J. Antibiot. 58(10): 615–620, 2005

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Fungal Metabolites, Sorbicillinoid Polyketides and Their Effects on the Activation of Peroxisome Proliferator-activated Receptor γ

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Received: June 22, 2005 / Accepted: October 4, 2005

Abstract A new sorbicillinoid polyketide, dihydrotrichodimerol (**2**), along with known trichodimerol (**1**), and rezishanones C (**3**) and D (**4**) were isolated by bioassay-guided fractionation from an unidentified fungal strain. Dihydrotrichodimerol (**2**) specifically activated peroxisome proliferator-activated receptor γ with an ED_{50} value of 80 ng/ml as measured by a transactivation assay using a chimeric hPPAR/GAL4 system without affecting peroxisome proliferator-activated receptors α and δ. On the other hand, compounds **1** and **2** suppressed the production of tumor necrosis factor-α and nitric oxide in LPS-stimulated RAW264.7 cells to a similar extent.

Keywords sorbicillinoid, trichodimerol derivative, peroxisome proliferator-activated receptor γ, tumor necrosis factor-α, nitric oxide

Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-activated transcription factors, a family that includes the receptors for steroid hormones, retinoids, thyroid hormone, and vitamin D. Three mammalian PPARs have been identified to date, termed PPARα, PPARγ, and PPARδ. PPARs function as regulators of lipid and lipoprotein metabolism, glucose homeostasis, cellular differentiation and also appear to control the inflammatory response [1, 2]. PPARγ agonists have therapeutic potential in the treatment of type 2 diabetes, inflammatory disease, and certain cancers [3].

In our search for biologically active agents of natural origin, the culture broth of an unidentified fungal strain exhibited significant activity in a reporter gene assay of peroxisome proliferator-activated receptor γ (PPARγ) [4]. Bioassay-guided fractionation of an EtOAc-soluble extract of fungus using this assay resulted in the isolation of a new sorbicillinoid polyketide, dihydrotrichodimerol (**2**), as well as three known sorbicillinoids, trichodimerol (**1**), and rezishanones C (**3**) and D (**4**) (Fig. 1) [5〜7]. Sorbicillinoid polyketides, unique metabolites from various terrestrial and marine fungi, have drawn recent interest in terms of their structural complexity and biological activity including antioxidant and tumor necrosis factor (TNF)-α inhibitory activities [5, 8〜11]. Synthetic and biosynthetic studies of trichodimerol (**1**) and related natural products have been reported by several groups [12〜16]. Here we described the isolation, structural characterization of compounds **1**〜**4** and the biological evaluation of the compounds isolated.

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† The same new compound (dihydrotrichodimerol) which was isolated independently appears in this issue (pages 621〜624).

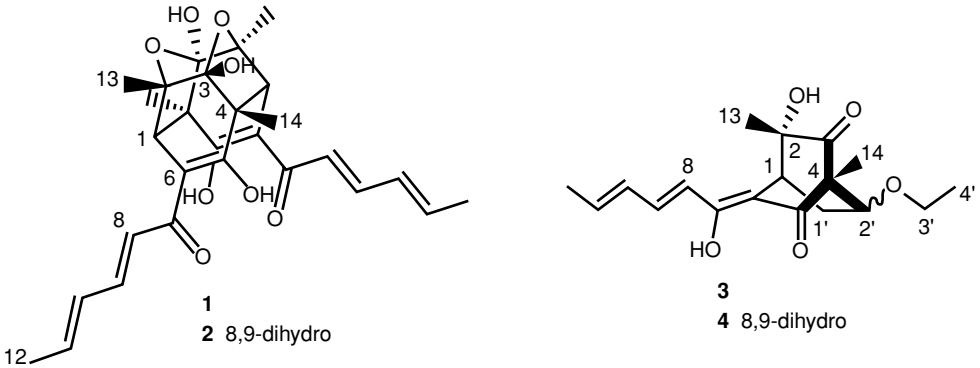

Fig. 1 Structures of compounds **1**~**4**.

Materials and Methods

General Experimental Procedure

All melting points were determined on an Electrothermal 9100 instrument without correction. Optical rotations were measured on a JASCO P-1020 polarimeter. UV and CD spectra were measured on a Shimadzu UV-1601 UV-visible and a JASCO J-7200 spectrophotometers, respectively. NMR spectra were recorded on Varian UNITY 400 or Bruker DMX 600 NMR spectrometers with $CDCl_3$. ESI-MS and HRESI-MS were obtained on a Platform quadrupole and Mariner mass spectrometers, respectively. HRFAB-MS was obtained on a JMS-HX110A/HX110A Tandem Mass spectrometer (JEOL). Preparative and recycling HPLC were carried out on a Waters semi-prep and LC-908 Japan Analytical systems, respectively.

