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Potential Anti-angiogenesis Effects of *p*-Terphenyl Compounds from *Polyozellus multiplex*

Ikuko Nagasawa,[†] Akira Kaneko,[†] Toshihiro Suzuki,[‡] Kazuto Nishio,[§] Kaoru Kinoshita,[†] Motto Shiro,[⊥] and Kiyotaka Koyama*[,†]

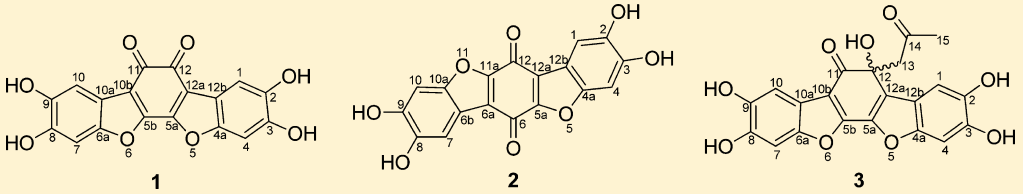
[†]Department of Pharmacognosy and Phytochemistry, Meiji Pharmaceutical University, Noshio 2-522-1, Kiyose-shi, Tokyo 204-8588, Japan

[‡]Department of Analytical Biochemistry, Meiji Pharmaceutical University, Noshio 2-522-1, Kiyose-shi, Tokyo 204-8588, Japan

[§]Department of Genome Biology, Kinki University School of Medicine, Ohno-Higashi 377-2, Osaka-sayama-shi, Osaka 589-8511, Japan

[⊥]X-ray Research Laboratory, Rigaku Corporation, Matsubara-cho 3-9-12, Akishima-shi, Tokyo 196-8666, Japan

Supporting Information



1 2 3

ABSTRACT: One novel *p*-terphenyl compound, polyozellic acid (**1**), and its acetone adduct (**3**), along with a known *p*-terphenyl compound, thelephoric acid (**2**), were isolated from the mushroom *Polyozellus multiplex*. Their molecular structures were determined by spectroscopic analysis, X-ray crystallographic analysis, and chemical modification. In some assays related to angiogenesis, compounds **1** and **2** in particular showed inhibitory effects on proliferation, tubule formation, and invasion of human umbilical vein endothelial cells. The quinone moiety within these molecules possibly contributes to their antiangiogenesis activity.

Angiogenesis, the formation of new vessels from an existing vascular network, is an important process in the growth of tumors and an essential component of the metastatic pathway. Therefore, tumor angiogenesis inhibitors are considered an effective strategy for the treatment of cancer. Several growth factors including vascular endothelial growth factor (VEGF), fibroblast growth factor, platelet-derived growth factor, epidermal growth factor, placental growth factor, angiopoietin-1, angiogenin, interleukin 8, and their associated receptor tyrosine kinases intricately regulate angiogenesis. Of these, VEGF is one of the most critical regulators of tumor angiogenesis. VEGF is activated by the hypoxic conditions in the tumor and then binds to the VEGF receptor 2 (VEGFR2/KDR/Flk-1), a transmembrane receptor tyrosine kinase on the membrane of endothelial cells. This activates VEGFR2 by autophosphorylation of the tyrosine kinase and consequently mediates signaling-based proliferation, invasion, and tubule formation of vascular endothelial cells.[1−3] Inhibition of the VEGFR2 signaling pathway is a promising therapeutic approach against tumor-induced angiogenesis. A range of multikinase inhibitors have been developed, with a focus on targeting VEGFR2 to treat cancer. We have previously reported that various natural products act as inhibitors of VEGFR2 tyrosine kinase or act on human umbilical vein endothelial cell (HUVEC) proliferation.[4−7]

Polyozellus multiplex is an edible Japanese mushroom with a black-purple fruiting body. A series of *p*-terphenyl compounds, polyozellin (**4**),[8] thelephoric acid (**2**),[9] and kynapcins-9 (**5**)[9] and -12,[10] and the benzofuran compounds kynapcin-13,[11] -24,[12] and -28,[11] have been isolated from the mushroom. All of these compounds have been reported as prolyl endopeptidase inhibitors. Polyozellin (**4**) has also been shown to have various physiological properties such as inhibition of TNF-α-induced interleukin 8[13] and antinitric oxide production activity.[14]

In this paper, we report the isolation, structural determination, and bioactivity against angiogenesis of novel *p*-terphenyl compounds including an *o*-quinone derivative and an artificial acetone adduct derivative isolated from acetone extracts of *P. multiplex*. We determined the structures of the new *p*-terphenyl compounds by spectroscopic analysis and chemical modification. Anti-VEGFR2 tyrosine kinase activities of these compounds were evaluated by ELISA, and antiproliferative activity in HUVECs was measured by a WST-8 assay. Furthermore, inhibitory effects of the compounds on tubule formation and invasion of HUVECs were examined.

