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New Anti-influenza Agents, FR198248 and Its Derivatives

II. Characterization of FR198248, Its Related Compounds and Some Derivatives

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We isolated FR198248 from the cultured broth of *Aspergillus terreus* No.13830 as a new anti-influenza agents. The structure of FR198248 was elucidated by several spectroscopic experiments as a novel tetrahydroxybenzaldehyde compound. Furthermore, we described the characteristics of FR198248, its related compounds and some derivatives.

The sialidase (neuraminidase) of influenza virus is involved in promoting the release of progeny virus from the surface of infected cells and is thought to enhance virus movement through the respiratory tract[1~4]. The selective sialidase inhibitor zanamivir (GG167) has been shown to inhibit infectivity of influenza virus *in vitro* and *in vivo*[5~8].

During studies on screening new viral sialidase inhibitors from microbial cultured broths, we found a fungus metabolite, FR198248, isolated from *Aspergillus terreus* No.13830. Its taxonomy, fermentation, isolation, physicochemical properties and biological activities have already been reported in the preceding paper[9]. We also investigated the characterization of its related compounds. Furthermore, we synthesized a series of derivatives of FR198248 to enhance anti-influenza activity. In this paper, we described the characteristics of FR198248, its related compounds and some derivatives.

Results and Discussion

Structure Elucidation of FR198248 (1)

FR198248 (**1**) was determined to have a molecular formula of $C_9H_{10}O_5$ on the basis of MS spectra, elemental analysis, ^1H NMR and ^{13}C NMR data. The ^{13}C NMR and DEPT data revealed the presence of six sp^2 quaternary carbons, one acetal methine, one oxygenated methylene and one methyl carbon, leaving four protons bonded to oxygen atoms. The presence of phenol was first inferred from a positive color reaction with $FeCl_3$ and the compound's acidic nature. In the ^1H NMR spectrum in DMSO-d_6 four exchangeable protons were clearly seen. Three broadened signals (δ_H 8.45, 8.15 and 8.10) were due to phenol hydroxyls and a doublet signal at 6.14 ppm was ascribable to a secondary alcohol. The doublet OH proton (J=6 Hz) was coupled to a methine proton at 6.27 ppm. This proton was directly connected to a carbon resonating at 99.9 ppm whose chemical shift was typical of an acetal carbon. The presence of the acetal substructure was further supported by formation of methanol adduct product (**2**) which was obtained by methanol treatment (see Experimental). Figure 2 shows the key HMBC correlations that allowed the assembly of the partial structures into the gross structure (**1**). From the above information, the structure of **1** was concluded to be 4-methyl-1,3-dihydro-2-benzofuran-1,5,6,7-tetraol. A full ^1H and ^{13}C assignment of FR198248 was listed in Table 1.

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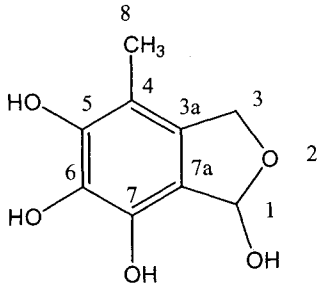
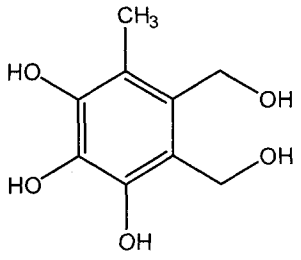
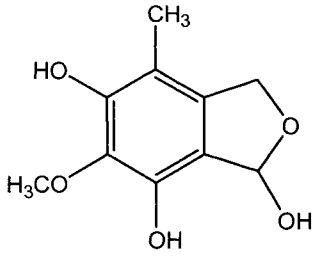
Fig. 1. Structures of FR198248 (**1**) and its related compounds.



FR198248 (**1**)



FR203655 (**3**)



Related compound **4**

Structure Elucidation of Related Compound **3**

A comparison of ^{1}H and ^{13}C NMR data of **3** with those of **1** indicated that **3** lacked the acetal and had generated a new methylene carbon. The molecular formula of $C_9H_{12}O_5$ (two hydrogens more than **1**) was consistent with the MS and ^{1}H and ^{13}C NMR data (see Experimental). The HMBC correlations of **3** are shown in Figure 2 together with **1**. The structural assignment of **3** was secured by the following chemical correlation. Reduction of **1** with $NaBH_4$ gave an alcohol whose NMR data was identical with that of **3**. Thus the structure of **3** was elucidated to be 2,3,4-trihydroxy-6-(hydroxymethyl)-5-methylbenzyl alcohol.

