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FR191512 , a Novel Anti-influenza Agent Isolated from a Fungus Strain No.17415

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Structure Elucidation

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In the course of our screening for anti-influenza agents of microbial origin, FR191512 was isolated from the cultured broth of fungus strain No.17415 as colorless powder. The structure of FR191512 was determined by several spectroscopic experiments as a novel polyphenolic compound. This compound showed potent antiviral activity against influenza A virus.

Influenza viruses contain two envelope glycoproteins on their surface: hemagglutinin and sialidase (neuraminidase, EC 3.2.1.18). The hemagglutinin (HA) is known to mediate the binding to the cellular receptors such as glycoproteins containing sialic acid residue[1,2]. The sialidase catalyzes the cleavage of the sialic acid from the viral glycoconjugate receptors[3]. Therefore, this enzyme, destroying the cell surface receptor for influenza virus, is one of the most important targets for anti-influenza therapy[4].

During the course of our screening for the isolation of anti-influenza agents from soil microorganisms, we discovered the novel inhibitor, FR191512, from the cultured broth of fungus strain No.17415. FR191512 is a polyketide-derived natural product and a unique polyphenol compound (Fig. 1).

In this paper, we describe the taxonomy, fermentation, isolation, physico-chemical properties and structure elucidation of this agent.

Materials and Methods

Taxonomy

The producing fungus, strain No.17415, was originally isolated from a decayed leaf sample collected at a forest in Nagano, Japan. The morphological characteristics were determined from the cultures on MIURA's LCA medium[5]. The culture has been deposited to the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM P-15188.

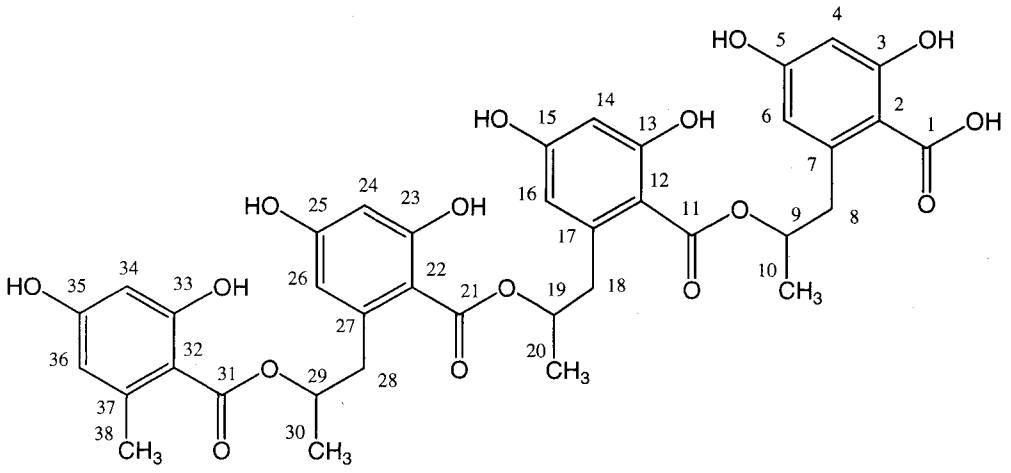
Detection of FR191512

Detection of FR191512 from the fermentation broth and the fractions under purification was monitored by HPLC using a reverse phase column YMC-ODS-AM(AM 303, 250×4.6 mm i.d., YMC Co., Ltd.). The solvent system was 70% aqueous methanol containing 0.05% TFA and the flow rate was 1.0 ml/minute. The detection wave length was set at 210 nm.

Analytical Procedures

Melting points were determined on a Yanagimoto micro melting point apparatus. Optical rotations were measured on a Jasco DIP-140 polarimeter in a 10 cm microcell. IR spectra were recorded on a Jasco A-102 infrared spectrometer. FAB-MS and MS/MS spectra were measured on a VG ZAB-SE mass spectrometer. NMR spectra were acquired on a Bruker DRX500 or Varian Gemini 300

Fig. 1. Structure of FR191512.



spectrometer.

Results

Taxonomy of Strain No.17415

This organism grew restrictedly on various culture media, and formed grayish colonies. On some agar media, strain No.17415 formed sporodochial anamorph; phialides on stromata and slimy conidial drops. Its mycological characteristics were as follows.

