NOTE

Tolypoalbin, a new tetramic acid from *Tolypocladium album* TAMA 479

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INTRODUCTION

Many of the species in the order Hypocreales (Ascomycota, Sordariomycetes) are known to be associated with other organisms such as plants, insects and fungi as pathogens, saprobes or symbionts.¹ Recent multigene phylogenetic analyses inferred that the ancestral hypocrealean fungi mainly had taken plant-based nutrients^{1,2} and the species diversity was expanded in association with the host range expansion from plants/debris to animals, insects and fungi.³ Hypocrealean fungi with such diverse lifestyles are believed to produce a variety of bioactive compounds during the interactions with host organisms.^{4,5} *Chaunopycnis* in the family Ophiocordycipitaceae is a saprobic fungus collectable from soil, plant litter and lichens.⁶ The first description of this genus was made in 1980.⁷ To date, 16 secondary metabolites are known from this genus, including 12 indole-diterpenes represented by terpendole E.^{8,9} Very recently, *Chaunopycnis* was integrated into the genus *Tolypocladium*. Accordingly, *C. alba*, *C. ovalispora* and *C. pustulata* were renamed as *T. album*, *T. ovalisporum* and *T. pustulatum*, respectively.¹⁰

In the course of our investigation on metabolite diversity in hypocrealean fungi,^{11,12} a *Tolypocladium* strain was found to produce a new tetramic acid derivative (tolypoalbin, **1**) and its known congener (F-14329, **2**).¹³ Herein, we describe the isolation and structure elucidation of the new compound **1**.

The producing strain TAMA 479 was isolated from a soil sample collected at Machida, Tokyo, Japan. On the basis of morphological and cultural characteristics and ITS sequence similarity, this fungus was identified as *Tolypocladium album*. The strain was cultured in A-3M liquid medium at 30 °C for 12 days, and the whole culture broth was extracted with 1-butanol. The crude extract (7.0 g from 1 l) was subjected to consecutive fractionation using silica gel and ODS column chromatographies, followed by reverse-phase HPLC to yield tolypoalbin (**1**, 46 mg) and F-14329 (**2**, 40 mg) (Figure 1).

Compound **1** was obtained as an optically active, yellow amorphous solid ($[\alpha]_D^{24}$ –158 (c 0.05, MeOH). High-resolution ESITOFMS

analysis in the positive ion mode gave a molecular ion peak $[M+Na]^+$ at m/z 380.1832, corresponding to the molecular formula $C_{21}H_{27}NO_4$ (Δ 0.0 mmu, calcd for $C_{21}H_{27}NO_4Na$) which was consistent with the 1H and ^{13}C NMR data (Table 1). The UV absorption maxima at 220 and 281 nm (MeOH) and characteristic ^{13}C NMR signals at δ_C 194.0, 193.6, 175.3 and 100.5 were suggestive of the presence of tetramic acid/tetronic acid functionality.^{14,15} This tautomeric structure was also supported by the observation that some of the 1H and ^{13}C NMR signals were detected doubly in a ratio of 4:1 in $CDCl_3$ while these signal duplications were not seen in $DMSO-d_6$. Because some down-field carbon signals were not detected in $DMSO-d_6$, structure elucidation was carried out with the major isomer present in $CDCl_3$. The ^{13}C NMR spectrum of **1** showed the presence of 21 carbons including 3 carbonyl carbons, 8 olefinic or aromatic carbons, an upfield sp^2 quaternary carbon, 3 sp^3 methines, 3 sp^3 methylenes and 3 methyl groups (Table 1).

A linearly connected eight-carbon fragment from H_3 -13 to H_3 -15 was identified in the COSY spectrum. To this fragment were attached a methyl group at C-9 and a carbonyl carbon at C-7 by a COSY correlation between H -9 and H_3 -14 and HMBs from H -8 and H_3 -15 to C-6 (δ_C 194.0), providing an acyl side chain. Since the proton signals for H -11 and H -12 were multiplet in the intact 1H NMR spectrum, the coupling constant between H -11 and H -12 could not be read. However, irradiation at the methyl proton H -13 made the splitting pattern look simpler as a doublet for H -12 ($J_{H-12/H-11}$ 15.0 Hz) and a double of triplet for H -11 ($J_{H-11/H-12}$ 15.0 Hz and $J_{H-11/H-10}$ 6.0 Hz): thereby the geometry was established as *E* (Supplementary Information, Figure S4). A 1,4-disubstituted benzene ring was implied by the presence of COSY-correlated protons at δ_H 7.03 and 6.77 each accounting for two protons. These proton signals were broadened in $CDCl_3$ due to the tautomerization of the tetramic acid unit but were clearly split into a doublet (J = 8.5 Hz) in $DMSO-d_6$. HMBs from H -18 and H -22 to C-20 and from H_2 -16 to C-17, C-18, and C-22 completed the disubstituted benzene ring and