Structure Elucidation of Related Compound **4**

The molecular formula of $C_{10}H_{12}O_5$ was derived from ESI-MS and ^{1}H and ^{13}C NMR data. A methoxy signal was observed in the ^{1}H (δ 3.73 (3H, s)) and ^{13}C (δ 61.1) NMR spectra. The difference between **1** and **4** regarding ^{13}C NMR data in CD_3OD was found at C-3a, C-5, C-6 and C-7. This finding indicated that the position of attachment of the methoxy group was at C-6 and thus the structure of the related compound (**4**) was elucidated to be 6-methoxy-4-methyl-1,3-dihydro-2-benzofuran-1,5,7-triol.

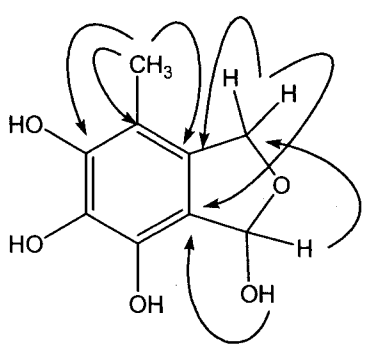
Chemical Modification

The trimethyl ether derivative of **1** (**5**) was obtained by the reaction of the phenolic hydroxyl groups of **1** with methyl iodide. The tetraacetate of **1** (**6**) was obtained by the reaction of **1** with acetic anhydride in anhydrous pyridine. Sodium borohydride was used for the source of hydride ion to reduce the carbonyl groups of **1**, to the reduction product of **1** (**3**). Furthermore, the pentaacetate of **3** (**7**) was synthesized by the reaction of **3** with Ac_2O in pyridine. The structures of these derivatives are shown in Figure 3.

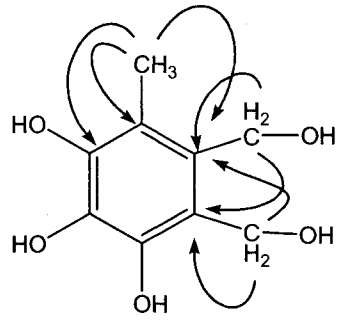
Biological Activities

The structure of FR198248 was elucidated as a novel tetrahydroxy benzaldehyde derivative and furthermore the

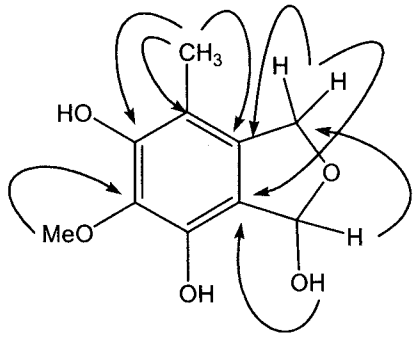
Fig. 2. Key HMBC correlations of **1**, **3** and **4**.



1



3



4

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR assignment of **1**.

Position	in DMSO-d_6		in CD_3OD	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	99.9	6.27 (br d, 6)	108.1	6.18 (d, 2)
1-OH		6.14 (d, 6)		
3	70.1	4.90 (dd, 12, 1)	73.0	5.00 (dd, 12, 2)
		4.67 (d, 12)		4.83 (d, 12)
3a	129.7	–	131.8	–
4	107.5	–	109.8	–
5	145.2	–	147.2	–
6	132.4	–	133.9	–
7	139.0	–	140.5	–
7a	117.8	–	115.7	–
8	11.9	1.90 (3H, s)	11.8	2.00 (3H, s)

Table 2. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR assignment of **4** in CD_3OD.

Position	δ_C	δ_H (J in Hz)
1	108.1	6.15 (d, 2)
3	72.9	5.01 (dd, 12, 2) 4.86 (d, 12)
3a	136.6	–
4	109.9	–
5	151.1	–
6	136.5	–
7	144.8	–
7a	115.8	–
8	11.7	1.99 (3H, s)

structures of other two related compounds were also determined. To analyze the importance of hydroxyl groups for the activity of this compound, we decided to modify those hydroxyl groups and synthesized five more derivatives. *In vitro* anti-influenza viral study was performed using plaque reduction procedure to estimate IC_{50} value of each compound. The test result and cellular toxicity of each compound against host, MDCK cells were summarized in Table 3. The selective index values calculated as CC_{50}/IC_{50} from each derivative were also indicated.

