A New Phloroglucinol Derivative from the Brown Alga *Eisenia bicyclis*: Potential for the Effective Treatment of Diabetic Complications

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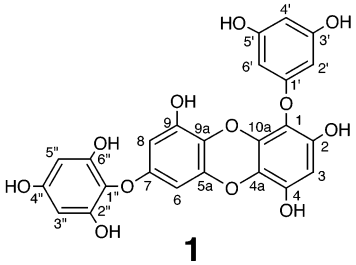
Received July 17, 2003

A new phloroglucinol derivative (**1**) and two known compounds (**2** and **3**) were isolated from the brown alga *Eisenia bicyclis*. These isolates exhibit inhibitory activity on glycation and α-amylase. Their structures were determined on the basis of spectroscopic data.

Eisenia bicyclis (Kijillman) Setchell (Laminariaceae) is a very common brown alga that inhabits the middle Pacific coast around Japan. It is consumed as the raw material for sodium alginate. Nakamura et al. reported the isolation of eckol and dieckol from this raw material and described the antioxidant activity of these compounds.[1] In a previous paper, we described the inhibitory effect of *E. bicyclis* on aldose reductase,[2] which reduces glucose conversion to sorbitol in cells. Accumulation of sorbitol in cells leads to the development of various chronic complications of diabetes, such as cataracts, neuropathy, and retinopathy. Meanwhile, carrier proteins in blood vessels, structural proteins, and enzymes in the body are modified by glucose in a process called glycation. It is known that accumulation of these glycated proteins (AGE) causes diabetic complications. An inhibitor of glycation has been shown to retard the development of diabetic complications in experimental diabetes.[3] We already reported the isolation of flavonoids that exhibited glycation inhibitory activity from crude drug.[4,5] Furthermore, it is widely accepted that the most challenging goal in the management of patients with diabetes is to achieve blood glucose levels as close to normal as possible. α-Amylases, endoglucanases that catalyze hydrolysis of the internal α-1,4-glucosidic linkage in starch and other related polysaccharides, have also been targets for suppression of postprandial hyperglycemia. In the present paper, we report the isolation of one new and two known phloroglucinol derivatives from *E. bicyclis* and discuss the inhibitory effects of these compounds on glycation and α-amylase.

E. bicyclis was extracted with methanol under reflux to give the methanol extract. This extract was subjected to Diaion HP-20, followed by centrifuged partition chromatography (CPC) and reversed-phase HPLC, to afford three phloroglucinol derivatives, a new compound (**1**), eckol (**2**), and dieckol (**3**).

Compound **1** had the molecular formula $C_{24}H_{16}O_{12}$ as determined from its HRFABMS, ^{13}C NMR, and ^{13}C DEPT spectral data. The ^1H NMR spectrum of **1** showed an AB_2 system at δ 5.71 (2H, J = 2.1 Hz), 5.79 (1H, J = 2.1 Hz), an AB system at δ 5.78 (1H, J = 2.8 Hz), 6.00 (1H, J = 2.8 Hz), and two singlets at δ 6.13 (1H) and 5.85 (2H) as well as eight phenolic OH protons at δ 9.00 (1H), 9.13 (2H), 9.14 (2H), 9.20 (1H), 9.40 (1H), and 9.61 (1H). The ^{13}C NMR spectrum indicated the presence of eight nonsubstituted and 16 O-bearing aromatic carbons. The ^{13}C NMR spectrum is very similar to that of eckol (**2**),[6] except for four extra signals, indicating that **1** was composed of four phloroglucinol units. The molecular weight of **1** was 124 more than eckol (496 vs 372). Detailed assignment of the protons and carbons was accomplished by means of the HMQC, HMBC, and NOE experiments (Figure 1). The position of the additional phloroglucinol moiety was determined to be C-7 from the fact that the ^{13}C NMR signals for the basic skeleton in **1** were almost identical with those of **2** except for C-7 and C-9a. These signals were observed at low field compared to those of **2**. A similar phenomenon