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connected a methylene carbon (C-16) to C-17. In addition, the upfield shift for C-19/C-21 (δ_c 115.8) and the downfield shift for C-20 (δ_c 155.0) and C-18/C-22 (δ_c 130.3) suggested the presence of an electron withdrawing group at C-20. The presence of a hydroxy group at C-20 was confirmed by HMBCs from an exchangeable proton (δ_H 9.17) to C-19, C-20, and C-21 (these correlations were detected in DMSO- d_6 but not in CDCl₃). The remaining sp^3 methine carbon (δ_c 63.5) and sp^2 quaternary carbons (δ_c 193.6, 175.3, 100.5) were assembled into a tetramic acid unit in consideration of the characteristic chemical shifts and observed tautomerism, consistent with the HMBCs from H-5 to C-1 and C-4.¹⁶ The *p*-hydroxybenzyl group was connected to C-5 on the basis of a COSY correlation between H-5 and H₂-16 and the acyl side chain was connected to C-3, completing the planar structure of **1** (Figure 2).

tolypaalbin (**1**) : R=H (5*S*, 7*S*^{*}, 9*S*^{*})
F-14329 (**2**) : R=OH (absolute configuration unknown)

Figure 1 Structures of tolypaalbin (**1**) and F-14329 (**2**).

It is proposed that the relative configuration of two methyl groups branched in 1,3-positions in the carbon chain can be assigned on the basis of the chemical shift difference ($\Delta\delta$) between the geminal methylene protons flanked by the methyl branches.¹⁷ If the two methyl groups are placed in *syn* relationship, then the $\Delta\delta$ displays larger values (typically >0.4 p.p.m.). The $\Delta\delta$ is small (typically 0.0–0.1 p.p.m.) if the methyl groups have *anti* relationship. According to this empirical rule, the relative configuration between C-14 and C-15 methyls was proposed to be *syn* ($\Delta\delta$ =0.63 p.p.m.).

The absolute configuration at C-5 was determined by using Marfey's method. LC-MS analysis of the FDLA (1-fluoro-2,4-dinitro-phenyl-5-leucinamide) derivatives prepared after the conventional acid hydrolysis (6 M HCl, 110 °C, 12 h) gave two peaks for L- and D-Tyr in a ratio of 2:1, implying the partial racemization at C-5 during acid hydrolysis. To avoid the epimerization, **1** was alcoholized with EtOH/1 M HCl at 110 °C for 15 min¹⁸ and subsequently hydrolyzed in 0.5 M KOH for 2 h at room temperature. The hydrolysate was similarly derivatized with L-FDLA and analyzed by LCMS. The extracted ion chromatogram for the FDLA-derivatized Tyr (m/z 474, [M-H][−]) gave only one peak for L-Tyr (Supplementary Information, Figure S1). Therefore, the stereochemistry of C-5 in **1** was determined to be *S*.

Compound **2** was obtained as a yellow amorphous solid. High-resolution ESITOFMS gave an [M+Na]⁺ at m/z 396.1781 appropriate for a molecular formula of C₂₁H₂₇NO₅ (Δ 0.0 mmu, calcd for C₂₁H₂₇NO₅Na), corresponding to the addition of one oxygen atom to **1**. Detailed 2D NMR analyses confirmed that **2** was identical

