Anti-plasmin Inhibitor. VI.[1] Structure of Phlorofucofuroeckol A, a Novel Phlorotannin with Both Dibenzo-1,4-dioxin and Dibenzofuran Elements, from *Ecklonia kurome* OKAMURA[2]

Yoshiyasu FUKUYAMA,*[,a] Mitsuaki KODAMA,[a] Iwao MIURA,[b] Ziunei KINZYO,[b,3] Hideo MORI,[b] Yasuo NAKAYAMA,[b] and Masayuki TAKAHASHI[b]

Faculty of Pharmaceutical Sciences, Tokushima Bunri University,[a] Yamashiro-cho, Tokushima 770, Japan and Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd.,[b] Kagasuno, Kawauchi-cho, Tokushima 771-01, Japan. *Received May 23, 1989*

Phlorofucofuroeckol A (1), a novel phlorotannin with both dibenzo-1,4-dioxin and dibenzofuran elements, has been isolated from the brown alga *Ecklonia kurome* OKAMURA as a potent anti-plasmin inhibitor. Its structure has been elucidated to be 1,11-di(3,5-dihydroxyphenoxy)-benzofuro[3,2-*a*]dibenzo[*b*,*e*][1,4]dioxin-2,4,8,10,14-pentaol on the basis of the spectral data, in particular, by means of negative nuclear Overhauser effect (NOE) and long-range carbon–proton couplings ($^2J_{CH}$ and $^3J_{CH}$). Phlorofucofuroeckol A inhibited the action of α_2-macroglobulin (IC_{50} = 1.0 μg/ml) and α_2-plasmin inhibitor (IC_{50} = 0.3 μg/ml), the main plasmin inhibitors in plasma.

Keywords phlorofucofuroeckol A; eckol; polyphenol; phlorotannin; *Ecklonia kurome*; NMR; negative NOE; anti-plasmin inhibitor; α_2-macroglobulin; α_2-plasmin inhibitor

We have demonstrated that the edible brown alga *Ecklonia kurome* OKAMURA elaborates a series of biologically active novel phlorotannins with a dibenzo-1,4-dioxin unit,[4,5] and they have been featured to be active in inhibiting the action of α_2-macroglobulin and α_2-plasmin inhibitor, the main plasmin inhibitors in plasma.[6] The compounds generally consist of phloroglucinol moieties linked by aryl–aryl or aryl–ether bonds, or of a mixed type.[1] In our search for anti-plasmin inhibitor among natural products, we found an additional active substance in the methanol extract of the title plant. A combination of chromatographies on silica gel and Sephadex LH-20 led to the isolation of a new eckol-type phlorotannin (1) having a furan ring, named phlorofucofuroeckol A.[7] In this paper, we report the isolation and the structural elucidation of this new compound.

Phlorofucofuroeckol A (**1**) failed to show a molecular ion peak in the electron impact mass spectrum (EIMS) and the field desorption mass spectrum (FDMS), but the EIMS of its trimethylsilyl (TMS) derivative and the FDMS of its peracetate (**1a**) revealed the molecular ion peaks at *m/z*

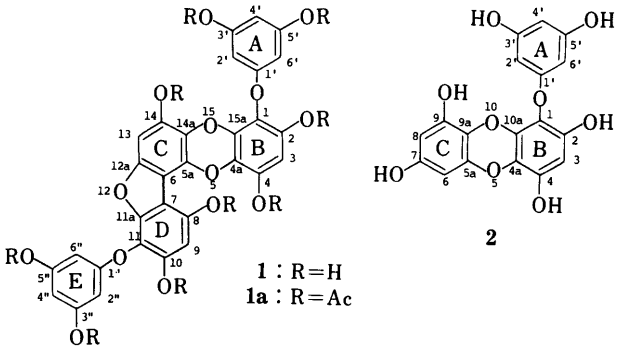


Fig. 1

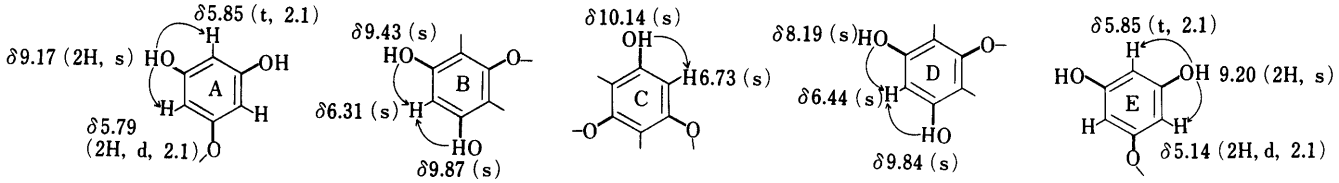


Fig. 2. The Phloroglucinol Units Existing in **1** and ^{1}H-NMR Data (DMSO-d_6) for **1**; multiplicity and *J* Values (in Hz) are given in parentheses, and the observed negative NOEs are indicated by arrows