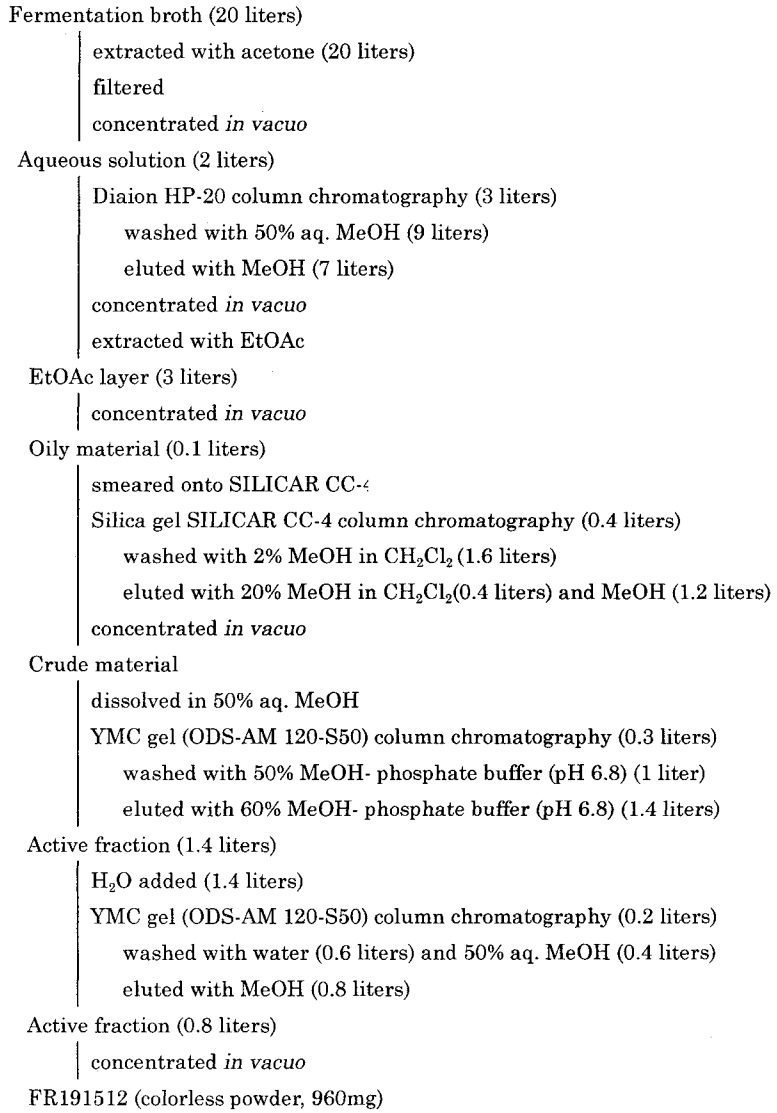
Culture characteristics on various agar media are summarized in Table 1. Culture on potato dextrose agar grew rather restrictedly, attaining 2.5~3.0 cm in diameter two weeks later at 25°C. This colony surface was felty, somewhat raised and showed several colors, dull green to greenish gray at the center, brownish orange at the middle and pale yellow at the margin. The reverse was dark green at the center and yellowish white at the margin. Conidial structures were not observed. Colonies on corn meal agar grew very restrictedly, attaining 0.5~1.5 cm in diameter under the same conditions. The surface was plane, felty, dark green to dull green. The reverse was dark green at the center and yellowish white at the margin. Sporodochia and conidia were often produced in or on the media. The strain did not grow on CZAPEK's solution agar.

The morphological characteristics determined from the culture on MIURA's LCM medium were as follows. Sporodochia of strain No.17415 consisted of stromata and phialides. The stromata were formed in the agar media, composed of loosely aggregations on hyphae, irregular to discoid, sessile, confluent, up to 3000 μm in diameter and pale yellow at first but becoming black with sporulation. The conidiophores were micronematous, hyaline, smooth, simple or branched, and phialides at the tips. The phialides were hyaline, smooth, cylindrical to filiform, (6~)10~18(~20)×2~4 μm in size, and produced conidia in slimy drops. The conidia were pale brown, but black in mass, smooth, one-celled, ellipsoidal to oblong, rounded at the apical end, slightly constricted at the center, with a small projection and gelatinous appendage at the basal end, and 7~10×3.5~5 μm. The vegetative hyphae were smooth, septate, hyaline and branched. The hyphal cells were cylindrical and 1.0~4.0 μm in width. The chlamydospores were absent.

Strain No.17415 was able to grow at the temperature range from 9 to 34°C, with the growth optimum at 26 to 28°C. These temperature data were determined on potato dextrose agar (made by NISSUI).