Table 1 ¹H and ¹³C NMR data for tolypaalbin (**1**) and F-14329 (**2**) in CDCl₃

Position	Tolypaalbin (1)			F-14329 (2)		
	δ_c^a	δ_H mult (J in Hz) ^b	HMBC ^{b,c}	δ_c^a	δ_H mult (J in Hz) ^b	HMBC ^{b,c}
2	175.3, qC	—		175.5, qC		
3	100.5, qC	—		100.4, qC		
4	193.6, qC	—		194.5, qC		
5	63.5, CH	3.99, br.d (8.0)	2, 4, 16, 17	65.7, CH	3.98, d (7.0)	2, 4, 16, 17
6	194.0, qC			194.8, qC		
7	34.0, CH	3.80, m	8, 15	34.3, CH	3.70, m	3, 6, 9, 15
8	40.3, CH ₂	1.81, m ^{d,e} 1.18, m ^d	6, 9	40.26, CH ₂ ^g	1.75–1.85, m ^e 1.17, m	6, 7, 9 6, 7, 9, 10, 14, 15
9	31.3, CH	1.38, m		31.3, CH	1.36, m	7, 11, 14
10	40.4, CH ₂	1.98, m ^d 1.81, m ^{d,e}	8, 9, 11, 12, 14 9, 11, 12, 14	40.31, CH ₂ ^g	1.96, m 1.75–1.85, m ^e	8, 9, 11, 12, 14 9, 11, 12, 14
11	129.3, CH	5.38, m ^{d,e}	10, 13	129.2, CH	5.36, m ^{d,e}	10, 12, 13
12	126.4, CH	5.41, m ^{d,e}	10, 13	126.6, CH	5.41, m ^{d,e}	10, 11, 13
13	18.0, CH ₃	1.64, d (5.5)	11, 12	18.0, CH ₃	1.65, d (6.0)	11, 12
14	19.5, CH ₃	0.87, d (7.0)	9	19.5, CH ₃	0.86, d (7.0)	8, 9, 10
15	18.4, CH ₃	1.17, d (6.5)	6, 7, 8	18.3, CH ₃	1.12, d (7.0)	6, 7, 8
16	37.4, CH ₂	3.19, dr. d (14.0) 2.66, dd (14.0, 8.0)	5, 18, 22 4, 5, 17, 18, 22	74.2, CH	4.76, d (7.0)	4, 17, 18, 22
17	128.0, qC			130.0, qC		
18/22	130.3, CH	7.03, br. s	16, 20, 18, 22	128.4, CH	7.17, br d (7.3)	16, 19, 20, 21, 22
19/21	115.8, CH	6.77, br. s		115.6, CH	6.70, br d (7.3)	17, 19, 20, 21
20	155.0, qC			156.4, qC		
4-OH					6.03, br. s	3, 4, 5
20-OH ^f		9.17, s	19, 20, 21			

^aRecorded at 100 MHz.

^bRecorded at 500 MHz.

^cHMBCs are from proton(s) to the indicated carbon.

^d¹H shifts are assigned by HSQC experiments.

^eOverlapping signals.

^fObserved in DMSO- d_6 .

^gInterchangeable.

Figure 2 COSY and key HMBCs for **1**.

Figure 3 Effect of tolypoalbin (**1**) and F-14329 (**2**) on activation of PPAR γ in luciferase ligand assay (V=vehicle (DMSO)).

with F-14329, which was described in a patent as a neutral fat absorption inhibitor from *Chaunopycnis alba* SANK 21805.¹³

Adipocyte differentiation activity of **1** and **2** was evaluated for murine ST-13 preadipocyte cells by Oil Red O staining.¹⁹ Treatment of ST-13 cells with 40 μM of **1** for 11 days induced the differentiation of 58% preadipocyte cells into mature adipocytes in association with the lipid droplets accumulation comparing with the control (Supplementary Information, Figure S2). Compound **2** was not active at the same concentration. The differentiation of preadipocytes into mature adipocytes leads to the secretion of adiponectin, a peptide hormone that can improve insulin resistance in type 2 diabetes mellitus patients. Adipogenesis is controlled by complex network of transcription factors such as peroxisome proliferator-activated receptor γ (PPAR γ), a ligand-dependent transcription factor considered as a master regulator for adipogenesis.²⁰ Thiazolidinediones, antidiabetic drugs used for type 2 diabetes, act as an agonist of PPAR γ . To confirm whether **1** is a PPAR γ agonist, it was tested in a reporter gene assay where the luciferase gene is expressed in response to the binding of PPAR γ to the ligand.¹⁹ In this bioassay, **1** activated luciferase luminescence in the range of 25–100 μM in a concentration-dependent manner whereas no such effects were observed with **2** (Figure 3). Antimicrobial activity of **1** was relatively weak with an MIC value of 40 μg ml⁻¹ against *Micrococcus luteus* and not active against *Escherichia coli* and *Candida albicans* at 40 μg ml⁻¹.

In summary, we isolated a new tetramic acid, tolypoalbin (**1**) along with a known tetramic acid F-14329 (**2**) from a hypocrealean fungus *Tolypocladium album* TAMA 479. Compound **1** induced the differentiation of murine ST-13 preadipocytes while its hydroxylated derivative **2** was inactive. In addition, **1** activated PPAR γ -mediated

transcription in the same concentration range as the adipocyte differentiation was observed. Although a range of biological properties such as antibacterial, fungicidal, anti-HIV and herbicidal activities are known for tetramic acids,²¹ **1** is the first example of this class that has adipogenic activity.