The antiviral activities of mono methylated products of **1** (**2** and **4**) obtained from this fungal cultured broths showed similar potency as **1**. This result means all hydroxyl groups are not necessary for the activity of **1**. To investigate activity in the case of modification to increase the number of hydroxyl group of **1**, we synthesized reduced form derivative and examined the activity against influenza virus. The obtained benzylalcohol derivative (**3**) showed slightly weaker activities than **1** but still retained antiviral activity. This result suggests the possibility that some hydroxylbenzylalcohol derivatives may also work as antiviral agent. However, either methylation (**5**) or acetylation (**6** and **7**) at the all positions of hydroxyl groups of **1** or **3** occurred drastic reduction of the activity. The important role of phenolic hydroxyl group is clear from these results, but further study is needed to identify the essential hydroxyl group position of **1**.

Some groups have reported about anti-influenza viral activities of natural products having multi-hydroxyl groups. Polyphenol compounds from plant leaf extracts is one of the example of them and the report showed that tea polyphenols had weak inhibitory activities of viral sialidase, but more potentially prevented infection of influenza virus to MDCK cells[10]. This phenomenon may correlate with the biological characteristics of FR198248. There is another report about small molecular compound named 10-norparvulenone exhibiting both sialidase inhibition and anti-influenza viral activity[14]. We also demonstrated unique antiviral polyphenolic compound, FR191512 in the previous report[15,16]. Over all, as these compounds have not common structural features, so their modes of actions remain to be solved.

In conclusion, the biological characteristics of these derivatives revealed so far, that phenolic hydroxyl groups of these compounds work as key role for activity *in vitro*. In the preliminary study, we reported that the mode of action of **1** could be ascribed to inhibit of virus adsorption level[9]. Further investigation is proceeding to clarify the structural and activity relationship of these compounds.

Experimental

General

EI-MS spectra were measured on a VG ZAB-SE mass spectrometer. ESI-MS (Electrospray Ionization MS) spectra were measured on a VG QUATTRO mass spectrometer. NMR spectra were acquired on a Bruker DRX500 or Varian Gemini300 spectrometer. IR spectra were recorded on a Jasco A-102 infrared spectrometer.

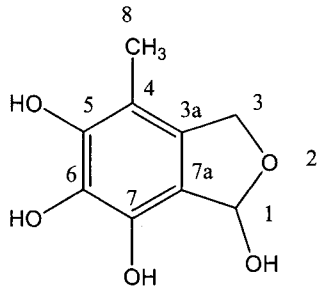
HPLC Analysis of FR198248

Detection of FR198248 from the fermentation broth and the fractions under purification were monitored by HPLC using a reverse phase column of YMC-ODS-AM (AM 303, 250×4.6 mm i.d., YMC Co.,Ltd.). The solvent system was a mixture of 10% aqueous methanol and 10% aqueous acetonitrile. The flow rate was 1.0 ml/minute. The detection wave length was set at 210 nm.

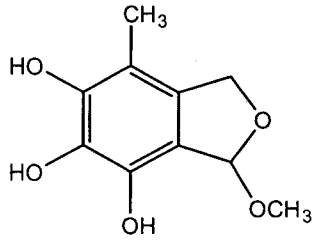
4-Methyl-1,3-dihydro-2-benzofuran-1,5,6,7-tetraol (1)

The isolation procedure was described in the previous paper. The product was obtained as a colorless powder: m.p. 112~117°C (dec.); IR (KBr) 3440, 3230, 1640, 1510,

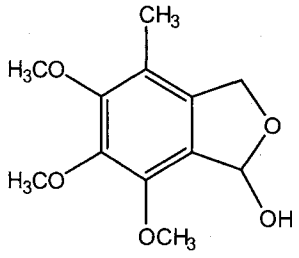
Fig. 3. Structures for derivatives of **1**.



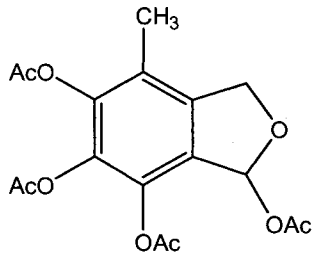
FR198248 (**1**)



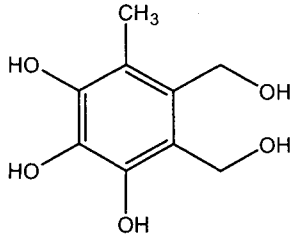
FR202306 (**2**)



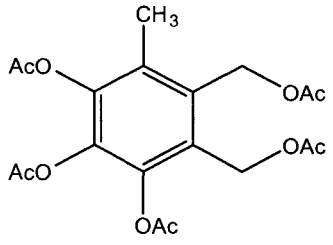
Trimethyl ether derivative of **1** (**5**)



Tetraacetate of **1** (**6**)



Reduction derivative of **1** (**3**)



Pentaacetate of **3** (**7**)

Table 3. Antiviral activities of FR198248 (**1**) and related compounds by plaque reduction assay.

