Phenylnaphthalene Compounds from the Subterranean Part of *Vitex rotundifolia* and Their Antibacterial Activity Against Methicillin-Resistant *Staphylococcus aureus*

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A careful investigation of the subterranean part of *Vitex rotundifolia* has shown that this plant contains five novel lignans having a 1-phenylnaphthalene-type skeleton together with four known lignans. These structures were elucidated on the basis of spectroscopic data. Furthermore, some of the isolated compounds showed antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA).

Vitex rotundifolia L.f (Verbenaceae) grows at the seaside areas of Japan and Southeast Asia. Its fruit ("Mankeishi" in Japanese) has been used as folk medicine in China and Japan for curing colds and other inflammation. From the fruits, some iridoids and diterpenoids have been isolated,[1,2] and from its leaf, an iridoid having insect repellent activity was reported.[3] There are no reports of a chemical study on the subterranean part of this plant, except for our previous paper in which we reported three phenylnaphthalene-type lignans (**1**−**3**).[4] In this paper, the structural elucidation of five new phenylnaphthalene-type lignans, vitrofolal D (**4**), vitrofolal E (**5**), vitrofolal F (**6**), **7**, and **8**, are described. Further, the antibacterial activity of these compounds against methicillin-resistant *Staphylococcus aureus* (MRSA) and a hypothetical biosynthetic pathway are also explained.

Results and Discussion

The dried subterranean part of *V. rotundifolia* was extracted with MeOH, and the MeOH extract was partitioned between AcOEt and water to obtain an AcOEt extract. By purification of the AcOEt extract with column chromatography and HPLC, compounds **1**−**9** (Chart 1) were isolated.

Compounds **1**−**3**, which are vitrofolal A, B, and C, respectively, have been reported previously.[4]

Vitrofolal D (**4**) is a red, amorphous solid. It showed strong UV absorption at 259 nm, indicating a condensed ring, and an IR absorption at 1684 cm^{-1}, which was attributed to a carbonyl group. The high-resolution MS of **4** revealed a molecular formula of $C_{21}H_{16}O_6$. From the ^{13}C NMR spectrum of **4**, 16 aromatic carbons were observed along with two carbonyl carbons and three methoxyl groups. There were no high-field signals in the ^1H NMR spectrum. The ^1H and ^{13}C NMR spectral patterns indicated that the structure of **4** was very similar to that of vitrofolal C (**3**), with the exception that a signal (δ_C 73.0) for a carbon bearing a hydroxy group in the ^{13}C NMR spectrum of **3** was replaced by a carbonyl carbon signal at δ_C 194.0 in that of **4** (Table 1). In the HMBC spectrum of **4**, H-8 showed long-range correlation to the carbonyl carbon. NOE correlations between a formyl proton (δ 11.65) and H-5, and between H-5 and H-4, when considered together with the ^1H NMR spectrum, suggested that the substitution pattern for the naphthalene ring in **4** was the same as observed for **3**. Consequently, vitrofolal D was deduced to be 2-hydroxy-1,9,10-trimethoxy-7-benzo[*c*]fluorenone-6-carbaldehyde, as shown in structure **4**. Compounds **3** and **4** are the first examples of naturally occurring compounds with the benzo[*c*]fluorene moiety.

Table 1. ^{13}C NMR Spectral Data for **3** and **4** in Pyridine-d_5 (δ_C)

position	**3**	**4**
1	141.0	140.6
2	151.8	151.9
3	119.7	121.8
4	128.2	128.6
4a	129.5	132.0
5	131.3	127.8
6	128.0[a]	128.0
6a	146.1	131.5
7	73.0	194.0
7a	127.8[a]	140.8
8	107.8	97.2
9	148.4	148.7
10	148.7	154.1
11	110.2	110.9
11a	132.4	139.3
11b	140.2	125.8
11c	126.3	125.6
6-CHO	192.1	189.9
1-OMe	60.3	60.6
9-OMe	54.8	54.8
10-OMe	54.8	54.8

[a] Assignments may be reversed.

