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CRM-51006, a New Phospholipase C (PLC) Inhibitor, Produced by Unidentified Fungal Strain MT51005

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Transmembrane signal transduction is a complex process which extracellular stimuli are translated into intracellular physiological response. Depending on the interaction of specific ligands with the cell-surface receptors in most of eukaryotic cells, phosphoinositide specific phospholipase C (PI-PLC) plays a pivotal role in the transmembrane signal transduction pathways[1]. In the course of our screening program for new cell signaling inhibitors, CRM-51006 (**1**) having inhibititory activity on PI-PLC was isolated from the culture broth of unidentified fungal strain MT51005. The structure of **1** was related with those of previously reported compounds, anguilosporal (**2**)[2] and CRM-51005 (**3**)[3] (Fig. 1). In this paper, we report the isolation and structure elucidation, biological activities of **1**.

PLCγ1 enzyme was purified to homogeneity (over 95% purity of PLCγ1) from bovine cerebellum through column chromatography and the enzyme activity of PLC was assayed using [^{3}H]-PI as the substrate according to the method of RHEE *et al.*[4]. For the measurement of total inositol phosphates (IP_t) produced by the PLCγ1 activated with platelet-derived growth factor (PDGF) stimulation in cell, NIH3T3γ1 cells (6×10^5 cells/ml) were prelabeled with 1 μCi/ml of *myo*-[2-^{3}H]-inositol in inositol-free DMEM for 24 hours. The labeled cells were washed with phosphate buffered saline (PBS) and incubated in inositol-free DMEM containing 20 mM HEPES, pH 7.5, 20 mM LiCl and 1 mg/ml BSA for 15 minutes and then test compounds were added. After stimulation with PDGF for 30 minutes, inositol phosphates were extracted with ice-cold 5% $HClO_4$ and total inositol phosphates (IP_t) were measured by using a Bio-Rad AG 1-X8 anion exchange column[5]. *In vitro* cytotoxicity against various cancer cell lines was tested by the protocol of Developmental Therapeutic Program of National Cancer Institute in order to evaluate the drug's selectivity for particular tumor types[6]. The cells were fixed by gently layering 50 μl of cold 50% trichloroacetic acid and incubated at 4°C for 1 hour. The medium was removed and the cells were stained with 0.4% (w/v) sulfurhodamine B (SRB). The OD of the well was read on a 96-well microplate reader at 570 nm.

The producing fungus, strain MT51005, was isolated from a soil sample collected in Jeju island, Korea. The strain was deposited at the Korean Collection for Type Culture (KCTC), under accession No. KCTC 8787P. The cultivation of fungi was carried out at 25°C in a 15-liter fermentation tank containing 10 liters of sterile YM medium (yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, glucose 1.0%) with agitation at 200 rpm for 7 days. After the fermentation broth of strain MT51005 was centrifuged, the mycelial cake was separated from the culture broth and extracted with acetone. The supernatant was past through Diaion HP-20 column chromatography, followed by washing with 30% MeOH, the absorbent on column was eluted by acetone. After the combined organic layers were concentrated *in vacuo*, its residue was chromatographed on silica gel (Kiesegel 60, 0.063～0.2 mm, Merck) using a mixture of $CHCl_3$/MeOH (10/1). The active fraction was chromatographed on ODS RP-18 (70～230 mesh, YMC Co.) with MeOH/H_2O (7/3～9/1, stepwise). The active compounds were purified by preparative HPLC (YMC-Pack ODS-AM, i.d. 6.0 mm×250 mm, 254 nm, Shimadzu LC-6AD) using a solvent system of MeOH/H_2O (8/2) to give three related compounds, **1** (12 mg), **2** (430 mg), and **3** (132 mg).