Compound	IC_{50} (μM)	CC_{50} (μM)	SI[a]
1	11.0	500	45.5
2	14.0	270	19.3
3	39.2	250	6.38
4	11.2	490	43.8
5	CT[b]	240	---
6	265	370	1.40
7	285	371	1.30

[a] SI (Selectivity index) was calculated by the formulation of CC_{50} /(IC_{50} in plaque reduction assay)

[b] CT : Cytotoxicity was observed at the presence of 280 μM of the compound.

1490, 1430, 1400, 1300, 1110, 1010, 940, 920 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 6.18 (1H, d, J=2 Hz), 5.00 (1H, dd, J=12 and 2 Hz), 4.83 (1H, d, J=12 Hz), 2.00 (3H, s).; ^{13}C NMR (125 MHz, CD_3OD) δ 147.2 (s), 140.5 (s), 133.9 (s), 131.8 (s), 115.7 (s), 109.8 (s), 108.1 (d), 73.0 (t), 11.8 (q).; EI-MS *m/z* 180 $(M-H_2O)^+$; *Anal* Calcd for $C_9H_{10}O_5$: C 54.55, H 5.09; Found C 54.22, H 5.07.

1-Methoxy-4-methyl-1,3-dihydro-2-benzofuran-5,6,7-triol (**2**)

3 g of **1** was dissolved in anhydrous methanol (150 ml) and the mixture was filtered. The filtrate was concentrated to 50 ml and the solution was allowed to stand at 5°C overnight. The precipitate was collected and dried to give 1.8 g of **2** in pure form. Colorless prisms: m.p. 99~104°C (dec.); IR (KBr) 3490, 3340, 2940, 1640, 1510, 1500, 1400, 1370, 1300, 1260, 1120, 1080 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 8.57 (1H, s), 8.28 (1H, s), 8.16 (1H, s), 6.08 (1H, d, J=2 Hz), 4.88 (1H, dd, J=12 and 2 Hz), 4.76 (1H, d, J=12 Hz), 3.20 (3H, s), 1.91 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 145.7 (s), 139.3 (s), 132.4 (s), 130.1 (s), 114.6 (s), 107.7 (s), 106.2 (d), 71.3 (t), 52.9 (q), 11.9 (q); EI-MS *m/z* 180 $(M-MeOH)^+$; *Anal* Calcd for $C_{10}H_{12}O_5$: C 56.60, H 5.70; Found C 56.84, H 5.86.

2,3,4-Trihydroxy-6-(hydroxymethyl)-5-methylbenzyl-alcohol (**3**)

Compound **3** was obtained as a hygroscopic powder. : m.p. 102~107°C; IR (KBr) 3430, 3270, 1630, 1510, 1480, 1380, 1310, 1110, 1050 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 6.15 (1H, d, J=2 Hz), 5.01 (1H, dd, J=12 and 2 Hz), 4.86 (1H, d, J=12 Hz), 1.99 (3H, s).; ^{13}C NMR (125 MHz, CD_3OD) δ 145.2 (s), 138.9 (s), 134.0 (s), 130.0 (s), 117.0 (s), 110.3 (s), 74.3 (t), 73.3 (t), 12.2 (q); EI-MS *m/z* 182 $(M-H_2O)^+$.

6-Methoxy-4-methyl-1,3-dihydro-2-benzofuran-1,5,7-triol (**4**)

The isolation procedure was described as follows; The filtrate (2.5 liters) of the cultured broth of *Aspergillus terreus* No.13830 was passed through a column of Sepabeads SP-207 (Mitsubishi chemical Co., Ltd.) and eluted with 60% aqueous MeOH. After dilution of the eluate, the active solution was applied to a column of ODS-AM (ODS-AM 120-S50, YMC CO., Ltd.) and eluted with 30% aqueous MeOH. The eluate was concentrated *in vacuo* to dryness to give compound **4** as pale yellowish powder (5.7 mg). Obtained as a hygroscopic powder; IR (KBr) 3387, 2940, 1627, 1499, 1468, 1393, 1377, 1295, 1237, 1160 cm^{-1}; ^1H NMR and ^{13}C NMR data were shown in Table 2; ESI-MS *m/z* 195 $(M-H_2O+H)^+$.