EXPERIMENTAL PROCEDURE

Fermentation

Strain TAMA 479 grown on a slant culture was inoculated into 500 ml K-1 flasks each containing 100 ml of the V-22 seed medium (soluble starch 1.0%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, Tryptone (Difco Laboratories, Sparks, MD, USA) 0.5%, CaCO₃ 0.3%, K₂HPO₄ 0.1%, and MgSO₄·7H₂O 0.05% (pH 7.0)). The inoculated flasks were placed on a rotary shaker (200 r.p.m.) at 30 °C for 4 days. A globular colony produced in the seed culture was transferred into 500 ml K-1 flasks each containing 100 ml of the A-3M production medium (glucose 0.5%, glycerol 2.0%, soluble starch 2.0%, Pharmamedia (Traders Protein, Memphis, TN, USA) 1.5%, yeast extract 0.3%, Diaion HP-20 (Mitsubishi Chemical, Kanagawa, Japan) 1.0% (pH 7.0)). The inoculated flasks were placed on a rotary shaker (200 r.p.m.) at 30 °C for 12 days.

Extraction and isolation

At the end of the fermentation period, 100 ml of 1-butanol was added to each flask. They were allowed to shake on a rotary shaker (200 r.p.m.) for 1 h. The mixture was centrifuged at 5000 r.p.m. for 10 min and the organic layer was separated from the aqueous layer containing mycelium. Evaporation of the organic solvent provided approximately 7.0 g of extract from 2 l of culture. The crude extract was chromatographed on a silica gel column with a step gradient of CHCl₃/MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1 and 0:1 v/v).

The 4:1 fraction was evaporated followed by further purification by silica gel column chromatography using *n*-hexane/EtOAc (4:1, 2:1, 1:1 and 0:1 v/v) and CH₃Cl/MeOH (5:1 and 0:1 v/v). Fractions 4–6 (*n*-hexane/EtOAc 0:1 and CH₃Cl/MeOH fractions) were combined and finally purified by preparative reverse-phase HPLC (CAPCELL PAK C₁₈ MGII S5 20 × 150 mm, 16 ml min⁻¹) with MeCN–0.1% formic acid (60:40) to yield tolypoalbin (**1**, 46 mg, *t*_R 12.8 min). Similarly, the fraction eluted with CHCl₃/MeOH (2:1) was evaporated and subjected to HPLC purification (CAPCELL PAK C₁₈ MGII S5 20 × 150 mm, MeCN–0.1% formic acid (55:45), 16 ml min⁻¹). The collected fraction was concentrated to dryness to give F-14329 (**2**, 40 mg, *t*_R 12.0 min).

Tolypoalbin (**1**): yellow amorphous solid, [α]_D²⁴ –158 (c 0.05, MeOH); IR λ_{max} (ATR) 3204, 1652, 1603, 1595, 1515, 1448, 1237 cm⁻¹; UV (MeOH) λ_{max} (log ε) 229 (4.09), 242 (4.04), 282 (4.14) nm, (MeOH/0.01 M HCl) λ_{max} (log ε) 223 (4.08), 280 (4.13) nm, (MeOH/0.01 M NaOH) λ_{max} (log ε) 243 (4.25), 281 (4.22) nm; ¹H and ¹³C NMR (Table 1); HR-ESITOFMS *m/z* [M+Na]⁺ 380.1832 (found for C₂₁H₂₇NO₄Na, 380.1832).

F-14329 (**2**): colorless amorphous solid, [α]_D²⁴ –140 (c 0.05, MeOH) {lit. [α]_D²⁴ –220 (c 1.0, MeOH)}; IR λ_{max} (ATR) 3242, 1651, 1606, 1598 cm⁻¹; UV (MeOH) λ_{max} (log ε) 228 (4.00), 244 (3.92), 282 (4.03) nm, (MeOH/0.01 M HCl) λ_{max} (log ε) 224 (4.00), 280 (4.00) nm, (MeOH/0.01 M NaOH) λ_{max} (log ε) 243 (4.16), 280 (4.11) nm; ¹H and ¹³C NMR (Table 1); HR-ESITOFMS *m/z* [M+Na]⁺ 396.1781 (found for C₂₁H₂₇NO₅Na, 396.1781).

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