The molecular formula of **1** was confirmed as $C_{15}H_{22}O_3$ from HREI-MS [found *m/z* 250.1751, calcd for 250.1673] and the ^{13}C-NMR data. The ^{1}H-NMR, ^{13}C-NMR, and DEPT data of **1** indicated the presence of a 1,2,3,4-substituted benzene ring (δ 6.38 and δ 7.61, d, J=8.9 Hz) with two OH group bearing carbons (δ 163.99 and δ 164.96), an ethyl group (δ 1.08 and δ 2.63, J=7.5 Hz), a carbonyl group (δ 210.97), and a 1′-methylpentyl group.

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Fig. 1. Chemical structures of CRM-51006 (**1**), anguillosporal (**2**) and CRM-51005 (**3**).

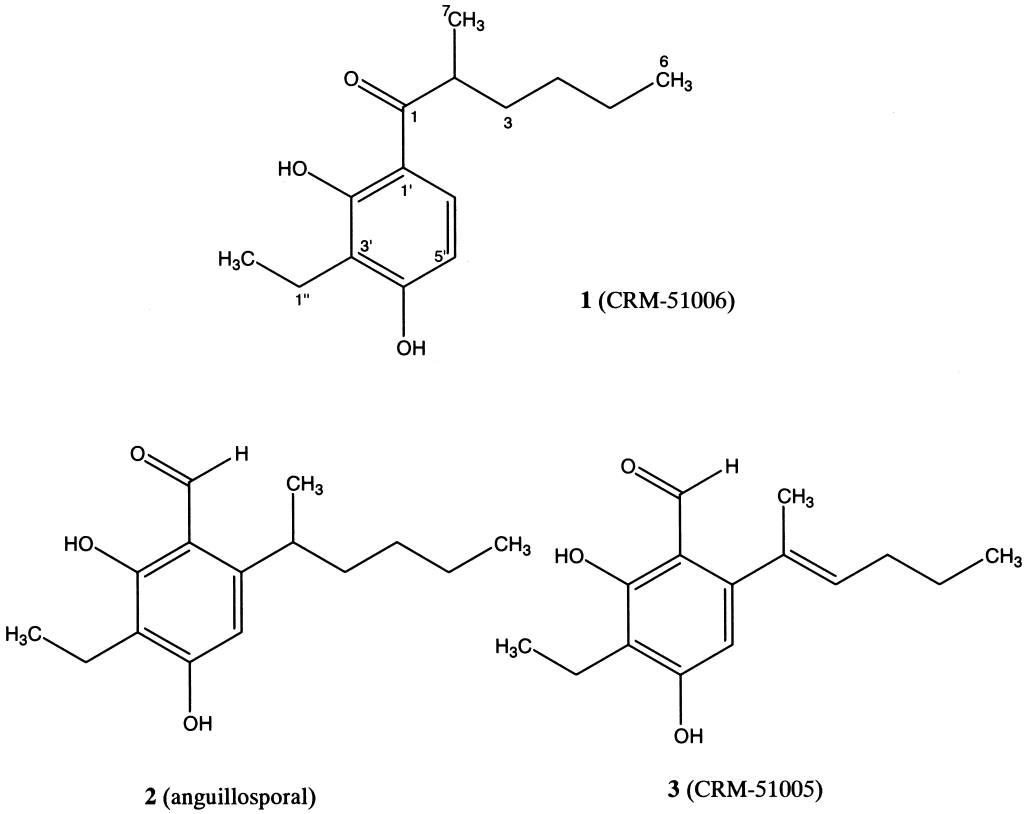
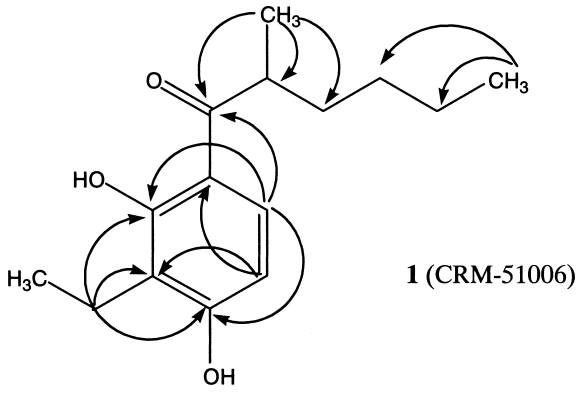


Fig. 2. Significant correlations observed in the HMBC and NOESY spectra of CRM-51006 (**1**).