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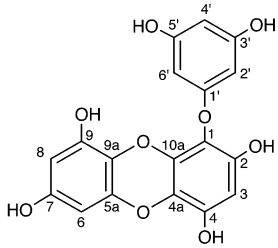

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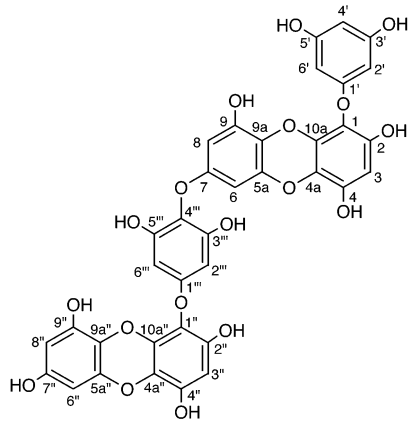

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10.1021/np030323j CCC: $27.50
Published on Web 12/25/2003

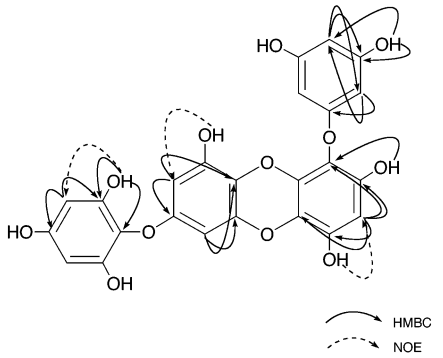

Figure 1. HMBC and NOE of compound **1**.

was reported by Fukuyama et al.[4] on elucidation of another phloroglucinol derivative, 2-phloroeckol. Consequently, it was concluded that **1** was 1-(3′,5′-dihydroxyphenoxy)-7-(2″,4″,6″-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin (**1**).

Compound **2** had the molecular formula $C_{18}H_{12}O_9$ as determined from EIMS, ^{13}C NMR, and ^{13}C DEPT spectral data. In the ^1H NMR spectrum of **2**, an AB_2 system at δ 5.71 (2H, $J = 2.0$ Hz), 5.79 (1H, $J = 2.0$ Hz), an AB system at δ 5.78 (1H, $J = 2.6$ Hz), 5.95 (1H, $J = 2.6$ Hz), and a singlet at δ 6.13 (1H) were observed in addition to six phenolic OH protons at δ 9.12 (2H), 9.15 (2H), 9.42 (1H), and 9.52 (1H). The ^{13}C NMR spectrum indicated the presence of six nonsubstituted and 12 O-bearing aromatic carbons. The ^1H and ^{13}C NMR signals were assigned with the aid of HMQC, HMBC, and NOE experiments. According to the above data and values in the literture,[6] **2** was concluded to be eckol.

Compound **3** had the molecular formula $C_{36}H_{22}O_{18}$ as determined from FABMS, ^{13}C NMR, and ^{13}C DEPT spectral data. The ^1H NMR spectrum of **3** showed an AB_2 system at δ 5.78 (2H, $J = 2.0$ Hz), 5.86 (1H, $J = 2.0$ Hz) and two AB systems at δ 5.88 (1H, $J = 2.7$ Hz), 6.08 (1H, $J = 2.7$ Hz) and δ 5.87 (1H, $J = 2.7$ Hz), 6.05 (1H, $J = 2.7$ Hz). Furthermore, three singlet signals in the ^1H NMR at δ 6.01 (2H), 6.02 (1H), and 6.22 (1H) and nine phenolic OH proton signals at δ 9.18 (2H), 9.24 (1H), 9.26 (1H), 9.31 (1H), 9.38 (2H), 9.48 (1H), 9.53 (1H), 9.63 (1H), and 9.73 (1H) were observed. The ^{13}C NMR signals indicated the presence of nine nonsubstituted and 23 O-bearing aromatic carbons. The above data for **3** were consistent with the literature values for dieckol.[1,6]