Fermentation

An unidentified fungal strain B00853 isolated from a soil sample collected in a field in Yuseong, Daejeon, was cultured in a seed agar medium, which was composed of glucose 2%, malt extract 2%, peptone 0.1%, agar 1.8%. The production medium was composed of soluble starch 2.2%, yeast extract 0.2%, polypeptone 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05% and KH_2PO_4 0.1% and adjusted to pH 5.6. For production cultures, 300 ml of production medium in 1000-ml baffled flasks was inoculated with 1×1 cm seed agar piece of fully grown mycelia of the fungus, and the flasks were incubated at 28°C and 180 rpm for 7 days.

Bioassay for Activation of Peroxisome Proliferator-Activated Receptors

The ligand binding domains (LBDs) of hPPARα(amino acids 167~468), hPPARδ(amino acids 167~441), and hPPARγ(amino acids 163~477) were generated by PCR amplification using *Pfu* polymerase (Stratagene, La Jolla, CA, USA) and gene specific primers flanked with restriction enzymes *Bam*HI and *Xba*I, respectively. The LBDs were subcloned in-frame into the pFA-CMV (Stratagene, La Jolla, CA, USA) vector. The 4xGAL4-luciferase reporter plasmid (pFR-Luc) was from Stratagene. The ability of the compound to activate PPARs was determined by a transactivation assay. The pFA-GAL4-PPARs chimera expression constructs, containing the LBD of human PPARs, were prepared by standard methods for the vector. At 75~90% confluence, NIH3T3 cells were transiently co-transfected with the vectors for pFA-GAL4-PPAR, pFR-Luc and pRL-CMV (Promega, Madison, WI, USA) using Lipofectamine plus reagent according to the instructions of manufacturer (Invitrogen). Following 24 hours incubation, the cells were treated with various concentrations of the test compounds and incubated for 16 hours. Luciferase assay was performed using a Dual-luciferase reporter assay system according to the instructions of the manufacturer (Promega). Luciferase activity was determined in a Microlumat Plus luminometer (EG&G Berthold, Bad Wildbad, Germany) by injecting 100 μl of assay buffer containing luciferin and measuring light emission for 10 seconds. The results were normalized to the activity of *Renilla* expressed by cotransfected *Rluc* gene under the control of a constitutive promoter [4, 17].

Bioassay of Nitric Oxide Production

RAW264.7 cells were seeded in 96 well plates at 1×10^5 cells/well. After 3 hours, the cells were treated with various concentrations of compounds and stimulated for 24 hours with or without 1 μg/ml of LPS (Sigma Chemical Co., St. Louis, MO, USA). As a parameter of nitric oxide (NO) synthesis, nitrite concentration was measured in the supernatant of RAW264.7 cells by the Griess reaction as previously described [18].

Bioassay of TNF-α Production

RAW264.7 cells were seeded in 96 well plates at a

density of 1×10^4 cells/well, pretreated with different concentrations of compounds for 1 hours; then the cells were stimulated with LPS (1 μg/ml) for 18 hours. TNF-α production in the supernatant of RAW264.7 cells was quantitated using OptELATM assay kit according to the manufacture's instructions (Pharmingen, San Diego, CA, USA) as previously described [18].

Results and Discussion

Isolation and Characterization of Fungal Metabolites

An unidentified fungal strain was grown in 8 liters liquid media at 28°C for 7 days. The cultured broth was extracted with EtOAc twice and these extracts were filtered through a fritted funnel *in vacuo* to remove insolubles. The volume of the filtrate was reduced *in vacuo* and then partitioned between EtOAc and H_2O to give the organic extracts (2.6 g). Fractionation of the EtOAc-soluble extract was initiated by silica gel chromatography using a $CHCl_3$ - MeOH gradient as mobile phase, and fractions obtained were pooled based on TLC and ESI-MS analysis. Fraction 1, eluted with $CHCl_3$ - MeOH (99 : 1), was passed through a Sephadex LH-20 column using $CHCl_3$ - MeOH (1 : 1) and further purified by recycling HPLC [YMC ODS-A, 250×20 mm i.d., 50% MeCN - H_2O (0.05% TFA), 10 ml/minute] to afford compound **3** (2.7 mg, 0.10% w/w) and compound **4** (3.6 mg, 0.14% w/w). Fraction 4, eluted with $CHCl_3$ - MeOH (50 : 1), was passed through a Sephadex LH-20 column using $CHCl_3$ - MeOH (1 : 1) and further purified by HPLC [YMC ODS-A, 250×20 mm i.d., MeCN - H_2O (0.05% TFA) gradient, 10 ml/minute] to afford compound **1** (5.8 mg, 0.22% w/w) and compound **2** (9.8 mg, 0.38% w/w).