⟶ : ^{1}H-^{13}C long-range correlations from HMBC spectrum.

Assignments of carbons bearing protons were made with the aid of HMQC data (Table 1). HMBC experiment was performed to establish the regiochemistry of the substituents in the 1,2,3,4-substituted benzene ring. HMBC correlation of H-6′ with carbonyl carbon at δ 210.97 placed the carbonyl group at C-1 position. The presence of 1′-methylpentyl moiety was verified by the analysis of HMBC data, and in turn this group was connected to carbonyl group (C-1) on the basis of HMBC correlations of H-7 with C-1. HMBC correlations of H_3-2″ with C-3′ and of H-1″ with C-2′, C-3′ and C-4′, together with chemical shift considerations, permitted the location of an ethyl group and two hydroxy groups at C-3′, C-2′, and C-4′, respectively. This assignments were also supported by the observation of HMBC correlations of H-5′ with C-1′ and C-3′ as well as HMBC correlations of H-6′ with C-2′, and C-4′. Based on these spectral data, the structure of **1** was determined as 1-(3′-ethyl-2′,4′-dihydroxyphenyl)-2-methyl-1-hexanone according to IUPAC rule and named as the CRM-51006. CRM-51006 is a new fungal metabolite closely related to anguillosporal (**2**) and CRM-51005 (**3**).

Table 1. NMR Data of CRM-51006 (**1**).

Position	δ ^{1}H(mult., *J*, Hz)[a]	δ^{13} C[b]	HMBC
1		210.97	
2	3.50 (sextet, 6.8)	40.81	
3	1.45 (m)	35.40**	
	1.78 (m)		
4	1.29 (m)	31.10	
5	1.29 (m)	24.18	
6	0.88 (t, 6.9)	14.59	24.18, 31.10
7	1.16 (d, 6.8)	18.71	35.40, 40.81, 210.97
2'-OH	13.37 (s)*		
4'-OH	5.30 (s)*		
1'		113.54	
2'		164.96	
3'		119.18	
4'		163.99	
5'	6.38 (d, 8.9)	108.48	113.54, 119.18
6'	7.61 (d, 8.9)	130.66	163.99, 164.96, 210.97
1"	2.63 (q, 7.5)	16.89	13.82, 119.18, 163.99, 164.96
2"	1.08 (t, 7.5)	13.82	16.89, 119.18

[a](CD_3OD, 500 MHz). [b](CD_3OD, 125 MHz).

*These signals were detected in $CDCl_3$.

** The Carbon data attached two diastereotopic proton peaks was confirmed by HMQC

CRM-51006 inhibited PLCγ1 activity in dose-dependent manner with an IC_{50} of 2 μg/ml (8 μM). Activity of this compound was either greater or similar in comparison with the previously reported inhibitors against PLCγ1 such as vinaxanthone, Q12713, hispidospermidine, caloporoside, and CRM-51005. When the effect of this compound on the PLCγ1 activity induced by PDGF stimulus in NIH 3T3γ1 cells was examined, **1** has an IC_{50} of 0.8 μg/ml, which showed the similar inhibitory activity with fluvirucin B_2 previously reported as a PLC inhibitor with an IC_{50} of 0.7 μg/ml in A431 cells by UI, *et al.*[7]. CRM-51006 (**1**) was also evaluated for antitumor activity against cancer cell lines *in vitro*. The GI_{50} (μg/ml) values against various cancer cells indicated that this compound had potent cytotoxicity against T47D (breast cancer, 0.88), PC-3 (prostate cancer, 0.86) and OVCAR-4 (ovary cancer, 0.36). These results suggest that the cytotoxicity of could be related to the inhibition of PLC activity in these cells.

Acknowledgements

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