Some diabetic complications are caused by AGE, which are produced through a series of reactions called glycation. Glycation involves the nonenzymatic glycosylation of amino groups in proteins, lipids, and nucleic acids to form Schiff bases and Amadori products as intermediates. The inhibitory effects of the isolated compounds on glycation were tested by ELISA. Percent inhibition was calculated to be 91.1% for **1**, 96.2% for **2**, 86.7% for **3**, and 76.0% for aminoguanidine at 1 mM. It is believed that an α-amylase inhibitor that can suppress postprandial hyperglycemia may be effective at preventing the complications of diabetes. The inhibitory effects of the compounds isolated on α-amylase were tested by the method of Murata et al.,[7] with slight modification. Percent inhibition was calculated to be 89.5% for **1**, 87.5% for **2**, and 97.5% for **3** at 1 mM. These results suggest that the phloroglucinol derivatives may have an effect on the complications of diabetes.

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanako micro-melting point apparatus and were uncorrected. UV spectra were obtained with a Shimadzu UV 1600 spectrophotometer. EIMS and FABMS (magic bullet matrix) were obtained with a JEOL JMS-700 mass spectrometer. ^1H and ^{13}C NMR spectra were measured on JEOL AL-300, AL-400, and LA-500 spectrometers using tetramethylsilane as an internal standard. All chemical shifts (δ) are given in ppm, and the samples were solubilized in DMSO-d_6.

Table 1. ^1H NMR Data for Compound **1** in DMSO-d_6

3	6.13 (1H, s)
6	5.78 (1H, d, 2.8)
8	6.00 (1H, d, 2.8)
2′,6′	5.71 (2H, d, 2.1)
4′	5.79 (1H, t, 2.1)
3″	5.85 (1H, s)
5″	5.85 (1H, s)
6″	
8″	
2‴,6‴	
2-OH	9.20 (1H, s)
4-OH	9.40 (1H, s)
7-OH	
9-OH	9.61 (1H, s)
3′,5′-OH	9.14 (2H, s)
2″-OH	9.13 (1H, s)
4″-OH	9.00 (1H, s)
6″-OH	9.13 (1H, s)
7″-OH	
9″-OH	
3‴,5‴-OH	

[a] Recorded at 500 MHz. Coupling constants (Hz) are in parentheses.

Plant Material. *E. bicyclis* (Kijillman) Setchell was purchased from Daichu Shokuhin Co., Ltd (Mie, Japan). A voucher specimen (No. NP 021213) was deposited with the laboratory of the Department of Natural Medicine and Phytochemistry at Meiji Pharmaceutical University.

Extraction and Isolation. *E. bicyclis* (10 kg) was extracted with MeOH (18 L) three times for 3 h under reflux, and the solvent was evaporated in vacuo to give MeOH extract (329 g). This extract was subjected to Diaion HP-20 ($H_2O \rightarrow$ MeOH $\rightarrow$ acetone $\rightarrow$ AcOEt) to yield the corresponding fractions. The MeOH fraction (123.9 g) was subjected to Diaion HP-20 (aqueous MeOH $\rightarrow$ MeOH $\rightarrow$ acetone) to give fractions 1−10. Fraction 4 (6.2 g) was chromatographed over TOYOPEARL HW-40F [MeOH] to afford fractions 11−14. Fraction 13 (927.8 mg) was subjected to CPC (*n*-butanol−*n*-propanol−H_2O, 4:1:5) to give fractions 15−23. Fraction 21 (696 mg) and fraction 22 (62.4 mg) were subjected to reversed-phase HPLC (C-8, 18, 22) eluting with a MeOH−H_2O mixture. **3** (8.6 mg) was isolated from fraction 21, and **1** (4.5 mg) and **2** (3.2 mg) were isolated from fraction 22.