The characteristics of strain No.17415 resembled those of the genus *Myrothecium* Tode ex Fr. 1821, however, it was differed that the colonies of *Myrothecium* species were white and cottony, and formed sporodochia consisting macronematous conidiophores on the media. Thus, because of an uncertainty in the classification of the strain, it has been referred to simply as fungus strain No.17415. Additional studies related their taxonomic identifications are being pursued.

Fig. 2. Isolation procedure of FR191512.



Fermentation

An aqueous seed medium (160 ml) containing glycerol 2%, Pharmamedia (TM: Cotton seed flour, Traders Protein Co., Ltd.) 2%, dried yeast 1%, peptone 1%, KH_2PO_4 0.1% and Tween 80 0.1% was poured into a 500-ml Erlenmeyer flask and sterilized at 120°C for 30 minutes.

A loopful of fungus strain No.17415 was inoculated from a slant culture into the flask. The flask was cultured on rotary shaker (220 rpm, 5.1 cm throw) at 25°C for 6 days. The resultant seed culture was inoculated to 20 liters of sterile production medium consisting of corn starch 2%, glucose 1%, Pharmamedia 1%, soybean meal 0.5%, dried yeast 0.5%, Adecanol LG-109 (deforming agent, Asahi Denka Co., Ltd.) 0.05%, and Silicone KM-70 (deforming agent, Shin-Etsu Chemical Co., Ltd.) 0.05% in a 30-liter jar-fermenter.

Fermentation was carried out at 25°C for 6 days under aeration of 20 liters/minute and agitation of 250 rpm.

Isolation and Purification

The isolation procedure of FR191512 is outlined in Fig. 2. The cultured broth (20 liters) was extracted with an equal volume of acetone by intermittent mixing. The acetone extract was filtered with an aid of diatomaceous earth and concentrated *in vacuo* to 2 liters. This extract was passed through a column (3 liters) of Diaion HP-20 (Mitsubishi

Table 1. Culture characteristics of the strain No.17415.

Medium	Culture characteristics
Malt extract agar	G: Fairly rapidly, 3.5-4.0 cm
(Blaskeslee 1915)	S: Circular, plane, felty, not formed anamorph, greenish gray (28F2) to dull green (28E2) at the center and olive (1F3) at the margin
	R: Olive (1F3-4)
Potato dextrose agar	G: Rather restrictedly, 2.5-3.0 cm
(Difco 0013)	S: Circular to irregular, felty, somewhat raised, not formed anamorph, dull green (27E3) to greenish gray (26E2) at the center, brownish orange (7C3) at the middle, pale yellow (4A3) at the margin
	R: Dark green (27F3) at the center, yellowish white (4A2) at the margin
Czapek's solution agar	G: No growth
Sabouraud dextrose	G: Very restrictedly, 1.0-1.5 cm
agar(Difco 0190)	S: Circular, felty, raised, thick, not formed anamorph,brownish gray (4F4), producing pale reddish soluble pigment
	R: grayish brown (9F3)
Emerson Yp Ss agar	G: Very restrictedly, 0.5-1.0 cm
(difco 0739)	S: Irregular, scanty, not formed anamorph, brownish gray (6F2) and yellowish white (4A2)
	R: Dull brown (7F2) and yellowish white (4A2)
Corn meal agar	G: Very restrictedly, 0.5-1.5 cm
(Difco 0386)	S: Irregular to circular, felty, plane, formed anamorph abundantly, dark green (29F3) to dull green (29E4)
	R: Dark green (28F4-5) at the center, yellowish white (4A2) at the margin
MY20 agar	G: Restrictedly, 1.5-2.0 cm
	S: Irregular to circular, felty, plane, formed anamorph, gray (6C1) at the center, orange white (5A2) at the margin
	R: Brownish gray (4F2) at the center, yellowish white (3A2) at the margin

These characteristics were observed after 14 days of incubation at 25℃.