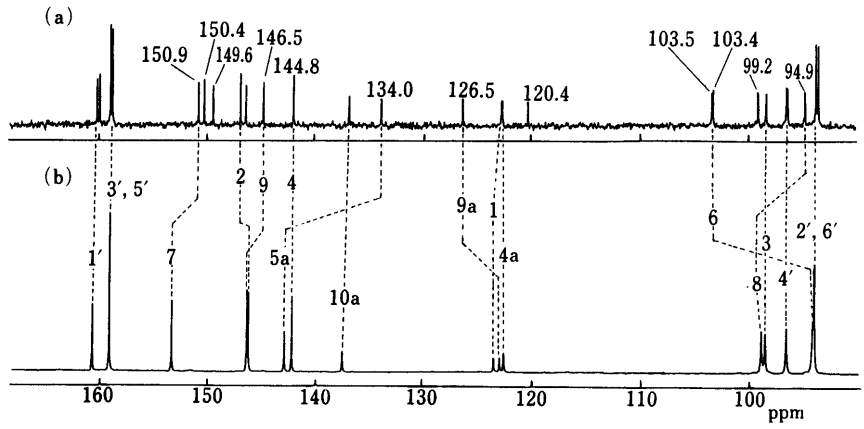


Fig. 3. ^{13}C-NMR Spectra (DMSO-d_6) of **1** (a) and **2** (b)