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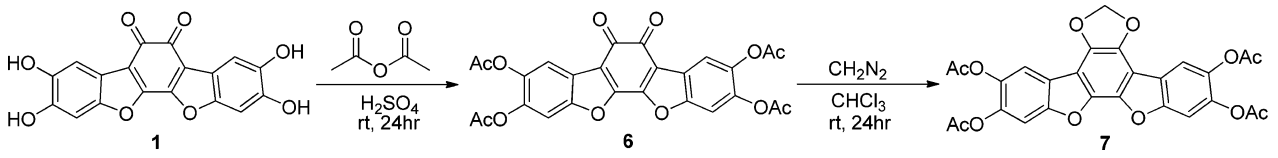
Scheme 1. Derivatization of 1



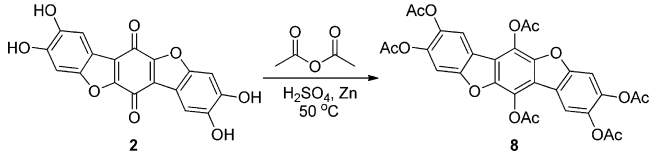
RESULTS AND DISCUSSION

Dry fruiting bodies of *P. multiplex* were extracted with $CHCl_3$, MeOH, and then acetone at room temperature. Various column chromatographic separations of the acetone extract afforded one novel compound (**1**), its artificial acetone adduct (**3**), and a known compound (**2**).

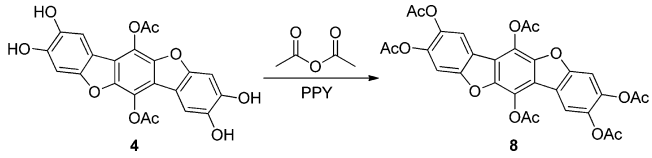
We were unable to determine an accurate molecular weight of compound **1**, a black powder, by FABMS. IR absorptions at 3375 and 1645 cm^{-1} suggested the presence of hydroxyl and carbonyl groups, respectively. The ^1H NMR spectrum showed only two protons [δ_H 7.12 (s, H-4, 7) and δ_H 7.23 (s, H-1, 10)] bonded to aromatic carbons. The ^{13}C NMR spectrum showed nine carbon resonances attributable to one carbonyl [δ_C 175.7 (C-11, 12)], six sp^2 quaternary carbons [δ_C 114.9 to 151.0 (C-2, 3, 4a, 5a, 5b, 6a, 8, 9, 10a, 10b, 12a, 12b)], and two aromatic methine carbons [δ_C 98.9 (C-4, 7) and 104.6 (C-1, 10)]. These chemical shift patterns were very similar to those of *p*-terphenyl compounds such as polyozellin (**4**) isolated from *P. multiplex* and boletopsins, leucomelons, and Bl series terphenyl compounds isolated from *Boletopsis leucomelas*.[5] We suspected that compound **1** probably has a *p*-terphenyl skeleton, but since the number of carbons identified was not enough for composition of such a skeleton, we considered whether compound **1** might be highly symmetrical. HMBC correlations between H-1 (10) and C-2 (9), C-3 (8), and C-4a (6a) and between H-4 (7) and C-2 (9), C-3 (8), C-4a (6a), and C-12b (10a), their chemical shifts, and spin−spin splitting patterns established partial structures of two 1,2-dihydroxybenzenes. Finally, two possible structures, thelephoric acid (**2**), one of the primary constituents isolated from *P. multiplex*, and its *o*-quinone form, were considered. Despite their relatively simple structures, it is difficult to determine their substitution patterns because the compounds are symmetrical. In such a situation, chemical modification often assists in the elucidation of structure. Some papers have reported that by the reaction of diazoalkanes with phenanthraquinone (*o*-quinone), both the epoxidized product and the methylenedioxy product are obtained.[15,16] The reaction of compound **1** acetate (**6**) with diazomethane was carried out, and a main product (**7**) containing a methylenedioxy group in the structure was obtained (Scheme 1). As a result, the structure of compound **1** was determined to be a novel *o*-quinone derivative of thelephoric acid (**2**), because if it was a *p*-quinone derivative, it should not be able to produce a methylenedioxy bridge moiety. Compound **1** was named polyozellic acid.