Trichodimerol (**1**)

Yellow powder; mp 140~142°C; $[\alpha]_D$ −387° (*c* 0.05, MeOH), UV (MeOH): λ_{max} (log ε) 242 (3.94), 359 (4.49) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 281 (−3.0), 342 (+1.6), 383 (−4.1); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; ESI-MS *m/z* 519 $[M+Na]^+$, 495 $[M-H]^-$.

Dihydrotrichodimerol (**2**)

Yellow powder; mp 82~83°C; $[\alpha]_D$+99° (*c* 0.01, MeOH), UV (MeOH): λ_{max} (log ε) 309 (4.19), 361 (4.31) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 282 (−3.9), 385 (−0.5); ^{1}H and ^{13}C

Table 1 ^{1}H NMR data of compounds **1**~**4**.

No.	1	2	3	4
1	2.99 s	2.92 s	3.15 t (2.4)	3.05 t (2.8)
8	6.13 d (15.0)	2.38 m, 2.45 m	6.20 m	2.45 m
9	7.31 dd (10.2, 14.7)	2.27 m	7.29 dd (10.4,14.8)	2.35 m
10	6.28 m	5.42 m	6.26 m	5.49 m
11	6.22 m	5.49 m	6.21 m	5.44 m
12	1.88 d (5.7)	1.64 d (6.0)	1.89 d (6.8)	1.64 d (6.4)
13	1.42 s	1.42 s	1.21 s	1.22 s
14	1.45 s	1.44 s	1.32 s	1.31 s
1′		2.98 s	1.68 dt (2.8, 13.6)	1.61 m
			2.79 ddd (2.0, 8.0, 13.6)	2.77 ddd (2.0, 7.6, 13.6)
2′			3.56 m	3.53 m
3′			3.36 m, 3.55 m	3.37 m, 3.55 m
4′			1.12 t (6.8)	1.13 t (6.8)
8′		6.14 d (14.4)		
9′		7.32 dd (10.8, 15.0)		
10′		6.29 m		
11′		6.20 m		
12′		1.88 d (6.6)		
13′		1.42 s		
14′		1.43 s		
5-OH	16.34 s	16.54 s		
7-OH			14.0 s	14.3 s
5′-OH		16.39 s		

Chemical shifts are shown in the δ scale with *J* values (Hz) in parenthesis.

NMR data, see Tables 1 and 2; ESI-MS *m/z* 521 $[M+Na]^+$, 497 $[M-H]^-$; HRFABMS *m/z* 521.2147, calculated for $C_{28}H_{34}O_8Na$, 521.2151.

Rezishanone C (**3**)

Pale yellowish powder; mp 55∼56°C; $[\alpha]_D$+176° (*c* 0.7, MeOH), UV (MeOH): λ_{max} (log ε) 345 (4.02) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 220 (−3.0), 306 (−5.6), 351 (+1.7); ^1H and ^{13}C NMR data, see Tables 1 and 2; ROESY correlations H-1/C-8, C-13; H-14/C-2′; ESI-MS *m/z* 321 $[M+H]^+$; HRESIMS *m/z* 321.1699, calculated for $C_{18}H_{25}O_5$, 321.1702.

Rezishanone D (**4**)

Pale yellowish oil; $[\alpha]_D$+197° (*c* 0.8, MeOH), UV (MeOH): λ_{max} (log ε) 293 (4.04) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 218 (−3.2), 289 (−5.3), 361 (−1.0); ^1H and ^{13}C NMR data, see Tables 1 and 2; ROESY correlations H-1/C-8, C-13, C-1′; H-14/C-2′; ESI-MS *m/z* 345 $[M+Na]^+$, 321 $[M-H]^-$; HRESIMS *m/z* 323.1865, calculated for $C_{18}H_{27}O_5$, 323.1858.

Table 2 ^{13}C NMR data of compounds **1**∼**4**.

