Communication

Isolation of a New Anti-Allergic Phlorotannin, Phlorofucofuroeckol-B, from an Edible Brown Alga, *Eisenia arborea*

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*Eisenia arborea* is an edible brown alga occasionally used as a folk medicine in gynecopathy in Japan. A new phlorotannin was isolated from the alga during our search for naturally occurring anti-allergic compounds from edible algae guided by the inhibitory effect on histamine release from rat basophile leukemia (RBL)-2H3 cells. The phlorotannin was called “phlorofucofuroeckol-B.” Its structure was determined by spectral analysis and chemical conversion. This paper describes the isolation, structure elucidation, and inhibitory effect of phlorofucofuroeckol-B on histamine release.

**Key words:** *Eisenia arborea*; histamine release; phlorotannin; RBL-2H3

Brown algae are very popular foods, and many people ingest them as a health food in Japan. These algae are known to contain several phlorotannins, viz., eckol,1 dieckol,1 6,6'-bieckol,2 8,8'-bieckol,2 and phlorofucofuroeckol-A (PFF-A).3 Phlorotannins have been reported to have certain biological activities, such as antioxidation,4 bactericide,5 anti-cancer,6 anti-diabetic complications,7,8 and chemoprevention against several vascular diseases.9,10 Although some anti-inflammatory activities of phlorotannins have also been reported,11,12 their inhibitory effect on histamine release from the stimulated cultured cells is still unknown.

*Eisenia arborea* is an edible brown alga that grows on the southern coast of Japan. It is popular as a health food and is occasionally used as a folk medicine in gynecopathy. In a previous study, we found that the crude extract of the alga had an inhibitory effect on histamine release from RBL cells and an anti-allergic effect on an allergic model animal, the Brown Norway rat (unpublished data). In the present study, our interest focused on the identification of the active principles in *E. arborea* that inhibit histamine release from RBL cells stimulated by antigen-antibody reaction.

*E. arborea* was collected from the Mugizaki coast of Mie Prefecture, Japan, and was powdered after drying in air. The powdered alga (500 g) was extracted with hexane, a mixture of hexane and ethyl acetate (4:1 and 1:1), to remove the lipids and pigments. The residual precipitate was extracted with M/C (methanol:chloroform = 1:2). The M/C extract was partitioned with water, and then the water layer was extracted with diethyl ether. After evaporation, the diethyl ether layer (430 mg) was dissolved in 10 ml of 1% methanol. The solution was directly subjected to reversed phase HPLC (RP-HPLC) purification at room temperature on a Develosil ODS-5 column (10 mm ID × 250 mm L, Nomura Chemical Co., Seto, Aichi, Japan). A gradient program (from 0.1% TFA to 30% acetonitrile containing 0.1% TFA, 120 min, followed by isocratic elution at the final concentration) was employed. The eluate was divided into 90 fractions. Since the 66th–73rd fractions (tR 132–150 min, yield, 19.0 mg) contained the desired bio-active compounds, it was subjected to RP-HPLC purification on the same column eluting with 47% methanol containing 0.1% TFA. The second chromatography yielded two major peaks, and one of them, the tR 41 min peak (data not shown), showed a strong inhibitory effect on histamine release in our bio-assay system. The yield of the active compound was 3.3 mg from 500 g of dried alga.

The active compound, a brownish yellow oil, showed a protonated molecular ion at m/z = 603 in MALDI-TOF-MS analysis. Its molecular formula was determined to be C30H18O14 on the basis of its high-resolution FAB-MS spectrum (positive, in glycerin,

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from the brown algae. Most of the chemical and molecular formula were same as those of PFF-A. The detailed structure of the active compound was assigned by HSQC analysis. Figure 1 shows the structure of the active compound, together with that of PFF-A for comparison. The figure indicates the similarity of their chemical shifts to those of PFF-A. Thus, the six corresponding moieties of PFF-A. HBMC analysis, in addition to the resemblances in the chemical shifts, clarified the partial structures, groups 1, 2, and 3, as shown in Fig. 1.

