h at 4°C to give 105 ml of the PTP.

The HRP was prepared as follows; five mg of the commercial horseradish peroxidase (Toyobo Co. Ltd., peroxidase [III]) was dissolved in a 0.1 M potassium phosphate buffer solution, pH 7.0 and diluted to 100 ml with the buffer.

Peroxidase activities of the two enzyme preparations were compared according to the *o*-dianisidine-activity test[37] with the activity expressed as Δ Abs. min^{-1} ml^{-1}, resulting in HRP at about 11 times greater

in activity than the PTP.

3.3.2 Spectral and visual examinations of enzymatic oxidation

For the spectral examinations, four compounds were used: acteoside, isoacteoside, tomentoside A, and Compound **9** and their difference visible-UV spectra were taken with an enzymatic oxidation. Solutions used for the spectral examinations were prepared from stock solutions (10 ml) of which for the first three compounds the concentrations were 0.25 mM in 0.2 M sodium phosphate buffer solution, pH 7.0 and for the last one it was 0.76 mM in the same buffer solution. The sample solution in the UV cell was composed of the buffer solution (2 ml), the stock solution (1 ml), a hydrogen peroxide solution (8.8 mM, 0.03 ml), and the above enzyme solution (0.2 ml). The reference solution in the UV cell was composed of the buffer solution (3.03 ml) and the above enzyme solution (0.2 ml). The measuring mixtures were held at 25±0.5°C for 5 min with stirring before an addition of the hydrogen peroxide solution. Spectra were taken at 1-, 3-, 5-, 10-, 20-, 30-, and 60-min after the addition of the hydrogen peroxide solution, scanned at 180- to 600-nm.

For the visual examinations, acteoside and Compound **9** were used: sufficient amounts of isoacteoside and tomentoside A were not available. Two solutions of a sample and a reference were prepared from the stock solutions (10 ml) of which the concentration was 6.5 mM in a 0.2 M sodium phosphate buffer solution, pH 7.0. The sample solution was composed of the stock solution (5 ml), a hydrogen peroxide solution (140 mM, 0.25 ml), and the above enzyme solution (0.5 ml). The reference solution was composed of the stock solution (5 ml), a hydrogen peroxide solution (140 mM, 0.25 ml), and the above buffer solution (0.5 ml). Photographs were taken at 1-, 3-, 5-, 10-, and 30-min; 1-, 3-, 6-, and 24-h after the addition of the hydrogen peroxide solution. The solutions were kept at 4°C for six months and then centrifuged, followed by washing with water (three times) to give the brown substances: the yields were 89- and 65-% based on the amount of acteoside for the HRP-catalyzed- and the PTP-catalyzed oxidation of it, respectively. Additionally, another experiment using acteoside and the HRP resulted in yields of such substances were 22- and 59-% (cumulative values) based on the amounts of acteoside in 24 h and four months at room temperature after the additions of hydrogen peroxide, respectively. The centrifugal supernatant of the reaction mixture in 24 h was analyzed for acteoside, glucose, and rhamnose by TLC: developing solvents, $CHCl_3$/MeOH/H_2O (30/10/1 v/v) for acteoside and acetonitrile/H_2O (5.2/1, v/v) for glucose and rhamnose. The relative flow rates (Rfs) were acteoside (0.30), glucose (0.26) and rhamnose (0.48). Also, the same supernatant was analyzed for glucose and rhamnose by HPLC: column, TSK gel Sugar AXI (TOSOH, 4.6 mmm I.D.×15 cm); mobile phase, 0.4 M sodium borate buffer (pH 8.5); flow rate, 0.45 ml min; pump pressure, 19- to 20-kg/cm^2; column temperature, 60°C. The retention times (Rts) (min) were glucose (124.36) and rhamnose (16.63). The TLC and HPLC analyses proved that the supernatant did not contain any of acteoside, glucose, and rhamnose.

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