Abbreviation G:growth, measuring colony size in diameter, S:colony surface, R:reverse

Chemical Ind. Co., Ltd.). The column was washed with water and 50% aqueous methanol, and then eluted with methanol. The eluate (7 liters) was concentrated *in vacuo* to 1 liter and extracted with ethyl acetate (3 liters). The mixture was dehydrated with Na_2SO_4 and concentrated *in vacuo* to 0.1 liters, and then the solution was smeared onto SILICAR CC-4 (Mallinckrodt Co., Ltd.). The solid matter was subjected to a column (0.4 liters) of SILICAR CC-4 gel. The column was washed with dichloromethane containing 2% methanol (1.6 liters), and then eluted with dichloromethane containing 20% methanol (0.4 liters) and methanol (1.2 liters). The active fraction (2.5 liters) was concentrated *in vacuo* to dryness. The resultant pale yellowish product was dissolved in a small volume of 50% aqueous methanol and was subjected to a column (0.3 liters) of YMC gel (ODS-AM 120-S50, YMC Co., Ltd.). The column was washed with 50% aqueous methanol containing 0.2% $(NH_4)_2HPO_4$-H_3PO_4 (pH 6.8) and eluted with 60% aqueous methanol containing 0.2% $(NH_4)_2HPO_4$-H_3PO_4 (pH 6.8). To the eluate (1.4 liters) was added an equal volume of water and applied to a column (0.2 liters) of YMC gel. After washing the column with water (0.6

Table 2. Physico-chemical properties of FR191512.

Appearance	Colorless powder
Nature	Acidic
Molecular formula	$C_{38}H_{38}O_{16}$
FAB-MS (*m/z*)	751
$(M+H)^+$ Calcd:	C 54.28, H 5.75 (for $C_{38}H_{38}O_{16} \cdot 5H_2O$)
Found:	C 53.93, H 5.16
UV λ_{max} in MeOH	215, 265, 307nm
$[\alpha]_D$ (23℃, MeOH)	−192° (C 1.0)
IR ν_{max} cm^{-1}	3380, 2980, 1650, 1620, 1450, 1360, 1310, 1260, 1200, 1170, 1050
Solubility : soluble	MeOH, DMSO
insoluble	$CHCl_3$, H_2O
TLC, SiO_2 (CH_2Cl_2 : MeOH = 10 : 1)	Rf 0.44
HPLC (YMC ODS-AM, 70% MeOH, 1.0ml/min)	Rt 15min.
mp.	135~138℃

liters) and 50% aqueous methanol (0.4 liters), the active fraction was eluted with methanol (0.8 liters). The eluate (0.8 liters) was concentrated *in vacuo* to dryness and FR191512 substance was obtained as colorless powder (960 mg).

Physico-chemical Properties

Physico-chemical properties of FR191512 are summarized in Table 2. FR191512 was readily soluble in methanol and dimethyl sulfoxide but insoluble in chloroform and water. FR191512 was negative to ninhydrin reagent and was positive to $FeCl_3$. It showed UV absorption at 215, 265 and 307 nm. It showed IR absorptions at 3380, 1650 and 1620 cm^{-1} due to hydroxyl and carbonyl groups, respectively. The FAB-MS spectrum showed a molecular ion peak at *m/z* 751 $(M+H)^+$.

Structure Elucidation

The molecular formula $C_{38}H_{38}O_{16}$ was derived from FAB-MS (*m/z* 751 $(M+H)^+$) and the ^{13}C NMR data (Table 3). The ^{13}C NMR and DEPT spectra revealed the presence of $4\times CH_3$, $3\times CH_2$, $3\times OCH$, $8\times =CH$, $16\times =C$ and 4 carbonyls, accounting for 29 carbon-bound protons, leaving 9 protons bonded to oxygen atoms. The molecular formula required 20 degrees of unsaturation, four of which were accountable in the form of four carbonyl groups. The remaining 16 degrees of unsaturation had to be accounted for by 24 olefinic carbons, indicating the presence of four benzene units. A broadened ^{13}C signal at 175.7 ppm gave the indication of a carboxylic acid group and positive color reaction of $FeCl_3$ was characteristic of a phenol. Treatment of FR191512 with trimethylsilyldiazomethane for 5 minutes yielded the methyl ester (^1H δ 3.90 (3H, s), FAB-MS *m/z* 787 $(M+Na)^+$). On acetylation with acetic anhydride in pyridine, it gave an octaacetyl derivative (FAB-MS *m/z* 1123 $(M+Na)^+$), suggesting the presence of 8 phenolic hydroxyl groups in FR191512. The chemical shift of C-29 (δ_H 5.52, δ_C 73.9) was typical for an 3 acyloxy methine. In ^1H-^1H COSY spectrum, the acyloxymethine proton was coupled to methylene 28-H_2 and methyl 30-H_3, indicating 2-acyloxypropyl moiety. In the same way, starting from 19-H (5.65 ppm) and 9-H (5.63 ppm) another two 2-acyloxypropyl moieties were deduced. A combined analysis of ^1H-^1H COSY, HMQC and HMBC revealed partial structures **a**, **b**, **c**, **d** (Fig. 3). The structure of orsellinic acid moiety C-31~C-38, a part of substructure **a**, was secured by a comparison of the ^{13}C NMR data with that of orsellinic acid ethyl ester[6]. The HMBC correlation between 29-H and C-31 revealed that orsellinic acid part was connected *via* ester bond to the 2-hydroxypropyl