Compound **2** was obtained as a black powder. Like compound **1**, we were not able to deduce its molecular weight by FABMS. The ^1H and ^{13}C NMR spectra showed two aromatic protons and nine carbon signals that were assigned to one carbonyl, six sp^2 quaternary carbons, and two aromatic methine carbons. NMR resonances and HMBC correlations of **2** were very similar to those of polyozellic acid (**1**). Consequently, compound **2** was determined to be thelephoric acid (**2**),[9] one of the main constituents of *P. multiplex*, containing a *p*-quinone moiety in the structure. This structure was also supported on the basis of a reductively acetylated derivative (**8**) of **2** being the same compound as an acetylated derivative (**8**) of polyozellin (**4**, identified in our laboratory) based on their spectroscopic data (Schemes 2 and 3).

Scheme 2. Reductive Acetylation of 2



Scheme 3. Acetylation of 4



The molecular formula of compound **3**, a reddish-brown powder, was determined to be $C_{21}H_{14}O_9$ by HR-FABMS. The IR spectrum of **3** showed absorptions at 3395 and 1635 cm^{-1} assignable to hydroxyl and carbonyl, respectively. The ^1H NMR spectrum of **3** showed the signals of four aromatic protons [δ_H 7.07 to 7.26 (s, H-4, 1, 7, and 10)], two methylene protons [δ_H 3.49 and 3.56 (each d, H-13)], one methyl [δ_H 1.97 (s, H-15)], and two broad signals [δ_H 6.24 (12-OH) and 9.38 (2, 3, 8, 9-OH)]. In the ^{13}C NMR spectrum, a total of 21 carbon signals including two carbonyl carbons [δ_C 194.8 (C-11) and 205.0 (C-14)], 13 quaternary carbons [δ_C 72.8 to 152.9], four methine carbons [δ_C 98.4 to 104.7 (C-4, 7, 1, and 10)], one methylene carbon [δ_C 53.2 (C-13)], and one methyl carbon [δ_C 30.2 (C-15)] appeared. These spectral data were very similar to those of kynapcin-9 (**5**),[9] which was isolated from *P. multiplex* (Figure 1). The definitive structure of **3** was obtained from X-ray crystallographic analysis of suitable brown needle crystals. Crystals of **3** were monoclinic, belonging to space group *P*2. As shown in an ORTEP drawing, X-ray analysis revealed that compound **3** is a racemate of an acetone adduct of **1** (Figure 2).

Compounds **1**−**3** and polyozellin (**4**) were evaluated using a tyrosine kinase assay kit with recombinant human VEGFR2. Compounds **1**−**4** showed inhibitory activity toward VEGFR2 tyrosine kinase with IC_{50} values as shown in Table 2. Since VEGFR2, a principal regulator of angiogenesis, is highly expressed in umbilical vein endothelial cells, we examined the effects of these terphenyl compounds on cell growth of HUVECs by a WST-8 assay. As a result, all compounds exhibited antiproliferative activity in HUVECs (Table 2).

To investigate their effects on angiogenesis, compounds **1**−**4** were examined for inhibitory effects on tubule formation of HUVECs. As shown in Figure 3, polyozellic acid (**1**) and thelephoric acid (**2**) suppressed the formation of the tubule

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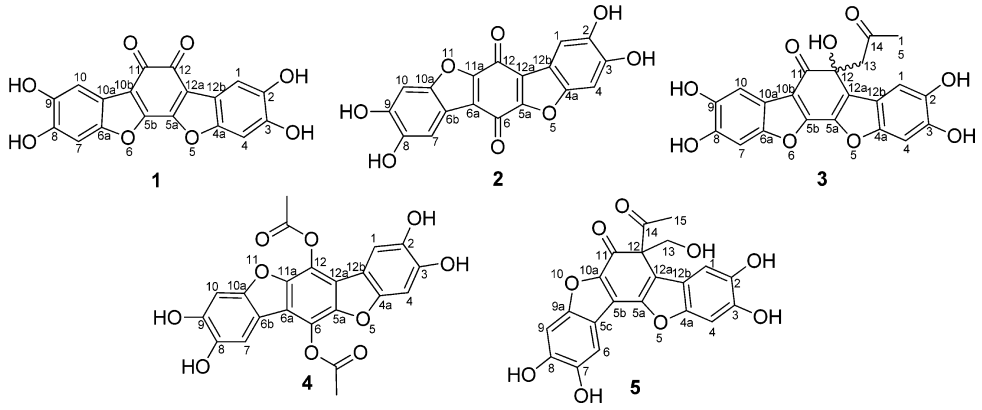

Figure 1. Structures of compounds **1**–**5**.