No.	1	2	3	4
1	57.5	57.8	39.7	40.2
2	78.9	79.0	74.6	74.3
3	104.1	104.1[a]	210.9	210.9
4	58.8	57.4	67.0	66.6
5	198.0	191.4	196.4	195.0
6	102.7	103.3	110.4	110.3
7	175.9	192.8	166.4	177.4
8	118.5	34.7	118.0	32.2
9	143.6	28.2	141.8	29.2
10	130.9	129.0	130.9	126.8
11	140.4	126.4	139.2	129.0
12	18.7	17.8	18.8	17.8
13	21.3	21.2	24.3	24.4
14	18.9	18.6	8.9	8.8
1′		57.6	30.6	30.9
2′		78.9	79.1	79.1
3′		104.2[a]	65.6	65.7
4′		58.7	15.1	15.1
5′		198.3		
6′		102.8		
7′		176.0		
8′		118.4		
9′		143.6		
10′		130.8		
11′		140.4		
12′		18.8		
13′		21.3		
14′		19.1		

Chemical shifts are shown in the δ scale; [a] Interchangeable.

Structure Determination

The structures of trichodimerol (**1**), and rezishanones C (**3**) and D (**4**) were identified by spectral data interpretation and comparison with literature values [5, 7, 19, 20]. Compound **2** was obtained as an amorphous yellow powder and was shown to possess a molecular formula of $C_{28}H_{34}O_8$ by positive HRFABMS. The ^1H and ^{13}C NMR spectra of compound **2** displayed the characteristic paired signals including those of trichodimerol (**1**), suggesting that compound **2** is a modified trichodimerol (**1**) and is based on a bisorbicillinoid skeleton [16, 21]. Thus, the absence of two olefinic methine signals and the presence of two sets of methylene signals at δ_H 2.38 (1H, m, H-8), 2.45 (1H, m, H-8), and 2.27 (2H, m, H-9), and the corresponding carbon signals at δ_C 34.7 (C-8), and 28.2 (C-9), indicated that one of the sorbyl chains of trichodimerol (**1**) is partially saturated. The position of hydrogenation was confirmed using the HMBC NMR experiment (H-8/C-7, C-9, C-10; H-9/C-7, C-8, C-10, C-11; H-10/C-8, C-9, C-11, C-12) (Fig. 2). Therefore, the structure of this new bisorbicillinoid, dihydrotrichodimerol (**2**), was assigned.

Biological Activities

All isolated compounds, trichodimerol (**1**), dihydrotrichodimerol (**2**), and rezishanones C (**3**) and D (**4**), were tested for the ability to activate peroxisome proliferator-activated receptors using a PPAR-GAL4 chimeric transactivation assay. These four compounds did not activate PPARα and PPARδ up to 10 μg/ml. However, dihydrotrichodimerol (**2**) selectively activated PPARγ with an ED_{50} value of 80 ng/ml (Fig 3, A). The maximum activity of dihydrotrichodimerol (**2**) activating PPARγ was about 75 % of rosiglitazone, a well-known selective PPARγ agonist [22]. Trichodimerol (**1**) has been reported to inhibit

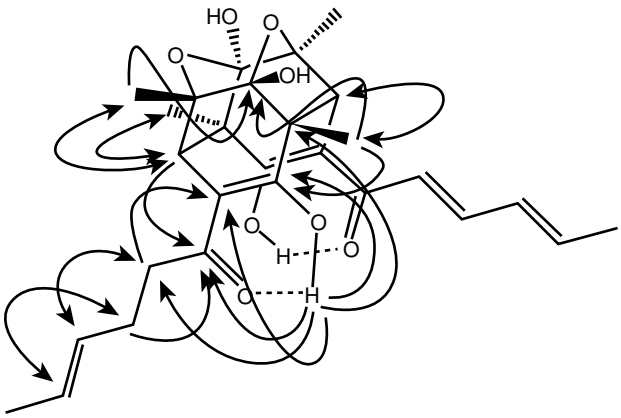

Fig. 2 Selected HMBC correlations of compound **2**.