Trimethyl Ether Derivative of **1** (**5**)

To a mixture of **1** (100 mg) and K_2CO_3 (142 mg) in DMF (1 ml) was added CH_3I (270 mg) and the mixture was heated at 50°C for 2 hours. The mixture was dried under a stream of nitrogen. The residue obtained was diluted with EtOAc and the mixture was washed with water and brine. The organic solution was evaporated to dryness and the residue was purified by silica gel column ($CHCl_3$-MeOH (50 : 1)) to give 8 mg of **5**: ^1H NMR (300 MHz, CD_3OD) δ 6.15 (1H, d, J=2 Hz), 5.01 (1H, dd, J=12 and 2 Hz), 4.86 (1H, d, J=12 Hz), 3.88 (3H, s), 3.80 (3H, s), 3.77 (3H, s), 1.99 (3H, s).

Tetraacetate of **1** (**6**)

To a solution of **1** (100 mg) in anhydrous pyridine (2 ml) was added acetic anhydride (1 ml) and the solution was allowed to stand at room temperature overnight. The mixture was dried to leave an oil. Purification of the oil by preparative TLC (Ethyl acetate-*n*-hexane (1 : 1)) gave 22 mg of an oil: ^1H NMR (300 MHz, CD_2Cl_2) δ 7.31 (1H, d, J=2 Hz), 5.23 (1H, dd, J=13 and 2 Hz), 5.07 (1H, J=13 Hz), 2.32 (3H, s), 2.27 (3H, s), 2.23 (3H, s), 2.08 (3H, s), 2.03 (3H, s); EI-MS *m/z* 306 $(M-AcOH)^+$.

Identification of **3** and $NaBH_4$ Reduction Product of **1**

To a solution of **1** (50 mg) in MeOH (5 ml) was added $NaBH_4$ (5 mg) and the mixture was stirred for 10 minutes at 5°C. The mixture was neutralized with addition of AcOH and diluted with water (100 ml) and applied to a column of HP-20. The column was washed with water and eluted with 50% aq. CH_3CN containing 0.05% TFA. The fraction obtained was lyophilized to afford 14 mg of the product whose ^1H NMR spectrum was superimposable with that of **3**.

Pentaacetate of **3** (**7**)

5 mg of **3** was dissolved in pyridine (0.5 ml) and acetic anhydride (0.5 ml) and the mixture was allowed to stand at room temperature overnight. The mixture was dried and the oil was separated by preparative TLC (Ethyl acetate-*n*-hexane (1 : 1)) to give 4 mg of **6**: ^1H NMR (300 MHz, CD_2Cl_2) δ 5.24 (2H, s), 5.18 (2H, s), 2.32 (3H, s), 2.30 (3H, s), 2.26 (3H, s), 2.22 (3H, s), 2.04 (3H, s), 1.99 (3H, s); ESI-MS *m/z* 433 $(M+Na)^+$.

Measurement of Anti-influenza Activity

Plaque assay was performed by a modification of the method described by HAYDEN *et al.*[11]. Confluent

monolayers of MDCK cells (1.1×10^6 cells/well) were inoculated with influenza virus diluted in Eagle's modification of minimal essential medium (pH 7.2~7.4) containing 1 μg of TPCK-treated trypsin to give approximately 100 plaques per well (6-well plate) in the absence or presence of varying concentrations of the test compound. Cells were left for 1 hour. at 37°C for virus to adsorb and overlaid with defined cell growth medium containing 1% Noble agar (Sigma Chemical Company), 1 μg of TPCK-treated trypsin per ml, 0.001% DEAE dextran with or without compound. After cells were incubated for 2 days at 37°C (humidified 5% CO_2), plaques were visualized by staining viable cells with neutral red, and then visualized plaques were counted.

In all cases, the IC_{50} was calculated as the concentration required to reduce virus induced plaque formation by 50%.

Cytotoxicity Test

The cytotoxic activity of FR198248 against MDCK cells measured. The concentration required to reduce cell viability by 50% (CC_{50}) was measured. The cytotoxicity was colorimetrically determined at 550 nm (and 660 nm as a reference) according to MTT method[12,13] after 5 days of incubation at 37°C in the presence of the test compounds.

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