1250 and 980, respectively, indicating the molecular formula $C_{30}H_{18}O_{14}$ for **1**, and this was supported by the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectral data. The infrared (IR) spectrum of **1** displayed the presence of hydroxyl groups (3350 and 3300 cm^{-1}) and an aromatic nucleus (1605 cm^{-1}), but had no carbonyl absorption. The EIMS of **1** showed prominent fragment peaks at *m/z* 232 and 126, presumably derived from the basic dibenzodioxin and phloroglucinol elements, respectively, commonly present in the eckols.[4] The ^{13}C-NMR spectrum of **1** indicated the presence of thirty aromatic carbons which consisted of nine methine and nineteen oxygen-bearing carbons as well as of two quaternary carbons (δ 103.4 and 103.5). These spectral data implied that compound **1** is made up of five phloroglucinol moieties, as are all the previously reported eckol-type phlorotannins.[4] The ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** contained the following signals: a set of AB_2 system signals [δ 5.85 (1H, t, J=2.1 Hz) and 5.79 (2H, d, J=2.1 Hz), and 5.85 (1H, t, J=2.1 Hz) and 5.14 (2H, d, J=2.1 Hz)], three singlet signals (δ 6.31, 6.44, and 6.73), and seven kinds of phenolic hydroxyl signals involving nine hydrogens [δ 8.19, 9.17 (2H), 9.20 (2H), 9.43, 9.84, 9.87, and 10.14]. Nuclear Overhauser effect (NOE) experiments were performed in order to correlate all the phenolic hydroxyl proton signals with the aromatic proton ones, since clear negative NOEs (−30—−40%) as shown in Fig. 2 were observed in dimethyl sulfoxide-d_6 (DMSO-d_6).[8] Selective irradiation of the phenolic hydroxyl protons at δ 9.17 and 9.20 resulted in decrease in the intensities of the AB_2 signals at δ 5.79 and 5.85, and at δ 5.14 and 5.85, respectively, indicating the presence of the two phloroglucinol units A and E. The singlet aromatic resonance at δ 6.31 was reduced in intensity upon irradiations of the phenolic hydroxyl protons at δ 9.43 and 9.87, whereas the intensity of another singlet one at δ 6.44 was decreased upon irradiation of the phenolic hydroxyl protons at δ 8.19 and 9.84, and these two singlet protons should therefore be placed to be *ortho* to the two hydroxyl groups which caused the negative NOEs. Thus, the additional two phloroglucinol units turn out to have the partial structures B and D. The remaining phloroglucinol unit C could be also identified from the distinct negative NOE observed for the aromatic proton at δ 6.73 upon selective irradiation of the phenolic hydroxyl proton at δ 10.14. Among the five phloroglucinol units, only A and E can be linked to the other units *via* an ether bond, but the assembly of the remaining units is unclear on the basis of the present data since they are made up of both ether and aryl–aryl bonds. This problem was resolved by comparison of the ^{13}C-NMR spectra of **1** and eckol (**2**) as shown in Fig. 3. The carbon signals (C-1, 2, 3, 4, 4a, 10a, 1′, 2′, 3′, 4′, 5′, 6′) due to rings A and B in **2** were found to correspond well to those in **1**. This means that **1** has a dibenzo-1,4-dioxin skeleton and its phloroglucinol elements A and B should be identical with the corresponding A and B rings in eckol. On the other hand, the carbon signals (C-5a, 6, 7, 8, 9, and 9a) due to ring C in **2** showed no correlation with those of ring C in **1**. If the methine carbon C-6 in **2** were replaced by one (δ 103.4) of the newly appeared quaternary carbons in **1**, the phloroglucinol unit C could be regarded as ring C of **2** and thus could belong to the left side ring of a dibenzo-1,4-dioxin element. Moreover, the additional phloroglucinol unit D is presumably bonded to unit C through an aryl–aryl linkage and then a furan ring could be formed by loss of H_2O between the two units C and D, taking account of the presence of the other quaternary carbon (δ 103.5) and of the number of hydroxyl groups. Finally, the assembly of the phloroglucinol unit E onto ring D led to the three possible structures for **1** as shown in Fig. 4. In order to identify the correct structure among the three, long-range selective proton decoupling (LSPD)[9] was carried out and the long-range C/H coupling constants ($^2J_{CH}$ and $^3J_{CH}$) through two and three bonds were measured.[6] The results summarized in Fig. 5 could allowed us to assign all the quaternary carbons. The aromatic proton at δ 6.73 on ring C coupled not only to C-14a (δ 126.5) and C-6 (δ 103.4) having $^3J_{CH}$ values of 6.9 and 5.3 Hz, but also to C-12a (δ 150.9) and C-14 (δ 144.8) having $^2J_{CH}$ values of 3.8 and 3.1 Hz, respectively. In addition, the hydroxyl proton at δ 10.14 on

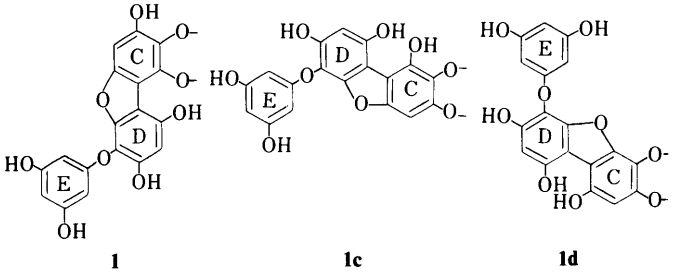
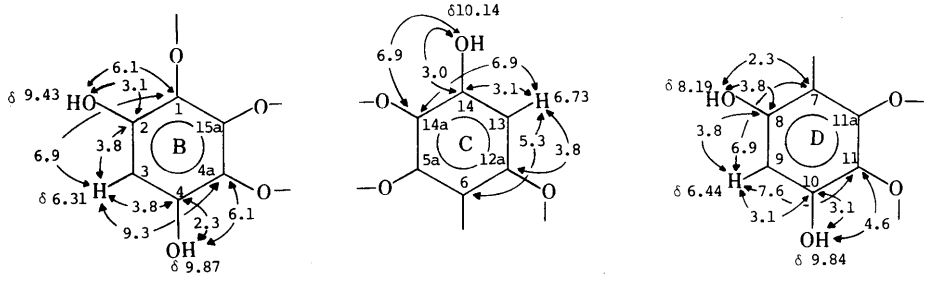