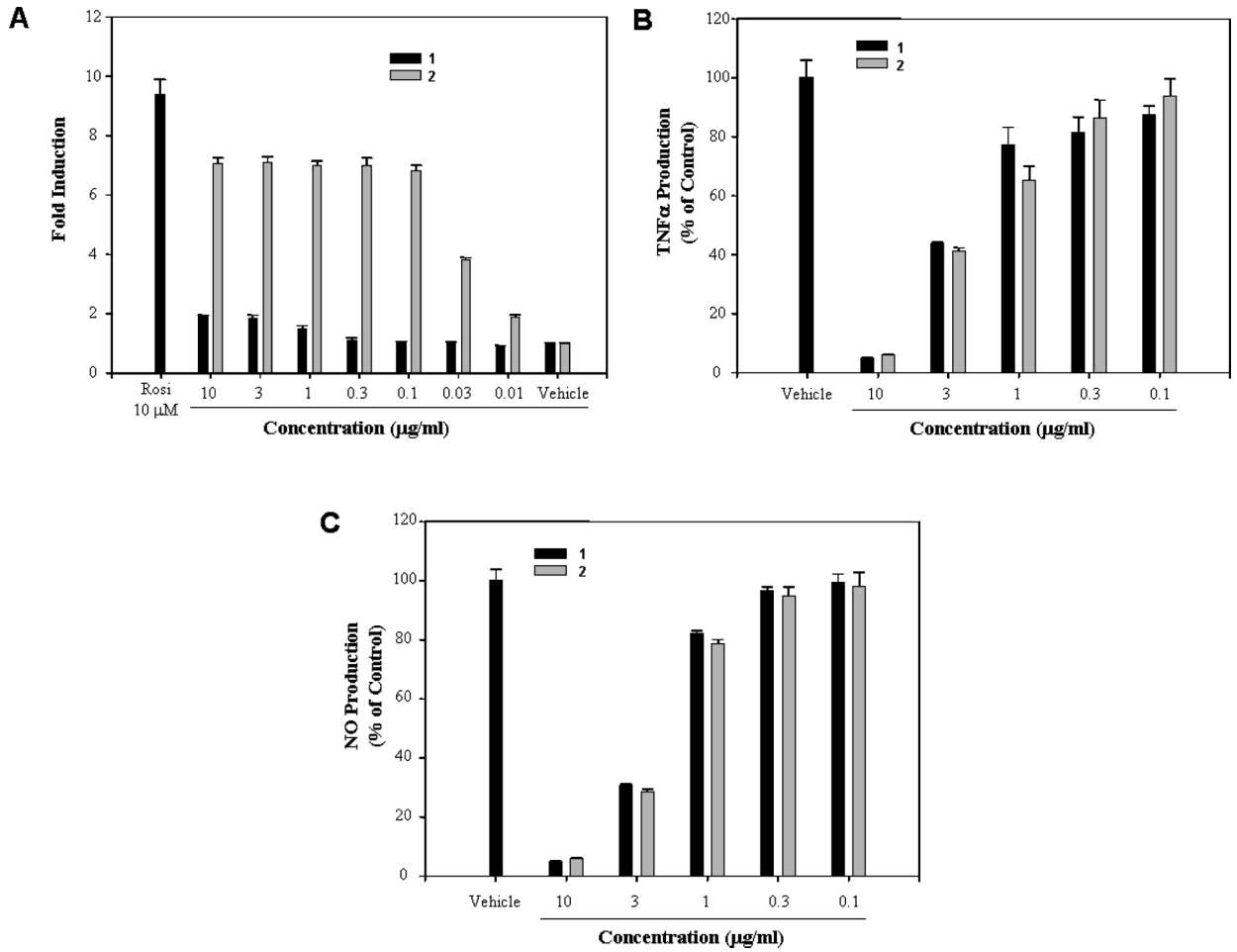

Fig. 3 Effects of compounds **1** and **2** on the activation of peroxisome proliferator-activated receptor γ (A), tumor necrosis factor (TNF)-α production (B), and nitric oxide (NO) production (C).

TNF-α production in LPS-stimulated RAW264.7 cells [11]. Therefore, we compared the effects of compounds **1** and **2** on the production of inflammatory mediators, TNF-α and nitric oxide (NO), in LPS-stimulated RAW264.7 cells. As shown in Fig. 3. (B and C), both compounds **1** and **2** suppressed the production of TNF-α and NO with similar IC_{50} values. Several reports have suggested that PPARγ ligands such as rosiglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 exert their anti-inflammatory activities with a PPARγ-independent mechanism in RAW 264.7 cells [22]. In this regard, trichodimerol (**1**) and dihydrotrichodimerol (**2**) could exert their anti-inflammatory activities independent of PPARγ. Therefore, further investigations are necessary to determine the exact mechanism of dihydrotrichodimerol (**2**) on the PPARγ activation.

Acknowledgements The work was supported by the KRIBB Research Initiative Program and the 21C Frontier Microbial Genomics and Application Center (Grant MG05-0303-2-0 to Y.-S.H.), the Ministry of Science and Technology, Republic of Korea. Korea Basic Science Institute is thanked for providing certain instruments used in this study.

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