The chemical shift values of the $^{13}$C carbons of group 5 were also very close to those of the corresponding moiety of PFF-A. The positions of C-8, C-9, C-11, and C-12 were assigned as shown in Fig. 1, according to the similarity of their chemical shifts to those of the corresponding signals of PFF-A and the HMBC correlations with H-10. The intact active compound did not show the HMBC cross peak between C-7a and H-10 at $\Delta = 60$ or 200 $\mu$s, but its permethylated derivative, measured in $\mathrm{DMSO-d_6}$, resembled those of PFF-A (Table 1), and these results indicate that the active compound might be an isomer of PFF-A.

The detailed structure of the active compound was determined by NMR analysis (Table 1). $^1$H-$^1$C correlations were assigned by HSQC analysis. Figure 1 shows the structure of the active compound, together with that of PFF-A for comparison. The figure indicates the similarity of their chemical shifts to those of the corresponding signals of PFF-A and the HMBC correlations with H-10. The intact active compound did not show the HMBC cross peak between C-7a and H-10 at $\Delta = 60$ or 200 $\mu$s, but its permethylated derivative, measured in $\mathrm{DMSO-d_6}$, resembled those of PFF-A (Table 1), and these results indicate that the active compound might be an isomer of PFF-A.

Table 1. NMR Spectral Data of PFF-B and Its Permethylated Derivative and Chemical Shifts of $^{13}$C of PFF-A and Eckol

| Group | No. | $^1$C | $^1$H | HMBC($^1$H-$^1$C) | $^1$C | $^1$H | HMBC($^1$H-$^1$C) | ROESY | PFF-A | Eckol
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<tr>
<td>1</td>
<td>1</td>
<td>160.3</td>
<td>94.6</td>
<td>6.17</td>
<td>1',3',4',5'</td>
<td>160.6</td>
<td>94.0</td>
<td>6.17</td>
<td>1',3',4',5'</td>
<td>3',5'-OMe, 14-OMe</td>
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<td>2, 2'</td>
<td>93.3</td>
<td>5.76</td>
<td>1,3',4',5'</td>
<td>158.9</td>
<td>161.5</td>
<td>3',5'-OMe, 14-OMe</td>
<td>94.2</td>
<td>6.09</td>
<td>2',3',5',6'</td>
<td>3',5'-OMe</td>
</tr>
<tr>
<td>3</td>
<td>159.0</td>
<td>160.4</td>
<td>94.0</td>
<td>6.09</td>
<td>1',3',4',5'</td>
<td>161.5</td>
<td>3',5'-OMe, 14-OMe</td>
<td>2',3',5',6'</td>
<td>3',5'-OMe</td>
<td>3',5'</td>
</tr>
</tbody>
</table>
| 4 | 96.3 | 5.82 | 2',3',5',6' | 122.2 | 125.3 | 113.4 | 141.7 | 126.4 | 136.9 | 200 m
| 5 | 98.6 | 6.19 | 1,2,4,4a,15a | 5a, 6a, 13a, 14a | 96.6 | 5.71 | 1 | 122.2 | 125.3 | 113.4 | 141.7 | 126.4 | 9a | 122.6 |
| 6 | 96.4 | 6.48 | 8,9,11,12,13a | 97.2 | 6.44 | 7a, 8, 9, 11, 12, 13a | 96.6 | 5.71 | 1 | 122.2 | 125.3 | 113.4 | 141.7 | 126.4 | 9a | 122.6 |
| 7 | 149.2 | 145.5 | 151.9 | 104.9 | 107.9 | 2',3',5',6' | 3',5'-OMe | 55.4 | 3.73 | 3',5' | 2',4',6' | 55.4 | 3.70 | 3',5' | 2',4',6' |
| methyl | 2-OMe | 57.1 | 3.79 | 2 | 3 | 2-OMe | 57.1 | 3.79 | 2 | 3 | 2-OMe | 57.1 | 3.79 | 2 | 3 | 2-OMe | 57.1 | 3.79 | 2 | 3 |
| 4-OMe | 56.8 | 3.91 | 4 | 3 | 4-OMe | 56.8 | 3.91 | 4 | 3 | 4-OMe | 56.8 | 3.91 | 4 | 3 | 4-OMe | 56.8 | 3.91 | 4 | 3 |
| 11-OMe | 56.6 | 3.97 | 11 | 10, 14 | 11-OMe | 56.6 | 3.97 | 11 | 10, 14 | 11-OMe | 56.6 | 3.97 | 11 | 10, 14 | 11-OMe | 56.6 | 3.97 | 11 | 10, 14 |
| 9-OMe | 56.9 | 3.86 | 9 | 10 | 9-OMe | 56.9 | 3.86 | 9 | 10 | 9-OMe | 56.9 | 3.86 | 9 | 10 | 9-OMe | 56.9 | 3.86 | 9 | 10 |
| 14-OMe | 61.7 | 3.57 | 14 | 2',6',11 | 14-OMe | 61.7 | 3.57 | 14 | 2',6',11 | 14-OMe | 61.7 | 3.57 | 14 | 2',6',11 | 14-OMe | 61.7 | 3.57 | 14 | 2',6',11 |