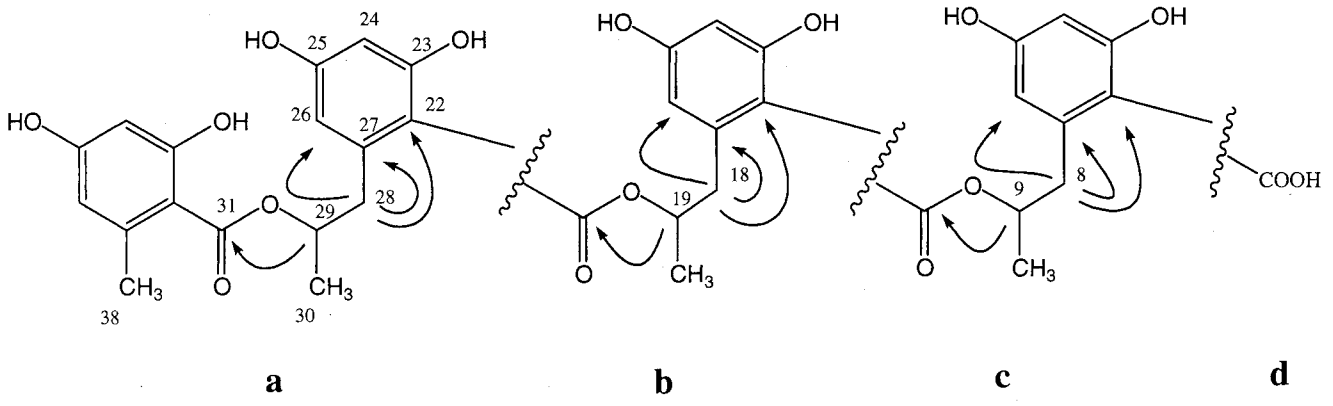
Table 3. The ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of FR191512 in CD_3OD.

position	δ_C (mult)	δ_H (mult, J (Hz))
1	175.7 (s)	
2	110.0 (s)	
3	166.1 (s)	
4	102.4 (d)	6.13 a
5	161.4 (s)	
6	111.9 (d)	6.13 a
7	144.1 (s)	
8	43.1 (t)	3.63 (dd, 13, 5) and 3.25 (dd, 13, 8)
9	75.5 (d)	5.63 (m)
10	20.5 (q)	1.40 (d, 6)
11	171.9 (s)	
12	106.2 (s)	
13	166.2 (s)	
14	102.7 (d)	6.13 a
15	163.2 (s)	
16	113.5 (d)	6.19 (d, 2)
17	143.2 (s)	
18	43.7 (t)	3.55 (dd, 14, 4) and 3.00 (dd, 14, 8)
19	74.2 (d)	5.65 (m)
20	20.6 (q)	1.44 (d, 6)
21	171.8 (s)	
22	106.4 (s)	
23	166.2 (s)	
24	102.8 (d)	6.13 a
25	163.3 (s)	
26	113.5 (d)	6.19 (d, 2)
27	143.2 (s)	
28	43.7 (t)	3.42 (dd, 14, 5) and 2.98 (dd, 14, 8)
29	73.9 (d)	5.52 (m)
30	20.7 (q)	1.37 (d, 6)
31	172.4 (s)	
32	106.0 (s)	
33	166.1 (s)	
34	101.7 (d)	6.10 (d, 2)
35	163.6 (s)	
36	112.4 (d)	6.16 (d, 2)
37	144.5 (s)	
38	24.7 (q)	2.42 (s)

a : coupling constant is not clear because of overlapping.