Fig. 4. The Possible Structures for Phlorofucofuroeckol A



C-1: 122.7 ppm
2: 147.0
3: 98.5
4: 142.0
4a: 122.8
15a: 136.9

C-5a: 134.0 ppm
6: 103.4
12a: 150.9
13: 94.9
14: 144.8
14a: 126.5

C-11a: 149.6 ppm
11: 120.4
10: 150.4
9: 99.2
8: 146.5
7: 103.5

Fig. 5. $^2J_{CH}$ and $^3J_{CH}$ Values (in Hz) between the Quaternary Carbon and the Protons through Two or Three Bonds in the Segments B, C, and D

ring C showed $^3J_{CH}$ (6.9 Hz) and $^2J_{CH}$ (3.0 Hz) couplings with C-14a (δ 126.5) and C-14 (δ 144.8), respectively. The observation of these C/H long-range couplings excludes the possibility of the structures **1c** and **1d** for the following reasons: in the case of **1c**, the single aromatic proton on ring C can not have NOE with the phenolic hydroxyl proton and should couple to all the quaternary carbons except for the OH-bearing carbon, whereas **1d** should have $^3J_{CH}$ coupling between the hydroxyl proton on ring C and the quaternary carbon signal at δ 103.4. Accordingly, the structure of phlorofucofuroeckol A (**1**) was elucidated as 1,11-di(3,5-dihydroxyphenoxy)-benzofuro[3,2-*a*]dibenzo-[*b*,*e*][1,4]dioxin-2,4,8,10,14-pentaol. Phlorofucofuroeckol A is the first example of an eckol-type phlorotannin[7] having a dibenzofuran ring. Compound **1** exhibited potent inhibitory activities against α_2-macroglobulin (IC_{50} 1.0 μg/ml) and α_2-plasmin inhibitor (IC_{50} 0.3 μg/ml), but its specificity was inferior to that of eckol (**2**).[5,6]

Experimental

Ultraviolet (UV) spectra were recorded on a Hitachi 340 spectrophotometer. IR spectra were measured with a Jasco A-202 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were obtained at 400 MHz (^{1}H-NMR) and 100.16 MHz (^{13}C-NMR), respectively, using a Bruker WH 400 spectrometer. Chemical shifts were expressed in δ (ppm) downfield from tetramethylsilane as an internal standard.

Extraction and Purification Fresh whole plants (600 kg) of *Ecklonia kurome* OKAMURA collected in Irino, Kochi prefecture, were immersed in methanol at room temperature for 6 d. After filtration, the methanol was evaporated off *in vacuo* to leave a gummy extract, which was partitioned between EtOAc and water. The course of purification was monitored by assay of the inhibitory activity towards the action of α_2-macroglobulin against plasmin.[5,6] The activity was concentrated into the EtOAc fraction (IC_{50} = 250 μg/ml). The EtOAc soluble portion (1.7 kg) mixed with Celite (3.4 kg) was dried under reduced pressure. The resultant solid was pulverized, packed into a glass column, and eluted successively with benzene (18 l), methylene chloride (36 l), ether (54 l), and methanol (20 l). The fraction (IC_{50} = 150 μg/ml, 552 g) eluted with ether was subjected to column chromatography on Sephadex LH-20 (3.5 kg). Each fraction (2 l) eluted with acetone was collected and evaporated *in vacuo* to yield eckol (**1**). The sixth fraction (IC_{50} = 10 μg/ml, 150 g) was rechromatographed on Sephadex LH-20 (3.5 kg) with methanol (20 l) to afford six fractions. Fraction 6 (IC_{50} = 1 μg/ml) was evaporated *in vacuo* to afford phlorofucofuroeckol (**1**) (2 g) as an amorphous material.