Vitrofolal E (**5**) also shows UV absorption assignable to a condensed ring. The molecular formula was determined to be $C_{19}H_{16}O_5$ by HRMS. The presence of a carbonyl function was suggested from the IR spectrum. In the ^{13}C NMR spectrum, its spectral pattern closely resembled that of vitrofolal A (**1**), suggesting vitroforal E has the same framework as **1** (Table 2). The ^1H NMR spectrum shows two hydroxyl groups (δ 6.16 and 5.74) and two methoxyl groups that show NOE correlations with H-5 and 2′, respectively. Furthermore, two singlet signals (δ 7.33 and 7.42) were present instead of *ortho*-coupled signals in compound **1**. A 1,3,6,7-tetrasubstituted naphthalene ring was revealed by the NOE correlation of the proton at δ 7.42 (H-8) with H-2′ and H-6′. From consideration of the above data, the structure of vitrofolal E (**5**) was established as 1-(4-hydroxy-3-methoxyphenyl)-7-hydroxy-6-methoxynaphthalene-3-carbaldehyde.

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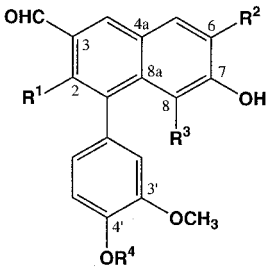
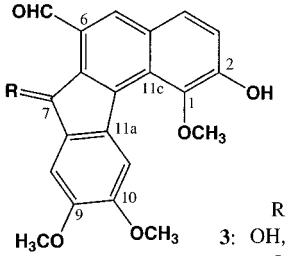
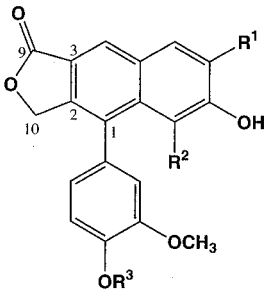
Chart 1



	R^1	R^2	R^3	R^4
1:	H	H	OCH_3	CH_3
2:	OH	H	OCH_3	CH_3
5:	H	OCH_3	H	H
6:	OH	OCH_3	H	H



	R
3:	OH, H
4:	O



	R^1	R^2	R^3
7:	H	OCH_3	CH_3
8:	OCH_3	H	CH_3
9:	OCH_3	H	H

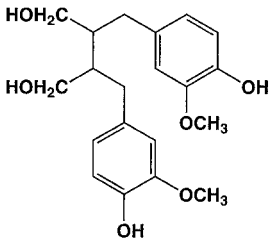


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Table 2. ^{13}C NMR Spectral Data for **1**, **5**−**8**, and **9** (δ_C)

position	**1**[a]	**5**[a]	**6**[b]	**7**[a]	**8**[a]	**9**[b]
1	137.3	139.9	122.0	129.8	132.3	132.4
2	127.2	122.8	151.6	141.5	137.8	138.3
3	131.5	132.1	119.8	120.9	121.1	121.3
4	134.5	131.7	133.7	127.2	124.4	124.4
4a	129.9	128.8	121.5	130.8	129.6	130.1
5	128.4	107.5	107.6	128.5	107.2	108.7
6	118.3	147.8	147.9	118.7	147.9	150.9
7	150.2	148.5	152.1	149.8	148.4	151.9
8	141.0	108.7	107.6	140.8	107.7	109.2
8a	128.8	132.1	135.0	129.2	132.3	133.2
9				171.3	171.7	172.2
10				69.9	69.6	70.0
1′	134.6	132.1	125.9	130.6	128.5	127.9
2′	113.7	112.4	114.3	112.9	112.3	123.7
3′	147.7	146.5	147.6	148.3	149.3	149.2
4′	148.4	145.3	146.5	148.6	149.0	148.4
5′	109.9	114.4	115.5	110.3	111.6	117.1
6′	122.0	122.9	125.9	120.9	121.7	123.0
3-CHO	191.9	192.3	195.4			
6-OMe		56.1	54.7		56.2	56.0
8-OMe	61.6			61.6		
3′-OMe	56.0	56.1	54.7	56.1	56.0	56.2
4′-OMe	56.1			55.9	56.0	

[a] Spectra were recorded in $CDCl_3$. [b] Spectra were recorded in pyridine-d_5.