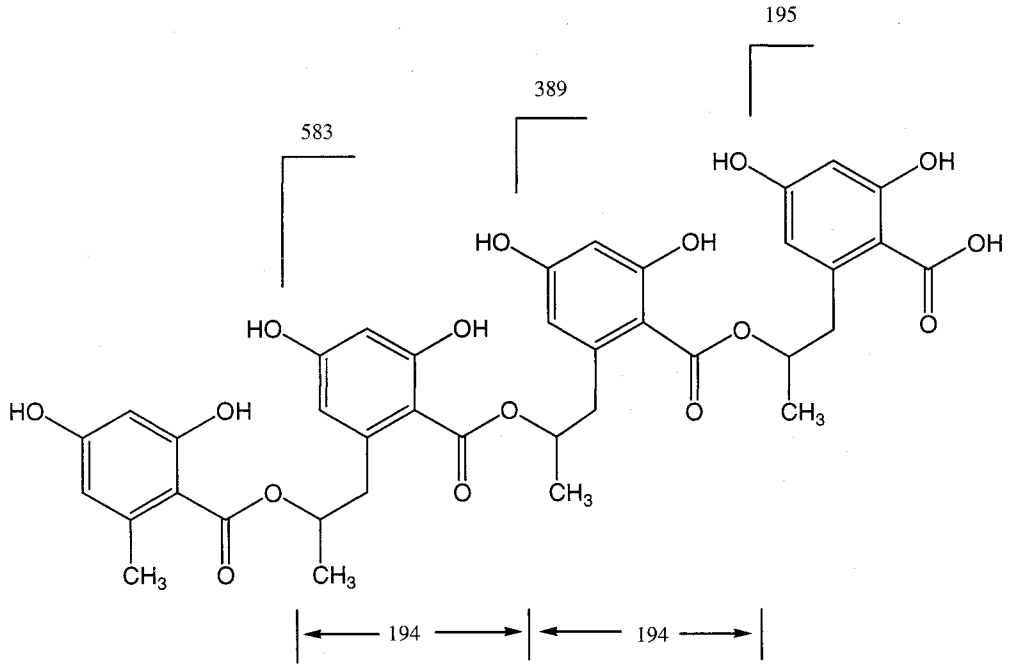
moiety. The methylene protons 28-H_2 (3.42 and 2.98 ppm) showed HMBC correlations to C-26 (δ 113.5 (d)), C-27 (δ 143.2 (s)) and C-22 (δ 106.4 (s)). The benzene proton 26-H (δ 6.19 (d, J=2 Hz)) was *meta*-coupled to 24-H (δ 6.13). The high-field shifted ^{1}H and ^{13}C chemical shifts of C-26 (113.5 ppm) and C-24 (102.8 ppm) indicated the placement of phenolic hydroxyl groups at C-23 and C-25. Elucidation of substructure **b** and **c** was achieved in a similar manner. The sum of the substructures **a**, **b**, **c** and **d** satisfied the molecular formula. There is only one reasonable connection between **a**, **b**, **c** and **d** and it would give the plane structure of FR191512 as shown in Fig. 1. The validity of the presence of 2,4-dihydroxy-6-(2-hydroxypropyl) benzoic acid moiety was obtained by the close agreement of the ^{13}C NMR data with the reported values of the relevant portion[7]. The structure was further strengthened by the MS/MS data as shown in Fig. 4. From the above information, the structure of FR191512 was

Fig. 3. Substructures of FR191512.



Arrows denote key HMBC correlations.

Fig. 4. MS/MS data of FR191512.



determined to be 2-(2-(6-(2-(6-(2-(2,4-dihydroxy-6-methylphenylcarbonyloxy)propyl)-2,4-dihydroxyphenylcarbonyloxy)propyl)-2,4-dihydroxyphenylcarbonyloxy)propyl)-4,6-dihydroxybenzoic acid. A full ^{1}H and ^{13}C assignment of FR191512 was made by ^{1}H-^{1}H COSY, HMQC and HMBC which listed in Table 3.

Discussion

A number of polyphenol compounds have been isolated from plant leaf extracts[8~10].

As far as we are aware, however, only a few polyphenol compounds that inhibit infection of influenza virus *in vitro*

and *in vivo* have been reported as secondary metabolites of microbial origin. In this time, we found a new polyphenol compound FR191512 from a fungus origin.

FR191512 was consisted of four units of dihydroxylated benzoic acid, one of orsellinic acid and three of a modified orsellinic acid. It is intriguing to note that both components are derived from polyketide[11], *i.e.*, tetraketide and pentaketide, respectively. There are some macrocyclic natural products including 2,4-dihydroxy-6-(2-hydroxypropyl) benzoic acid moiety such as NG-011, NG-012[7], BK223-B and BK223-C[12]. To the best of our knowledge, linear type like this is the first example. The biological properties of FR191512 including the anti-influenza activities *in vitro* and *in vivo* will be reported in the next paper[13].

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