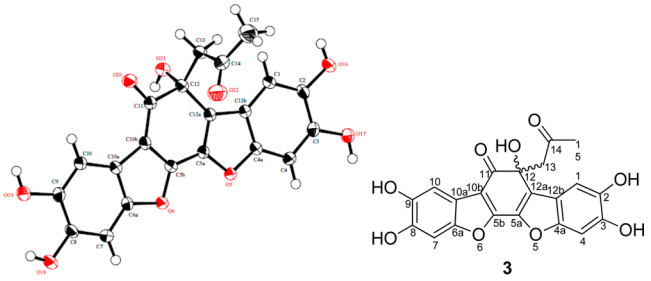

Figure 2. ORTEP drawing of **3** obtained by X-ray analysis.

network in a dose-dependent manner; in contrast, neither the acetone adduct (**3**) nor polyozellin (**4**) inhibited tubule formation. Cell invasion is another critical step for tumor angiogenesis. The effects of polyozellic acid (**1**) and thelephoric acid (**2**) on invasion of HUVECs were evaluated using a chamber assay, which measured the ability of cells to pass through a Matrigel matrix coated filter. As shown in Figure 4, **1**

Table 2. Inhibitory Activities of 1–4 against VEGFR2 Tyrosine Kinase and Proliferation of HUVECs

	IC_{50} (μM)	
compound	VEGFR2 tyrosine kinase inhibitory activity	HUVECs growth inhibitory activity
1	32	7.6
2	70	3.4
3	52	21.4
4	66	10.2
Ki8751 (positive control)	2.4	1.4

Table 1. ^{1}H and ^{13}C NMR Data for Compounds 1–3 [δ_H and δ_C (ppm), mult. (J in Hz)]

	polyozellic acid (**1**)		thelephoric acid (**2**)		acetone adduct (**3**)	
position	δ_C, multiplicity	δ_H (J in Hz)	δ_C, multiplicity	δ_H (J in Hz)	δ_C, multiplicity	δ_H (J in Hz)
1	104.6, CH	7.23, s	104.9, CH	7.34, s	104.3, CH	7.09, s
2	145.7, C		146.5, C		143.5, C	
3	146.5, C		149.4, C		145.9, C	
4	98.9, CH	7.12, s	98.6, CH	7.13, s	98.4, CH	7.07, s
4a	150.2, C		151.4, C		150.1, C	
5a	151.0, C		150.9, C		138.6,[a] C	
5b					152.9,[a] C	
6a					148.6, C	
7					98.7, CH	7.12, s
8					144.9,[b] C	
9					144.5,[b] C	
10					104.7, CH	7.26, s
10a					114.8, C	
10b					111.0, C	
11					194.8, C	
12	175.7, C		172.5, C		72.8, C	
12a	114.9, C		121.7, C		125.9, C	
12b	115.2, C		114.0, C		117.3, C	
13					53.2, CH_2	3.49, d (16.7)
						3.56, d (16.7)
14					205.0, C	
15					30.2, CH_3	1.97, s
13-OH						6.24, brs
2, 3, 8, 9-OH						9.38, brs

[a]May be interchanged. [b]Overlapping signals.

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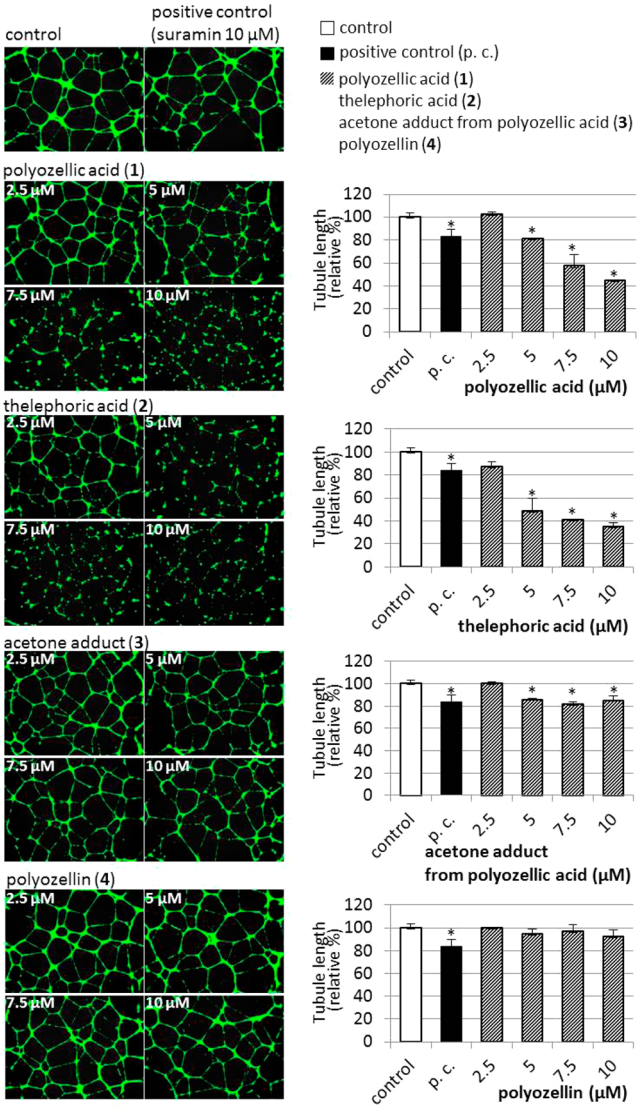

Figure 3. Polyozellic acid (**1**) and thelephoric acid (**2**) dose dependently suppressed tubule formation in HUVECs. Bars represent the means ± SD (n = 3). *p < 0.05 relative to control.

and **2** strongly inhibited HUVEC invasion at a concentration of 2.5 μM.