Table 3. Anti-MRSA Activity of Compounds **3**, **4**, **5**, and **9**

	MIC (μg/mL)			
strain no.	**3**	**4**	**5**	**9**
MRSA0001	>64	>64	>64	>64
0002	64	16	>64	8
0003	>64	32	>64	8
0004	>64	>64	>64	>64
0005	64	16	>64	8
0006	64	16	>64	8
0007	64	16	>64	16
0008	64	16	>64	8
0009	>64	>64	>64	>64
0012	>64	>64	>64	>64
0013	>64	>64	>64	>64
0016	64	16	>64	16
0017	>64	>64	>64	>64
0018	>64	>64	>64	>64
0019	>64	>64	>64	>64
0020	64	16	64	4
0021	>64	>64	>64	>64
0022	>64	>64	>64	>64

The molecular formula of vitrofolal F (**6**) was revealed as $C_{19}H_{16}O_6$. This compound shows NMR and UV spectra similar to those of vitrofolal E (**5**), except that in the ^1H NMR spectrum of **6** the signal for the proton corresponding to H-2 in **5** was absent and H-4 appeared as a singlet. An NOE correlation of the formyl proton with H-4 was evident, as was a long-range correlation with C-2 (δ_C 151.6). From consideration of the molecular formula and the spectral data, the structure of **6** was proposed as shown.

Although compound **7** has an NMR spectral pattern similar to that of vitrofolal A (**1**), the formyl group is absent. In the IR spectrum, an absorption for a lactone at 1757 cm^{-1} was observed. From these facts, it was presumed that compound **7** has the same skeleton as detetrahydroconidendrin (**9**).[5] In comparing the ^{13}C NMR signals of **7** with those of **1**, the signals of C-1, -2, -3, and -4 were altered remarkably, although the substitution pattern of methoxyl and hydroxy groups was the same as **1**, based on NOE correlation data. The long-range correlation of C-9 to H_2-10 and H-4, and C-2 to H_2-10, supported the presence of a lactone moiety, and the NOE correlation between H_2-10 and H-2′, -6′ indicated that the lactone ring was attached as shown.

Compound **8** has the same molecular formula and functional groups as **7** and thus is a structural isomer of **7**. The ^1H NMR spectrum of **8** was very similar to that of detetrahydroconidendrin (**9**), except that a methoxyl group was lacking. Consequently, the structure of compound **8** was revealed as shown and confirmed by 2D NMR data.

The antimicrobial activity of compounds **1**−**9** against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) ATCC 6538 was determined. For the evaluation, 18 strains of MRSA and six strains of MSSA were used. Using an agar disk diffusion test, we found that none of the compounds had effective activity against MSSA. However, in similar screening against MRSA, compounds **3**, **4**, and **9** showed antibacterial effect against eight out of 18 strains. The minimum inhibitory concentration (MIC) of these compounds was determined to be less than 64 μg/mL (Table 3).

MRSA is resistant to all β-lactams, penicillins, cephalosporins, carbapenem, and penems, because of its *mecA* gene, which produces an additional penicillin-binding

protein, called PBP2′ (or 2a), that has a low affinity for β-lactam antibiotics.[6,7] Since there is no such gene in MSSA, these strains are sensitive to β-lactams. We have shown that compounds **3**, **4**, and **9** possess antibacterial activity only against strains of MRSA and are inactive against MSSA. We could find no other report of a natural product showing selective anti-MRSA activity, nor have we observed such a remarkable difference in the activities specific to resistant vs sensitive strains in any of our previous studies.[8−10] Studies are underway in our laboratories to elaborate possible explanations for this observation.

