Phlorotannins Derived From the Brown Alga Colpomenia bullosa as Tyrosinase Inhibitors

Hideyuki Kurihara¹ and Kazuki Kujira²

Abstract
Tyrosinase catalyzes hydroxylation of L-tyrosine and dehydrogenation of L-DOPA in the melanin biosynthesis pathway. Tyrosinase inhibitors have potential use as cosmetic whitening agents and for preventing seafood deterioration. In this report, tyrosinase inhibitors extracted from brown alga Colpomenia bullosa (Scytosiphonaceae, Scytosiphonales) were investigated. Inhibitory principles were isolated from the extract and identified as phlorotannins, phloroglucinol (1), diphlorethol (2), triphlorethol C (3), which have not been isolated in a free form previously, and fucophlorethol C (4). Compounds 3 and 4 have not been reported previously as tyrosinase inhibitors. Triphlorethol C (3) was the most potent tyrosinase inhibitor among the phlorotannins isolated, whereas isomeric fucophlorethol C (4) displayed the weakest inhibitory activity. The results suggest that molecular structures of phlorotannins strongly affect their tyrosinase inhibitory activity.

Keywords
Colpomenia bullosa, Scytosiphonaceae, phlorotannin, phenolics, tyrosinase, inhibitor

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Tyrosinase (EC 1.14.18.1), a copper-containing oxidase, catalyzes hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and dehydrogenation of L-DOPA to L-dopaquinone, which is further converted to melanin.¹ Tyrosinase is responsible for the first step in melanin production by organisms. This pigment is distributed in human skin, eyes and hair and protects the body from ultraviolet radiation and free radicals.¹ However, dysfunctional or uncontrolled tyrosinase activity is associated with risks such as hyperpigmentation, melanoma, Parkinson’s disease, and alkaptonuria.² Melanin production is also associated with the deterioration of crustaceans such as lobster,³ with the resulting black-colored crustaceans having reduced market value. Thus, inhibiting tyrosinase activity can play an important role in protecting the deterioration of seafood.

Several natural and synthetic tyrosinase inhibitors are known.⁴ Various inhibitors such as kojic acid, arbutin and ellagic acid are used as whitening agents in cosmetics.⁴ There are numerous studies that have characterized tyrosinase inhibitors from marine organisms. Moreover, various phlorotannins derived from brown algae have been identified as tyrosinase inhibitors.⁵¹¹ Most of these phlorotannins have been obtained from Ecklonia and Agarum sp. belonging to Laminariales, Ishige sp. belonging to Ishigeales and Fucus sp. belonging to Fucales. During our investigation of tyrosinase inhibition, we have obtained tyrosinase inhibitors from algal extracts.¹²⁻¹⁴ In the present report, an extract of Colpomenia bullosa (C. bullosa) (Scytosiphonaceae, Scytosiphonales) showed relatively high inhibitory activity against tyrosinase when compared with that of other algal extracts. The inhibitory principles were isolated from the extract and identified as phlorotannins. The inhibitory activity of these previously unknown tyrosinase inhibitors was compared.

Results and Discussion
Dried methanol (MeOH) extracts from C. bullosa, which possess tyrosinase inhibitory activity, was separated into ethyl acetate (EtOAc)- and n-butanol (n-BuOH)-soluble fractions. Analytical thin layer chromatography (TLC) analysis with detection using the 2,4-dimethoxybenzaldehyde (DMBA) test revealed the presence of several low molecular weight

¹Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Japan
²Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Japan

Corresponding Author:
Hideyuki Kurihara, Faculty of Fisheries Sciences, Hokkaido University, Minato 3-1-1, Hakodate, Hokkaido 041-8611, Japan.
Email: kuri@fish.hokudai.ac.jp
phlorotannins in both fractions. Compounds 1-4 were isolated from the combined EtOAc- and n-BuOH-soluble fractions through a series of chromatographic operations (Figure 1). All compounds were positive for the DMBA test, indicating that these compounds were phlorotannins.

The determined molecular weight (126) of compound 1 is equivalent to trioxygenated benzene. The $^1$H NMR spectrum of 1 showed one aromatic signal, whereas the $^{13}$C NMR spectrum showed two aromatic signals. Thus, compound 1 possesses a $C_3$ symmetry element and was identified as phloroglucinol. The NMR data were similar to reported data, and analytical TLC evaluation showed that compound 1 is identical to authentic phloroglucinol.