In summary, we isolated compounds **1**−**3**, showing inhibitory activity toward VEGFR2 tyrosine kinase and cell proliferation in HUVECs. In a HUVEC tubule formation assay, compounds **1** and **2** suppressed the formation of tubule-like structures in a dose-dependent manner. Furthermore, **1** and **2** also markedly inhibited cell invasion. These results suggested that for antiangiogenesis effects a quinone moiety within the structure might play an essential role. As a possible mechanism of action, the compounds might interact with VEGFR2 and inhibit its activation, which is mediated by tyrosine autophosphorylation. Additional studies clarifying the detailed mechanisms controlling antiangiogenic activity by **1** and **2** are in progress. As a result, our study provided new findings that natural *p*-terphenyl derivatives from mushrooms have potential antiangiogenesis activity.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined on a Yanaco MP apparatus (Yanaco, Kyoto, Japan). UV spectra were recorded with a Shimadzu UV-240 spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were recorded with a Jasco IR Report-100 spectrophotometer (Jasco, Tokyo, Japan). ^{1}H NMR and ^{13}C NMR spectra were measured with JNM-AL400 MHz and JNM-LA500 MHz spectrometers (JEOL, Tokyo, Japan) using tetramethylsilane as the internal standard. Low- and high-resolution EIMS and FABMS spectra were measured with a JEOL JMS-700 spectrometer (JEOL, Tokyo, Japan). Column chromatography was performed using silica gel 60N (Kanto Chemical, Tokyo, Japan) and Sephadex LH-20 media (GE Healthcare, Little Chalfont, UK). HPLC was performed using a JASCO PU2080 PLUS pump equipped with a JASCO PU2075 PLUS detector (Jasco, Tokyo, Japan), with a Senshu Pak PEGASIL SP100 column (10 ⌀ × 250 mm, no. 0811077H) (Senshu Scientific, Tokyo, Japan).

Fungal Material. Fruiting bodies of *Polyozellus multiplex* were collected in Yamanashi Prefecture, Japan, in September 2010 and 2011. A voucher specimen (KI-2010) was deposited at the Department of Pharmacognosy and Phytochemistry, Meiji Pharmaceutical University. Species identification was confirmed by one of the authors (K. Koyama).

Extraction and isolation. Dried and fractured fruiting bodies of *P. multiplex* (5.2 g) were extracted two times each with $CHCl_3$, MeOH, and then acetone at room temperature. The acetone extract exhibited

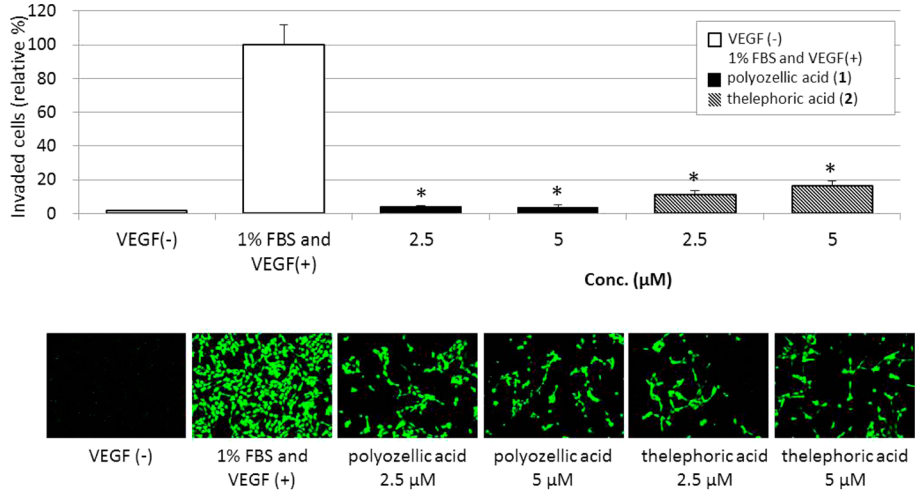

Figure 4. Polyozellic acid (**1**) and thelephoric acid (**2**) inhibited HUVEC invasion. Bars represent the means ± SD (n = 3). *p < 0.05 relative to control [1% FBS and VEGF(+)].