With regard to the biosynthesis of the isolated compounds, the following hypothesis is proposed.[11] Coupling of two coniferyl alcohols can yield secoisolariciresinol (**10**) via pinoresinol. Depending on the direction of cyclization of **10**, two regioisomeric series will result. Sequential oxidation can lead to the unsaturated ring and elimination of the CO_2 moiety to give rise to the basic skeleton of compounds **1**, **2**, **5**, and **6**. On the other hand, the benzofuran can be constructed from the bonding of the carbonyl carbon with another aromatic ring after formation of the naphthalene part of the molecule.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were acquired on a Bruker ARX-400 (^{1}H, 400 MHz; ^{13}C, 100 MHz), and all chemical shift values are given in ppm. ^{1}H NMR spectra were referenced to TMS, and ^{13}C NMR spectra were referenced to solvent signals with resonances at δ_C 77.1 ($CDCl_3$) and 135.5 (pyridine-d_5), relative to TMS. Column chromatography used Si gel 60 (230−400 mesh, Merck). Si gel HPLC, ODS HPLC, and GPC (Gel Permeation Chromatography) employed LiChrosorb Si 60 (7 mm, 250 × 20 mm, Merck), LiChrosorb RP-18 (7 mm, 250 × 20 mm, Merck), and H-2001 (1000 × 20 mm, Shodex), respectively.

Plant Material. *V. rotundifolia* was collected in 1997 at Tosa-domari beach in Tokushima Prefecture, Japan, and identified by Dr. K. Murakami, The University of Tokushima. Voucher specimens are deposited in the herbarium in the Faculty of Pharmaceutical Sciences, The University of Tokushima.

Extraction and Isolation. The isolation of vitrofolals A (**1**), B (**2**), and C (**3**) has been described previously.[4]

The subterranean part of *V. rotundifolia* was washed, then dried for 1 month at room temperature. The cut, dried plant material (12.1 kg) was extracted in MeOH with reflux to yield the MeOH extract, and it was partitioned between EtOAc and water to obtain an EtOAc-soluble part (106 g). This fraction was separated on a Si gel column eluted with $CHCl_3$−MeOH (4:1) to give fractions 1−9. Fraction 2 (19.9 g) was applied to a Si gel column that was eluted with hexane−AcOEt (4:1), following by benzene−acetone (10:1) to yield fractions 2.1−2.3. Fraction 2.3 was suspended in MeOH and filtered through filter paper (No. 2, Advantec), and the residue was washed several times with MeOH to afford vitrofolal D (**4**, 27.4 mg). The filtrate was combined with the washings and purified by HPLC (Si gel) eluting with $CHCl_3$−MeOH (4:1), hexane−AcOEt (4:1), and benzene−acetone (10:1) to obtain compounds **7** (6.9 mg) and **8** (5.1 mg). Fraction 3 (11.2 g) was separated by Si gel column chromatography eluting with hexane−AcOEt (1:1), to obtain fractions 3.1−3.4. Fraction 3.4 (1.6 g) was further chromatographed over Si gel, with $CHCl_3$−MeOH (100:1) as eluent, to yield fractions 3.4.1 and 3.4.2. Fraction 3.4.1 was purified by GPC ($CHCl_3$) and ODS HPLC (MeOH−water 5:1) to yield vitrofolal E (**5**, 13.1 mg) and F (**6**, 4.9 mg). The known compound detetrahydroconidendrin (**9**, 20.8 mg) was obtained from fraction 3.4.2.

Vitrofolal D (4): red amorphous solid; IR (KBr) ν_{max} 3600−2800, 2936, 1684, 1493, 1315, 1269, 1021 cm^{-1}; UV (dioxane) λ_{max} (log ϵ) 259 (4.2) nm; ^{13}C NMR data (see Table 1); ^{1}H NMR (pyridine-d_5) δ 11.65 (1H, s, 6-CHO), 8.46 (1H, s, H-5), 8.37 (1H, s, H-11), 7.81 (1H, d, J = 8.8 Hz, H-4), 7.63 (1H, d, J = 8.8 Hz, H-3), 7.42 (1H, s, H-8), 3.98 (3H, s, 10-OMe), 3.83 (3H, s, 1-OMe), 3.73 (3H, s, 9-OMe); HREIMS m/z 364.0930 [M]$^+$ (calcd for $C_{21}H_{16}O_6$, 364.0947).