$m/z = 603.0717, \Delta 2.0$ mmu). The molecular weight and molecular formula were same as those of PFF-A from the brown algae. Most of the $^{13}$C-NMR signals in the active compound measured in $\mathrm{DMSO-d_6}$ resembled those of PFF-A (Table 1), and these results indicate that the active compound might be an isomer of PFF-A.
carbons of group 5 were assigned to those of the oxygenated aromatic ring, as shown in Fig. 1.

In group 4, the chemical shifts of four carbons (C-5a, C-6, C-13, and C-14) were different from the corresponding signals of PFF-A, although the other two signals were close to those of PFF-A. Accordingly, this group must contain the structural difference between the active compound and PFF-A. The chemical shifts of C-5a, C-6, C-6a, and C-14a were very close to those of the corresponding signals of eckol, and those of the other two carbons (C-13 and C-14) were different from the corresponding signals. This result indicates that the active compound contained an eckol moiety, and that C-13 or C-14 can be modified or can exist close to the modification. The C-13 carbon possessed the HMBC correlation with the H-6 proton, which also showed HMBC cross peaks with C-5a, C-6a, and C-14a in the spectrum measured at $\Delta = 60\mu$s, and these carbons were assigned to positions ortho and meta to C-6. In the spectrum measured at $\Delta = 200\mu$s, the H-6 proton showed a correlation with C-14, thought to be the carbon para to C-6.

In the HMBC spectrum measured at $\Delta = 200\mu$s, the C-13 carbon in group 4 showed a correlation with the H-10 in group 5. This correlation, in addition to the chemical shift of C-13, indicated a connection between these two groups in forming a biphenyl structure. The carbon in group 5 that was connected to C-13 should be C-12, because it was not connected to either the hydrogen, as proved by its HSQC spectrum, or the oxygen atom, as indicated by its chemical shift value (104.9 ppm), close to that of C-13. Thus the biphenyl structure of groups 4 and 5 was elucidated.

To differentiate the free phenolic hydroxyl groups from the ether groups, the active compound was treated with diazomethane. The permethylated product showed a protonated molecular ion at $m/z = 729$ in MALDI-TOF MS. Its NMR spectral data, measured in CDCl$_3$,
are summarized in Table 1. These spectra showed that nine methyl groups were introduced into the active compound. Accordingly, nine oxygen atoms in the active compound were those of the free phenol groups, and the other five oxygen atoms, which were not methylated, were thought to be those of the ether groups. The introduced methyl groups were assigned by analyzing the $^1$H–$^1$H COSY, HSQC, HMBC, and ROESY spectra (Table 1 and Fig. 2).