Phlorofucofuroeckol (1) Colorless amorphous solid, EIMS *m/z* (rel. int.): 232 (80), 126 (100). EIMS of TMS derivative *m/z* (rel. int.): 1250 [M^+] (100), 1235 (8.6), 982 (9), 625 (45). UV λ_{max}^{MeOH} nm: 224 (sh), 244 (ε 58000), 292 (ε 6800), 304 (sh), 317 (ε 3100). IR ν_{max}^{KBr} cm^{-1}: 3350, 3300, 1605, 1470, 1360, 1110, 1060, 880. ^{1}H-NMR (DMSO-d_6) δ: 5.14 (2H, d, J = 2.1 Hz, 2″, 6″-H), 5.79 (2H, d, J = 2.1 Hz, 2′, 6′-H), 5.85 (2H, t, J = 2.1 Hz, 4′, 4″-H), 6.31 (1H, s, 3-H), 6.44 (1H, s, 9-H), 6.73 (1H, s, 13-H), 8.19 (1H, s, C_8-OH), 9.17 (2H, s, $C_{3',5'}$-OH), 9.20 (2H, s, $C_{2'',5''}$-OH), 9.43 (1H, s, C_2-OH), 9.84 (1H, s, C_{10}-OH), 9.87 (1H, s, C_4-OH), 10.14 (1H, s, C_{14}-OH). ^{13}C-NMR (DMSO-d_6) δ: 93.7 (d, C-2″, 6″), 93.9 (d, C-2′, 6′), 94.9 (d, C-13), 96.5 (d, C-4′), 96.5 (d, C-4″), 98.5 (d, C-3), 99.2 (d, C-9), 103.4 (s, C-6), 103.5 (s, C-7), 120.4 (s, C-11), 122.7 (C-1), 122.8 (s, C-4a), 126.5 (s, C-14a), 134.0 (s, C-5a), 136.9 (s, C-15a), 142.0 (s, C-4), 144.8 (s, C-14), 146.5 (s, C-8), 147.0 (s, C-2), 149.6 (s, C-11a), 150.4 (s, C-10), 150.9 (s, C-12a), 158.9 (s, C-3′, 5′), 159.0 (s, C-3″, 5″), 160.0 (s, C-1″), 160.3 (s, C-1′).

Phlorofucofuroeckol Nonaacetate (1a) A mixture of **1** (60 mg), pyridine (0.5 ml), and acetic anhydride (0.2 ml) was allowed to stand at room temperature. The reaction mixture was poured onto crushed ice and then extracted with ether. The combined ether layer were washed with 1 N HCl, saturated $NaHCO_3$, and brine, and dried over $MgSO_4$. Evaporation of the solvent afforded **1a** (50 mg) as an amorphous material. FDMS *m/z* (rel. int.): 981 (M^++H, 100), 980 (M^+, 80), 938 (5), 803 (10), 624 (15). EIMS *m/z*: 938 (M^+−42), 896 (M^+−42×2), 854 (M^+−42×3), 812 (M^+−42×4), 770 (M^+−42×5), 728 (M^+−42×6), 686 (M^+−42×7), 644 (M^+−42×8), 602 (M^+−42×9).

Acknowledgments We are indebted to Dr. M. Nishikawa and Mr. M. Inoue, Otsuka Pharmaceutical Co., Ltd., for the identification and collection of *E. kurome*.

References and Notes

1) Anti-plasmin Inhibitor. Part V: Y. Fukuyama, M. Kodama, I. Miura, Z. Kinzyo, H. Mori, Y. Nakayama, and M. Takahashi, *Chem. Pharm. Bull.*, **37**, 2438 (1989).
2) A part of this study was presented at the 49th Annual Meeting of the Chemical Society of Japan, April 1984.
3) Present address: *Faculty of Pharmaceutical Sciences, Kumamoto University, 5–1 Ooehonmachi, Kumamoto 862, Japan.*
4) Y. Fukuyama, I. Miura, Z. Kinzyo, H. Mori, M. Kido, Y. Nakayama, M. Takahashi, and M. Ochi, *Chem. Lett.*, **1985**, 739.
5) Y. Fukuyama, M. Kodama, I. Miura, Z. Kinzyo, M. Kido, H. Mori, Y. Nakayama, and M. Takahashi, *Chem. Pharm. Bull.*, **37**, 349 (1989).
6) Y. Nakayama, M. Takahashi, Y. Fukuyama, and Z. Kinzyo, *Agric. Biol. Chem.*, in press.
7) Phlorofucofuroeckol was isolated as its peracetate from *Ecklonia maxima* by Glombitza *et al.*, but the reported data can not distinguish the three possible structures. Hence, compound **1** has been named phlorofucofuroeckol A since its isomers are likely to be found in the future. K-W. Glombitza and H. P. Vogels, *Planta Med.*, **1985**, 308.
8) J. R. Kalman and D. H. Williams, *J. Am. Chem.Soc.*, **102**, 906 (1980).
9) H. Seto, T. Sasaki, H. Yonehara, and J. Uzawa, *Tetrahedron Lett.*, **1978**, 923.