The determined molecular weight (250) and formula ($C_{12}H_{10}O_6$) of compound 2 indicated that this species is a phloroglucinol dimer. The $^1$H NMR spectrum of 2 included singlet, broad singlet and triplet aromatic signals with integral values of 4H, 4H, and 2H, respectively. Unfortunately, the $^{13}$C NMR spectrum was not recorded because the amount of compound 2 isolated was too small to measure this spectrum in a reasonable timeframe. Nonetheless, there are three possible isomers of the phloroglucinol dimer (Figure 2). Among them, isomers 2a and 2b possess a $\sigma$ symmetry element. The predicted $^1$H NMR spectrum of 2a contains only one aromatic signal, whereas the predicted spectrum of 2b contains doublet and triplet aromatic signals. Neither of these predicted spectra match the observed $^1$H NMR spectrum. Thus, compound 2 was identified as diphlorethol.

The determined molecular weight (374) and formula ($C_{18}H_{14}O_9$) of compound 3 are equivalent to a phloroglucinol trimer. The peracetylated derivative 3a of 3 has a molecular weight of 668. The difference in molecular weight between 3 and 3a is 294, which matches seven acetyl groups. Thus, compound 3 possesses seven free phenolic hydroxy groups. The $^1$H NMR spectrum of 3 showed three aromatic signals. The $^{13}$C NMR spectrum of 3 showed 8 aromatic carbon signals. Based on NMR data, two symmetrical benzene ring moieties are symmetrically substituted to a third benzene ring moiety. Thus, each ring possesses a $\sigma$ symmetry element. Compound 3 was identified as triphlorethol C, which was first isolated as a peracetylated derivative. The $^1$H NMR spectrum of 3a is identical to literature data of hepta-$O$-acetyl triphlorethol C, thereby supporting the identification of 3. $^{13}$C NMR data were not measured because only a small amount of derivative 3a was available. This is the first report presenting the isolation of triphlorethol C as a free form.

Compound 4 was identified to be an isomer of compound 3 because they have the same molecular weight. TLC analysis and NMR data showed that compound 4 is authentic fucophlorethol C, which was isolated previously from the same alga.

Compounds 1-4 have been isolated from brown algae. Phloroglucinol (1), diphlorethol (2) and triphlorethol A, an isomer of compounds 3 and 4, are previously reported tyrosinase inhibitors. However, triphlorethol C (3) and fucophlorethol C (4) have not been reported as potential tyrosinase inhibitors.

The tyrosinase inhibitory activity of compounds 1-4 was examined (Table 1). Triphlorethol C (3) showed the most potent inhibition with the lowest IC$_{50}$ and $K_I$ values among the compounds examined. Diphlorethol (2) showed slightly weaker activity when compared with that of the well-known inhibitor...
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Table 1. Inhibition Parameters of Phlorotannin 1-4 Against Mushroom Tyrosinase Activity.

| Compound                 | Kᵢ (µM) | IC₅₀ (µM) | Inhibition type |
|--------------------------|---------|-----------|----------------|
| Phloroglucinol (1)       | NTb     | 439       | NT             |
| Diphlorethol (2)         | 11.4    | 11.4      | Non-competitive |
| Triphlorethol C (3)      | 1.42    | 2.61      | Competitive    |
| Fucophlorethol C (4)     | 281     | 439       | Competitive    |
| Kojic acid               | 5.1c    | 15.1      | Competitivec   |

aL-Tyrosine concentration was 184 µM.
bNot tested.
cLiterature data.21

The whole body (402 g semi-air-dried weight) of C. bullosa was cut and extracted twice with MeOH (5 L) at room temperature for 3 days. The extracting solution was filtered through filter paper. The solvent was removed under reduced pressure to yield dark extracts. Combined MeOH extracts (9.60 g) were separated into an EtOAc-soluble fraction (1.10 g) and an n-BuOH-soluble fraction (1.02 g). Both fractions containing low-molecular phlorotannins were evaluated by analytical TLC with DMBA reagent. Both fractions were then combined, and silica gel column chromatography was conducted with a combination of n-hexane and acetone to elute several active fractions. The low-polar fraction was separated by preparative TLC developed with toluene/EtOAc/acetic acid (AcOH) = 2/3/1 (v/v/v) to obtain compound 1 (1.63 mg). The remaining fractions were separated by preparative TLC developed with toluene/EtOAc/ AcOH = 2/3/1 (v/v/v), which was followed by preparative ODS HPLC with isocratic methanol/3% formic acid = 1/9 (v/v/v) to afford compounds 2 (1.01 mg), 3 (5.50 mg), and 4 (226 mg). All compounds showed a positive response in the DMBA test.

Phloroglucinol (1): colorless amorphous solid. FD-MS m/z 126.02 [M⁺] (calculated for C₃H₆O₃, 126.03). ¹H-NMR (CD₃OD, 400 MHz) δ ppm: 5.70 (3H, s, H-2a, 4a, 6a). ¹³C-NMR (CD₃OD, 400 MHz) δ ppm: 160.2 (3C, C-1a, 3a, 5a), 95.5 (5CH, C-2a, 4a, 6a).