Vitrofolal E (5): yellowish amorphous solid; IR (KBr) ν_{max} 3600−3000, 1684, 1508, 1270, 1211 cm^{-1}; UV (dioxane) λ_{max} (log ϵ) 320 (4.1), 267 (4.5) nm; ^{13}C NMR data (see Table 2); ^{1}H NMR ($CDCl_3$) δ 10.12 (1H, s, 3-CHO), 8.18 (1H, brs, H-4), 7.75 (1H, brs, H-2), 7.42 (1H, s, H-8), 7.33 (1H, s, H-5), 7.04 (1H, d, J = 7.9 Hz, H-5′), 6.16 (1H, s, 7-OH), 6.98 (1H, d, J = 7.9 Hz, H-6′), 6.97 (1H, s, H-2′), 5.74 (1H, s, 4′-OH), 4.09 (3H, s, 6-OMe), 3.93 (3H, s, 3′-OMe); HREIMS m/z 324.0981 [M]$^+$ (calcd for $C_{19}H_{16}O_5$, 324.0998).

Vitrofolal F (6): yellowish amorphous solid; IR (KBr) ν_{max} 3600−2800, 1652, 1509, 1287, 1211 cm^{-1}; UV (dioxane) λ_{max} (log ϵ) 333 (4.2), 269 (4.5), 239 (4.3) nm; ^{13}C NMR data (see Table 2); ^{1}H NMR (pyridine-d_5) δ 9.96 (1H, s, 3-CHO), 7.86 (1H, s, H-4), 7.14 (1H, s, H-8), 7.11 (1H, s, H-5), 6.99 (1H, d, J = 7.8 Hz, H-5′), 6.84 (1H, s, H-2′), 6.82 (1H, d, J = 7.8 Hz, H-6′), 3.60 (3H, s, 6-OMe), 3.34 (3H, s, 3′-OMe); HREIMS m/z 340.0939 [M]$^+$ (calcd for $C_{19}H_{16}O_6$, 340.0947).

Compound 7: brown amorphous solid; IR (KBr) ν_{max} 3600−2800, 2918, 1757, 1617, 1515, 1465, 1250, 1139, 1022 cm^{-1}; UV (dioxane) λ_{max} (log ϵ) 329 (3.6), 260 (4.4) nm; ^{13}C NMR data (see Table 2); ^{1}H NMR ($CDCl_3$) δ 8.43 (1H, s, H-4), 7.85 (1H, d, J = 8.9 Hz, H-5), 7.39 (1H, d, J = 8.9 Hz, H-6), 6.97 (1H, brs, H-5′), 6.97 (1H, s, H-6′), 6.90 (1H, brs, H-2′), 5.27, 5.11 (each 1H, ABq, J = 15.3 Hz, H_2-10), 3.98 (3H, s, 4′-OMe), 3.87 (3H, s, 3′-OMe), 3.18 (3H, s, 8-OMe); HREIMS m/z 366.1078 [M]$^+$ (calcd for $C_{21}H_{18}O_6$, 366.1103).

Compound 8: yellowish oil; IR (KBr) ν_{max} 3600−2800, 2922, 1761, 1622, 1516, 1488, 1258, 1141, 1025 cm^{-1}; UV (dioxane) λ_{max} (log ϵ) 319 (4.0), 257 (4.6) nm; ^{13}C NMR data (see Table 2); ^{1}H NMR ($CDCl_3$) δ 8.31 (1H, s, H-4), 7.32 (1H, s, H-5), 7.26 (1H, s, H-8), 7.03 (1H, d, J = 8.2 Hz, H-5′), 6.91 (1H, dd, J = 8.2, 1.7 Hz, H-6′), 6.86 (1H, d, J = 1.7 Hz, H-2′), 6.15 (1H, s, 7-OH), 5.23, 5.22 (2H, m, H_2-10), 4.09 (3H, s, 6-OMe), 3.98 (3H, s, 4′-OMe), 3.88 (3H, s, 3′-OMe); HREIMS m/z 366.1098 [M]$^+$ (calcd for $C_{21}H_{18}O_6$, 366.1103).