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75% VEGFR2 kinase inhibition at 100 μg/mL. The acetone extract was chromatographed on a silica gel column with a solvent system consisting of $CHCl_3$–MeOH (100:1 to 0:100) followed by acetone to yield eight fractions. Fraction 5 was chromatographed using four successive columns: [1] step-gradient silica gel with a solvent system consisting of $CHCl_3$–MeOH (20:1 to 0:100) followed by acetone; [2] step-gradient silica gel with a solvent system consisting of $CHCl_3$–EtOAc (7:1 to 1:2) followed by MeOH; [3] Sephadex LH-20 with MeOH; and [4] Sephadex LH-20 with MeOH to yield the acetone adduct (**3**) (12.5 mg). Fraction 6 was dissolved with MeOH, affording polyozellic acid (**1**) (insoluble part) (110.5 mg). Fraction 7 was also dissolved with MeOH, affording thelephoric acid (**2**) (insoluble part) (95.0 mg).

*Polyozellic acid (**1**):* black powder; mp >250 °C (dec); UV (EtOH) λ_{max} (log ε) 339 (3.82), 327 (4.28), 319 (4.19), 274 (4.01), 270 (4.00), 243 (3.93), 217 (4.27), 209 (4.23) nm; IR (KBr) ν_{max} 3375, 1645, 1624, 1601, 1463, 1285 cm^{-1}; ^1H NMR (DMSO-d_6), see Table 1; ^{13}C NMR (DMSO-d_6), see Table 1.

*Thelephoric acid (**2**):* black powder; mp >250 °C (dec); UV (EtOH) λ_{max} (log ε) 324 (3.85), 306 (4.14), 265 (4.10), 217 (4.25) nm; IR (KBr) ν_{max} 3410, 3250, 1625, 1522, 1265, 1250 cm^{-1}; ^1H NMR (DMSO-d_6), see Table 1; ^{13}C NMR (DMSO-d_6), see Table 1.

*Acetone adduct from polyozellic acid (**3**):* reddish-brown powder; mp 133 °C (dec); UV (MeOH) λ_{max} (log ε) 449 (4.04), 353 (3.60), 309 (4.09), 267 (4.04), 214 (4.33) nm; IR (KBr) ν_{max} 3395, 1635, 1595, 1480, 1362, 1332, 1290 cm^{-1}; ^1H NMR (DMSO-d_6), see Table 1; ^{13}C NMR (DMSO-d_6), see Table 1; FABMS *m/z* 411, 277, 185, 93, 75, 57; HR-FABMS *m/z* 411.0722 $[M + H]^+$ (calcd for $C_{21}H_{14}O_9$, 411.0716).

*Polyozellin (**4**):* pale brown powder; mp >250 °C; UV (MeOH) λ_{max} (log ε) 214 (4.43), 225 (4.45), 246 (4.38), 279 (4.18), 331 (4.49), 346 (4.65) nm; IR (KBr) ν_{max} 3391, 3250, 1780, 1749, 1618, 1200 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 2.57 (6H, s, $-OCOCH_3$), δ 7.19 (2H, s, H-1, 7), δ 7.19 (2H, s, H-4, 10), δ 9.35 (2H, brs, $-OH$), δ 9.75 (2H, brs, $-OH$); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 20.3 ($-OCOCH_3$), 98.8 (C-4, 10), 105.7 (C-1, 7), 112.7 (C-6b, 12b), 116.6 (C-6a, 12a), 130.6* (C-6, 12), 136.8* (C-5a, 11a), 143.2 (C-2, 8), 146.9 (C-3, 9), 150.1 (C-4a, 10a), 168.2 ($-OCOCH_3$), *may be interchanged; FABMS *m/z* 439; HR-FABMS *m/z* 439.0670 $[M + H]^+$ (calcd for $C_{21}H_{14}O_9$, 439.0665).

X-ray Crystallographic Data of Acetone Adduct (3). The acetone adduct (**3**) was crystallized from *n*-hexane–acetone to give brown needles. Crystal data: $C_{21}H_{14}O_9$, 0.5 (C_6H_{14}), 2 (C_3H_6O) (*n*-hexane and acetone disordered), space group $P2_1/c$ (#14), a = 10.8156(2) Å, b = 19.5038(4) Å, c = 13.286(1) Å, β = 93.594(7)°, V = 2797.2(2) Å^3, Z = 4, D_{calc} = 1.352 g/cm^3, R = 0.0581, wR2 = 0.1472. Crystallographic data for **3** reported in this paper have been deposited at the Cambridge Crystallographic Data Centre under reference number CCDC 969106. The data can be obtained free of charge. The crystal of acetone adduct (**3**) was cleared as a racemate by X-ray analysis. Refer to the Supporting Information for detailed data.