Diphlorethol (2): pale yellow amorphous solid. HR-FD-MS m/z 250.04671 [M⁺] (calculated for C₁₂H₁₀O₆, 250.0474). ¹H-NMR (CD₃OD, 400 MHz) δ ppm: 5.92 (4H, s, H-4a, 6a), 5.97 (4H, br, s, H-4a, 6a), 5.97 (2H, t, J = 1.4 Hz, H-4b).

Triphlorethol C (3): pale yellow amorphous solid. HR-FD-MS m/z 374.06467 [M⁺] (calculated for C₁₈H₁₆O₉, 374.06378). ¹H-NMR (CD₃OD, 400 MHz) δ ppm: 6.13 (1H, t, J = 2.2 Hz, H-2b), 5.92 (4H, s, H-4a, 6a, 4c, 6c), 5.89 (2H, d, J = 2.2 Hz, H-4b, 6b). ¹³C-NMR (CD₃OD, 100 MHz) δ ppm: 162.0 (2C, C-1h, 3b), 159.9 (1C, C-5b), 156.3 (2C, C-5a, 5c), 152.3 (4C, C-1a, 3a, 1c, 3c), 124.8 (2C, C-2a, 2c), 96.4 (2CH, C-4b, 6b), 96.3 (4CH, C-4a, 6a, 4c, 6c), 96.0 (1CH, C-2b).

Hepta-O-acetyl triphlorethol C (3a): compound 3 (2.28 mg) was dissolved in acetic anhydride (0.5 mL) and pyridine (0.5

Extraction, Isolation and Identification of Phlorotannins

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Hepta-O-acetyl triphlorethol C (3a): compound 3 (2.28 mg) was dissolved in acetic anhydride (0.5 mL) and pyridine (0.5
ml) and kept overnight at room temperature. The reaction mixture was evaporated under reduced pressure to obtain acetylated derivative 3a (3.20 mg). Pale yellow amorphous solid. FD-MS m/z 668.11 [M]+ (calculated for C_{28}H_{32}O_{10}, 668.14). 1H-NMR (CDCl_3, 400 MHz) δ (ppm): 6.93 (4H, s, H-4a, 6a, 4c, 6c), 6.43 (1H, t, J = 2.2 Hz, H-2b), 6.30 (2H, d, J = 2.2 Hz, H-4b, 6b), 2.26 (6H, s, acetyl), 2.20 (3H, s, acetyl), 2.07 (12H, s, acetyl).

Fucophlorethol C^4 (4): pale yellow amorphous solid. FD-MS m/z 374.08 [M]^+ (calculated for C_{18}H_{14}O_{9}, 374.06). 1H-NMR (CD_3OD, 100 MHz) δ (ppm): 159.4 (1C, C-5b), 159.2 (1C, C-1b), 159.1 (1C, C-5c), 158.4 (1C, C-3b), 157.4 (2C, C-1c, 3c), 156.6 (1C, C-5a), 152.0 (2C, C-1a, 3a), 124.2 (1C, C-2a), 102.4 (1C, C-2c), 101.9 (1C, C-2b), 97.8 (1CH, C-3b), 96.6 (2CH, C-4c, 6c), 95.8 (2CH, C-4a, 6a), 93.3 (1CH, C-6b).

Tyrosinase Activity Assay

The tyrosinase assay was performed by the method of Islam et al., with slight modifications. In brief, test compounds were dissolved in MeOH as the test solution. L-tyrosine was dissolved in 50 mM phosphate buffer (pH 6.8) to a concentration of 0.100 mg/mL (184 µM for inhibitor screening or determination of IC₅₀ values) or 0.100, 0.150 and 0.200 mg/mL as the enzyme solution. A mixture of test solution or MeOH as a control (15 µL), substrate solution (500 µL) and related pigmentations, as the substrate solution. Tyrosinase was dissolved in the phosphate buffer to a concentration of 200 units/mL, as the enzyme solution. A mixture of test solution or MeOH as a control (15 µL), substrate solution (500 µL) and the buffer (780 µL) was preincubated at 25 °C for 5 minutes. The reaction was initiated by adding enzyme solution (205 µL). As soon as possible, the reaction mixture was transferred to a cuvette and the absorbance at 490 nm was measured as the blank. The remaining reaction mixture was kept at 25 °C for 30 minutes to allow the tyrosinase reaction to progress. The absorbance of the reaction mixture was then measured at 490 nm immediately. Values are shown as the means ± standard errors of means (SEMs) from three independent experiments.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.
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