The positions of the O-methyl groups, except for 14-OMe, were determined by ROESY analysis, as shown in Table 1 and Fig. 2. The position of 14-O-Me was confirmed by HMBC correlations between the methyl proton and C-14 at $\Delta = 60$ $\mu$s, and between C-14 and H-6 at $\Delta = 200$ $\mu$s, and ROESY analysis. The proton signal of 14-O-Me showed weak but clear ROESY correlations with H-2$^0$ and H-6$^0$ in group 1, and with C-11-OMe in group 5, as shown in Fig. 2. These correlations suggest that C-14-O-Me was located close to H-2$^0$ or H-6$^0$ and C-11-OMe. One of the remaining ether oxygens, C-6a-O, was thought to be O-7, which connected C-6a and C-7a to form a dibenzofuran ring, because these two carbons were sterically close to each other. The ether oxygen, C-8-O, was considered to be the ether oxygen connecting the second phloroglucinol (group 2) to C-8 of the major skeleton.

Thus the structure of the isolated phlorotannin from *Eisenia arborea* that had significant inhibitory effect on histamine release from the RBL cells was found to be as shown in Fig. 1. It is a new structural isomer of PFF-A, and was designated phlorofucofuroeckol-B (PFF-B).

The anti-allergic effect of PFF-B was determined according to the procedure previously reported$^{(3)}$ with some modifications.

RBL-2H$^3$$^{(4)}$ cells precultured for 3 d in Eagle’s modified essential medium supplemented with 20% fetal bovine serum were sensitized with anti-dinitrophenyl (DNP) IgE for 18 h. The medium was then changed to Tyrode buffer (pH 7.2),$^{(15)}$ and the solution of the test sample was added. After incubation for 10 min, the cells were treated with an antigen, DNP-BSA, for 35 min. The antigen stimulated the sensitized cells to induce release of histamine together with $\beta$-hexosaminidase. Since the amount of released histamine was proportional to that of $\beta$-hexosaminidase,$^{(16)}$ it was estimated by the released enzymic activity. The stimulation reaction was stopped by cooling the reaction vessel on ice. The culture media were divided into two wells of a 96-well plate, and a substrate solution (1 mM p-nitrophenyl-$\beta$-D-glucosaminide) was added to each well. After incubation for 60 min at 37 $^\circ$C, 100 mM sodium bicarbonate was added to each solution to alkalize it and to develop a yellow color. The developed color (OD $^{405}$ nm) was measured to determine the amount of p-nitrophenol freed from the substrate by the action of the released enzyme.

To evaluate the effect of PFF-B on histamine release from RBL cells stimulated by the antigen-antibody reaction, epigallocatechin gallate (EGCg) was used as the positive control. As shown in Fig. 3, both PFF-B and EGCg inhibited histamine release from RBL cells in a dose-dependent manner, and their IC$_{50}$ values were 7.8 $\mu$M and 22.0 $\mu$M, respectively. Accordingly, PFF-B was 2.8 times stronger as an inhibitor than the typical natural inhibitor, EGCg. The IC$_{50}$ value for Tranilast, a clinically used anti-allergic drug, has been reported to be 46.6 $\mu$M$^{(17)}$ by the same assay system. Although we should be careful to compare the IC$_{50}$ values directly, because the IC$_{50}$ value of Tranilast was not determined in this experiment, the inhibitory effect of PFF-B might be strong enough for the clinical purposes. The strong effect on inhibition of histamine release of phlorotannin...
suggests that PFF-B, isolated from *E. arborea*, might be one of the reasons the alga showed anti-allergic activity on the allergy model rat, and that it might be a reason this alga has been popular as a health food and a folk medicine in Japan.

References