Derivatization of Polyozellic Acid (1). Polyozellic acid (**1**) (4.5 mg), acetic anhydride (3 mL), and H_2SO_4 as a catalyst were stirred at room temperature for 24 h. The reaction was quenched with a saturated solution of sodium hydrogen carbonate ($NaHCO_3$), and the products were extracted into $CHCl_3$. The organic layer was washed with a saturated solution of sodium chloride and dried over anhydrous Na_2SO_4. Filtration, concentration, and purification by silica gel column chromatography ($CHCl_3$) afforded 6.4 mg (50%) of **6** as a purple solid. Subsequently the reaction was carried out by adding gradually an ethereal solution of diazomethane to **6** (12.3 mg) and stirring at room temperature for 24 h. The final products were obtained by evaporation of the solvent and then separated by silica gel column chromatography ($CHCl_3$) and HPLC (*n*-hexane–$CHCl_3$) as **7** (3.7 mg, 29%).

*Polyozellic acid acetate (**6**):* black-purple powder; mp >250 °C; UV ($CHCl_3$) λ_{max} (log ε) 238 (4.09), 268 (4.39), 296 (4.54), 307 (4.60), 353 (3.71) nm^{-1}; IR (KBr) ν_{max} 1765, 1670, 1450, 1370, 1275, 1205 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 2.35 (6H, s, $-OCOCH_3$), 2.36 (6H, s, $-OCOCH_3$), 7.59 (2H, s, H-4, 7), 7.93 (2H, s, H-1, 10); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.5 ($-OCOCH_3$), 20.7 ($-OCOCH_3$), 108.1 (C-4, 7), 116.1 (C-1, 10), 116.5 (C-10b, 12a), 121.4 (C-10a, 12b), 141.5 (C-2, 9), 142.2 (C-3, 8), 153.4* (C-4a, 6a), 153.6* (C-5a, 5b), 167.9 (-$OCOCH_3$), 168.2 (-$OCOCH_3$), 174.7 (C-11, 12), *may be interchanged; FABMS *m/z* 521 $[M + H]^+$, 492, 450, 408, 366.

*11,12-(Methylenedioxy)polyozellic acid acetate (**7**):* colorless powder; mp >250 °C; UV ($CHCl_3$) λ_{max} (log ε) 248 (4.39), 271 (4.23), 280 (4.33), 309 (4.45), 322 (4.67), 346 (3.75), 365 (3.62) nm; IR (KBr) ν_{max} 1760, 1460, 1425, 1370, 1260, 1210, 1155, 1105 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.36 (6H, s, $-OCOCH_3$), 2.36 (6H, s, $-OCOCH_3$), 6.31 (2H, s, $-OCH_2O-$), 7.51 (2H, s, H-4, 7), 7.80 (2H, s, H-1, 10); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.6 ($-OCOCH_3$), 20.7 ($-OCOCH_3$), 103.3 ($-OCH_2O-$), 107.2 (C-4, 7), 108.6 (C-10b, 12a), 116.2 (C-1, 10), 120.0 (C-10a, 12b), 136.2* (C-11, 12), 136.3* (C-5a, 5b), 138.6 (C-2, 9), 141.6 (C-3, 8), 153.9 (C-4a, 6a), 168.3 ($-OCOCH_3$), 168.6 ($-OCOCH_3$), *may be interchanged; FABMS *m/z* 534 $[M]^+$, 492, 450, 408, 366; HR-FABMS *m/z* 534.0805 $[M]^+$ (calcd for $C_{27}H_{18}O_{12}$, 534.0797).

Reductive Acetylation of Thelephoric Acid (2). Thelephoric acid (**2**) (4.8 mg) was reductively acetylated using acetic anhydride (3 mL) in the presence of Zn and refluxed at 50 °C for 24 h. The end point of the reaction was monitored by TLC, and then the mixture was filtered, quenched with a saturated solution of sodium hydrogen carbonate ($NaHCO_3$), extracted with $CHCl_3$, washed with a saturated solution of sodium chloride, and dried over anhydrous Na_2SO_4. Filtration, concentration, and silica gel column chromatography ($CHCl_3$) gave 2 mg (24%) of **8** as a colorless solid.