Compound 1: light brown powder (lyophilized); UV λ_{max} (MeOH) 233.0 (ϵ 30700), 292.0 (ϵ 4900) nm; ^1H and ^{13}C NMR data, see Tables 1 and 2; positive FABMS *m/z* 497 $[M + H]^+$; HRFABMS *m/z* 495.0541 $[M - H]^+$ (calcd for $C_{24}H_{15}O_{12}$, 495.0563, Δ −2.2 nnu).

Compound 2: light brown powder (lyophilized); UV λ_{max} (MeOH) 232.0 (ϵ 26700), 291.0 (ϵ 4600) nm; ^1H NMR (DMSO-d_6, 400 MHz) δ 9.52 (1H, s, OH-9), 9.42 (1H, s, OH-4), 9.15 (2H, s, OH-2,7), 9.12 (2H, s, OH-3′, -5′), 6.13 (1H, s, H-3), 5.95 (1H, d, $J = 2.6$ Hz, H-8), 5.79 (1H, t, $J = 2.0$ Hz, H-4), 5.78 (1H, d, $J = 2.6$ Hz, H-6), 5.71 (2H, d, $J = 2.0$ Hz, H-2′, -6′); ^{13}C NMR data, see Table 2; EIMS *m/z* 372 $[M]^+$.

Compound 3: light brown powder (lyophilized); UV λ_{max} (MeOH) 233.5 (ϵ 59800), 292.0 (ϵ 6200) nm; ^1H NMR (DMSO-d_6, 300 MHz) δ 9.73 (1H, s, OH-9), 9.63 (1H, s, OH-9″), 9.53 (1H, s, OH-4‴), 9.48 (1H, s, OH-4′), 9.38 (2H, s, OH-3‴, -5‴), 9.31 (1H, s, OH-2″), 9.26 (1H, s, OH-2), 9.24 (1H, s, OH-7″), 9.18 (2H, s, OH-3′, -5′), 6.22 (1H, s, H-3″), 6.20 (1H, s, H-3), 6.08 (1H, d, $J = 2.7$ Hz, H-8), 6.05 (1H, d, $J = 2.7$ Hz, H-8″), 6.01 (2H, s, H-2‴, -6‴), 5.88 (1H, d, $J = 2.7$ Hz, H-6), 5.87 (1H, d, $J = 2.7$ Hz, H-6″), 5.86 (1H, t, $J = 2.0$ Hz, H-4′), 5.78 (2H, d, $J = 2.0$ Hz, H-2′, -6′); ^{13}C NMR data, see Table 2; positive FABMS *m/z* 743 $[M + H]^+$.

Table 2. ^{13}C Data for Compounds **1**−**3** in DMSO-d_6

	1[a]	**2**[b]	**3**[c]
1	122.2 s	122.9 s	122.1 s
2	145.9 s	145.6 s	145.9 s
3	98.3 d	97.9 d	98.2 d
4	141.9 s	141.5 s	141.7 s
4a	123.2 s	121.9 s	123.0 s
5a	142.4 s	142.2 s	142.2 s
6	93.5 d	93.6 d	93.4 d
7	154.5 s	152.6 s	154.0 s
8	98.2 d	98.3 d	97.9 d
9	146.1 s	145.7 s	145.7 s
9a	124.0 s	122.3 s	123.9 s
10a	137.1 s	136.8 s	137.0 s
1′	160.3 s	160.0 s	160.1 s
2′,6′	93.7 s	93.5 d	93.5 d
3′,5′	158.8 s	158.4 s	158.6 s
4′	96.2 d	96.0 d	96.1 d
1″	122.6 s		122.1 s
2″	151.2 s		145.7 s
3″	94.9 d		98.1 d
4″	154.8 s		141.8 s
4a″			123.1 s
5″	94.9		
5a″			142.4 s
6″	151.2 s		93.7 d
7″			152.8 s
8″			98.4 d
9″			145.8 s
9a″			122.4 s
10a″			136.9 s
1‴			155.7 s
2‴,6‴			94.4 d
3‴,5‴			150.9 s
4‴			124.1 s

[a] Recorded at 125 MHz. [b] Recorded at 100 MHz. [c] Recorded at 75 MHz.