Preparation of Bacterial Cells. Strains of methicillin-resistant *Staphylococcus aureus* (MRSA) were obtained from Tokushima University Hospital as clinical isolates, and methicillin-sensitive *S. aureus* (MSSA ATCC 6538) strains were purchased from ATCC. After culturing all strains on Mueller-Hinton agar (Difco, Detroit, MI) at 37 °C for 24 h, the cells were resuspended in Mueller Hinton broth (Difco) to give 10^8 colony-forming units/mL; the resuspended cells were then incubated as described above.

Agar Plate Diffusion Assays. The incubated broth was diluted with the medium to obtain 10^5 colony-forming units/mL of MRSA and MSSA, and Mueller-Hinton agar plates was inoculated with it. Susceptibility disks (ϕ 6 mm, Whatman AA disks) were impregnated with 100 μg of the isolated compound and placed on the agar plate. After 20 h of incubation at 37 °C, the inhibition zones were measured; inhibition zones with diameters exceeding 10 mm were considered positive.

Determination of Minimum Inhibitory Concentration (MIC). MIC was determined using Mueller-Hinton agar according to the method described by the Japanese Society for Antimicrobial Chemotherapy (1981).[12] Cell suspensions (1×10^6 colony-forming units/mL) of MRSA were inoculated onto agar plates using a replicating device. Plates were read after 20 h incubation at 37 °C.

References and Notes

(1) Asaka, Y.; Kamikawa, T.; Kubota, T. *Chem. Lett.* **1973**, 937−940.
(2) Tada, H.; Yasuda, F. *Heterocycles* **1984**, *22*, 2203−2205.
(3) Watanabe, K.; Takasa, Y.; Matsuo, N.; Nishimura, H. *Biosci. Biothech. Biochem.* **1995**, *59*, 1979−1980.
(4) Kawazoe, K.; Yutani, A.; Takaishi, Y. *Phytochemistry* **1999**, *52*, 1657−1659.
(5) Kuo, Y. H.; Wu, T. R.; Cheng, M. C.; Wang, Y. *Chem. Pharm. Bull.* **1990**, *38*, 3195−3201.
(6) Murakami, K.; Tomasz, A. *J. Bacteriol.* **1989**, *171*, 874−879.

(7) Chambers, H. F.; Sachdeva, M. *J. Infect. Dis.* **1990**, *161*, 1170−1176.
(8) Sato, Y.; Oketani, H.; Singyouchi, K.; Ohtsubo, T.; Kihara, M.; Shibata, H.; Higuti, T. *Biol. Phram. Bull.* **1997**, *20*, 401−404.
(9) Sato, Y.; Oketani, H.; Yamada, T.; Singyouchi, K.; Ohtsubo, T.; Kihara, M.; Shibata, H.; Higuti, T. *J. Pharm. Pharmacol.* **1997**, *49*, 1042−1044.
(10) Sato, Y.; Suzaki, S.; Nishikawa, T.; Kihara, M.; Shibata, H.; Higuti T. *J. Ethnopharm.* **2000**, *72*, 483−488.
(11) Lewis, N. G.; Davin, L. B. In *Comprehensive Natural Products Chemistry: Lignans*; Barton, D. H. R., Nakanishi, K., Eds.; Elsevier Science: Oxford, UK, 1999; Vol. 1, pp 639−712.
(12) Japanese Society for Antimicrobial Chemotherapy (1981) Revised method for measuring minimum inhibitory concentration: *Chemotherapy (Tokyo)* **1981**, *29*, 76−79.

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