*Thelephoric acid acetate (**8**):* colorless powder; mp >250 °C; UV ($CHCl_3$) λ_{max} (log ε) 327 (4.60), 313 (4.48), 277 (4.23), 266 (4.12), 243 (4.46), 237 (4.11) nm; IR (KBr) ν_{max} 1770, 1455, 1365, 1205 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.36 (12H, s, 2, 3, 8, 9-$OCOCH_3$), 2.51 (6H, s, 6, 12-$OCOCH_3$), 7.60 (2H, s, H-4, 10), 7.66 (2H, s, H-1, 7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.5 ($-OCOCH_3$), 20.7 ($-OCOCH_3$), 20.8 ($-OCOCH_3$), 107.7 (C-4, 10), 115.6 (C-1, 7), 118.1 (C-6b, 12b), 120.6 (C-6a, 12a), 132.5* (C-6, 12), 139.0** (C-2, 8), 139.1** (C-3, 9), 142.0* (C-5a, 11a), 153.7 (C-4a, 10a), 167.5 ($-OCOCH_3$), 168.0 ($-OCOCH_3$), 168.4 ($-OCOCH_3$), *may be interchanged, **may be interchanged; EIMS *m/z* (rel int %) 606 $[M]^+$ (14), 438 (100); HR-EIMS *m/z* 606.1013 $[M]^+$ (calcd for $C_{30}H_{22}O_{14}$, 606.1009).

Acetylation of Polyozellin (4). Acetylation according to the above procedure (acetylation of **1**) using polyozellin (**4**) (5.6 mg) in acetic anhydride (3 mL) with 4-pyrrolidinopyridine as a catalyst afforded 7.4 mg (97%) of **8** as a colorless solid. The structure of the product was characterized by its spectral data.

VEGFR2 Tyrosine Kinase Assay. VEGFR2 tyrosine kinase activity was assayed in 96-well plates using a universal tyrosine kinase assay kit (Takara Bio, Shiga, Japan) and recombinant VEGFR2 (Millipore, Billerica, MA, USA) according to a previous report.[5] Ki8751 (Millipore), VEGFR2 inhibitor, was used as positive control.

Cell Culture. HUVECs were purchased from Lonza Group, Lmtd., and routinely cultured using an EGM-2MV BulletKit (Lonza, Walkersville, MD, USA) at 37 °C in 5% CO_2. HUVECs from the second to eighth passages were used for experiments.

Cell Proliferation Assay. HUVECs (3×10^3 cells/well) were seeded in collagen-coated 96-well plates with EGM-2 and allowed to adhere for 12 h at 37 °C in 5% CO_2. The medium was removed and replaced with EGM-2 excluding VEGF and basic fibroblast growth factor and incubated for 12 h at 37 °C in 5% CO_2. VEGF (1 nM) and test compounds were added to each well and incubated for 72 h at 37 °C in 5% CO_2. Then 10 μL of WST-8 reagents was added to each well, and cells were incubated at 37 °C in 5% CO_2 for 2 h. The absorbance was measured at 450 nm using a plate reader. Ki8751 (Millipore), VEGFR2 inhibitor, was used as positive control.

Tubule Formation Assay on Matrigel. Matrigel matrix (BD Biosciences, NJ, USA) was added to 96-well plates with a volume of 27 μL in each well and incubated for 1 h at 37 °C to polymerize. HUVECs were plated at a density of 1.6×10^4 cells on the Matrigel with various concentrations of **1**–**3** (2.5, 5, 7.5, and 10 μM) or suramin as a positive control (10 μM). After a 16 h incubation in a 5%

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CO_2 atmosphere at 37 °C, cells were stained by calcein-AM. Images of tubule-like structures were taken using a fluorescence microscope at 4× magnification. Tubule-like structures were quantified by measuring the total tubule length using Angiogenesis Image Analyzer software (KURABO, Osaka, Japan).

Invasion Assay. FluoroBlok 24-well inserts (BD Biosciences) were coated with 0.2 mg/mL of Matrigel in a chamber for 1 h at 37 °C to polymerize. HUVECs were incubated for 5 h in serum-free EBM-2 containing 0.2% BSA. The starved cells were trypsinized and suspended in serum-free EBM-2 containing 0.2% BSA with compound **1** or **2** (2.5 and 5 μM). Cell suspensions were added to the Matrigel-coated inserts at a density of 1×10^5 cells in a volume of 0.25 mL. The bottom chambers were filled with 0.75 mL of EBM-2 containing 0.2% BSA, 20 ng/mL VEGF, 1% FBS, and individual test samples. After 22 h incubation in a 5% CO_2 atmosphere at 37 °C, the cells were labeled with calcein-AM and quantified by a fluorescence plate reader at 494/517 nm. Images of invaded cells were taken under a fluorescence microscope.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H, ^{13}C, HMQC, and HMBC spectra of compound **1**−**7**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +81-42-495-8913. Fax: +81-42-495-8912. E-mail: kiyotaka@my-pharm.ac.jp.

Notes

The authors declare no competing financial interest.

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