Inhibition Test on AGE Formation in Vitro. Lysozyme (10 mg/mL) was incubated with 100 mM fructose in the presence and absence of test compound for 7 days in 0.1 M phosphate buffer (pH 7.4) at 37 °C. After incubation, the level of CML was measured by CML-specific ELISA based on the method of Horiuchi.[8] Percent inhibition was calculated as follows: inhibition % = $[1 - (A_s - A_b)/(A_c - A_b)] \times 100$, where A_s is the CML level in the incubated mixture with sample, A_c is the CML level in the incubated mixture without sample as a positive control, and A_b is the CML level in the incubated mixture without sample and glucose that served as a blank control.

Inhibition Test on α-Amylase Activity. Soluble starch was dissolved in 2-(*N*-morpholino)ethanesulfonic acid (MES)−NaOH buffer (pH 6.5) containing 20 mM $CaCl_2$ and 100 mM NaCl and used as substrate solution. α-Amylase solution, test compound dissolved in MeOH, and substrate solution were mixed in a test tube and incubated for 15 min at 37 °C. After incubation, the reaction was stopped by the addition of 3,5-dinitrosalicylic acid (DNS) and boiling for 5 min. The absorbance of the reaction solution diluted by water was measured at 500 nm. The amount of liberated reducing sugar was obtained as units of maltose using a calibration curve for a reference standard. This method was based on detection of the reducing hemiacetal groups, produced during starch hydrolysis with DNS. Percent inhibition of each compound was calculated as follows: inhibition % = $[1 - (S_a - S_b)/(C - B)] \times 100$, where S_a is the units of maltose of the incubated mixture with sample, S_b is the maltose units of the unincubated mixture containing sample, C is the maltose units of incubated mixture without sample as a positive control, and B is the maltose units of unincubated mixture without sample as a blank control.

Acknowledgment. The authors are grateful to Dr. S. Terada of Zenyaku Kogyo Co., Ltd. for experimental support with the α-amylase inhibition test. This work was supported in part by a grant for the promotion of the advancement of education and research in graduate schools from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from the Japan−China Medical Association.

References and Notes

(1) Nakamura, T.; Nagayama, K.; Uchida, K.; Tanaka, R. *Fish. Sci.* **1996**, *62*, 923−926.
(2) Iwahori, Y.; Enomoto, S.; Okada, Y.; Tanaka, J.; Okuyama, T. *Nat. Med.* (*Tokyo*) **1999**, *53*, 138−140.
(3) Nakamura, S.; Makita, Z.; Ishikawa, S.; Yasumura, K.; Fujii, W.; Yanagisawa, K.; Kawata, T.; Koike, T. *Diabetes* **1997**, *46*, 895−899.
(4) Suzuki, R.; Okada, Y.; Okuyama, T. *J. Nat. Prod.* **2003**, *66*, 564−565.
(5) Suzuki, R.; Okada, Y.; Okuyama, T. *Chem. Pharm. Bull.* **2003**, *51*, 1186−1188.
(6) Fukuyama, Y.; Miura, I.; Kinzyo, Z.; Mori, H.; Kida, M.; Nakayama, Y.; Takahashi, M.; Ochi, M. *Chem. Lett.* **1985**, 739−742.
(7) Murata, T.; Furubayashi, M.; Tsukiyama, Y. Jpn. Kokai Tokkyo Koho 00 44,484, 2000.
(8) Horiuchi, S. *J. Biol. Chem.* **1992**, *226*, 7329−7332.

NP030323J