Preliminary Communication

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12T061C, a new Julichrome family radical scavenger from *Streptomyces* species

Abstract: The new radical scavenger 12T061C ($C_{20}H_{22}O_7$) has been identified from a culture of *Streptomyces* species. Spectroscopic elucidation showed that this compound is a new Julichrome family compound. 12T061C shows a potent radical-scavenging activity with an ED_{50} of 18 μM that is similar to those of the known antioxidant compounds α-tocopherol (ED_{50} 16 μM) and quercetin (ED_{50} 3 μM).

Keywords: 2,2-diphenyl-1-picrylhydrazyl (DPPH); Julichrome; radical scavenger; *Streptomyces* species.

DOI 10.1515/hc-2014-0126
Received July 22, 2014; accepted August 20, 2014

Introduction

We have recently identified several bioactive compounds that act as radical scavengers from a microorganism culture and from a vegetable [1–3]. Free radicals cause oxidative stress in various organs in the human body and also induce inflammatory diseases, whereas those present in food can cause deterioration of the nutrient factor as a result of lipid peroxidation [4, 5]. We have focused on the identification of new bioactive compounds based on their radical scavenging activity. Our screening program involves high-performance liquid chromatography (HPLC) with an electrochemical detection (ECD) as the first screening step. ECD can evaluate an oxidation-redox potential for each compound, such as antioxidants and radical scavengers. The next step involves a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay that, in this case, has allowed us to identify a new radical scavenger, namely 12T061C (**1**, Figure 1) from the culture broth of *Streptomyces* sp. 12T061. Herein we describe the cultivation of the strain, extraction, isolation, and structural elucidation of compound **1** and the determination of its radical-scavenging activity.

Around 300 *Streptomyces* sp. strains obtained from soil samples were evaluated in our initial screening program, 21 of which showed the positive peak in the HPLC-ECD analysis. The culture broths of these positive strains were extracted with EtOAc at pH 3.0 and then developed by thin-layer chromatography (TLC, silica gel, $CHCl_3$/MeOH, 95:15). The radical scavenging activity of each strain was monitored using a colorimetric assay involving DPPH solution, which was sprayed after development of the TLC plates. We selected two positive strains for further investigation in this secondary screening. The strain *Streptomyces* sp. 12T061 was one of the positive strains that produced some radical scavengers in the culture broth. This strain was inoculated into 9.6 L of medium (glucose, 30 g/L; malt extract, 2 g/L; yeast extract, 2 g/L; pH 7.3) in an Erlenmeyer flask and cultivated for 14 days at 30°C. The culture broth was then filtered, and the resulting filtrate was acidified to pH 3 and extracted with EtOAc. The crude extract was purified by silica gel column chromatography using a hexanes/acetone system as eluent. After washing with hexanes, spots attributable to radical scavengers were detected on the TLC plate. The active acetone fraction was further purified by Sephadex LH-20 chromatography (MeOH) and preparative HPLC. Finally, 30 mg of the new radical scavenger 12T061C (**1**) was isolated as a yellowish amorphous powder from this culture broth (9.6 L). Further details of the isolation procedure are provided in the experimental section. Actually, we found other active compounds in the culture of this strain in

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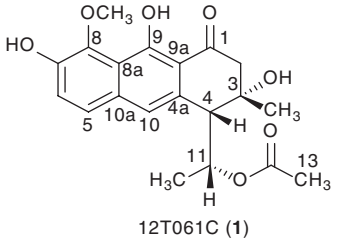

Figure 1 The structure and the numbering scheme of compound **1**.

addition to 12T061C. We are now investigating the purification, structure elucidation, and biological activity of these new active compounds.

The chemical structure of **1** was elucidated spectroscopically. The high-resolution fast atom bombardment mass spectrum (HR-FAB-MS) indicates that the molecular formula of **1** is $C_{20}H_{22}O_7$, and the IR spectrum of this compound confirms the presence of carbonyl and hydroxy groups. The ^{1}H NMR spectrum of **1** (400 MHz, DMSO-d_6) shows two aromatic OH signals in the range δ_H 9.0–15.0, three olefinic signals in the range δ_H 7.0–7.5, one hetero connecting signal at δ_H 5.61 (1H, qd, J = 6.1 and 1.5 Hz), one broad signal at δ_H 5.26, one methoxy signal at δ_H 3.80, asymmetric methylene signals (δ_H 2.84 and 2.58), one broad singlet at δ_H 2.82, and three methyl signals (δ_H 1.69, 1.20, and 1.16). The ^{13}C NMR (100 MHz, DMSO-d_6) and distortionless enhancement by polarization transfer spectra show one ketone signal at δ_C 204.53, one carbonyl signal at δ_C 169.09, ten olefinic carbon signals in the range of δ_C 110–165, three signals for hetero connecting carbons in the range of δ_C 60–70, two signals for carbons in the range of δ_C 40–60, and three methyl signals in the range of δ_C 10–30. The ^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear multiple-quantum coherence, and heteronuclear multiple-bond correlation (HMBC) spectra suggest a planar structure for **1** (Figure 2). The presence of the naphthalene system is supported by the measurement of ^{1}H-^{1}H COSY and HMBC. The coupling between H-5 and H-6 in the ^{1}H-NMR spectrum and ^{1}H-^{1}H COSY is clearly seen. These two olefinic protons are correlated with several olefinic carbons, respectively (Figure 2). The two aromatic OH signals are key to this structure elucidation of this moiety. In the HMBC experiment, the cross-peaks from an aromatic OH proton (δ_H 9.45) at C-6, C-7, and C-8 are seen. The carbon C-8 is also correlated with the C-8-methoxy methyl in that HMBC experiment. Another aromatic OH proton (δ_H 14.59) is correlated with C-8a, C-9, and C-9a in this spectrum. These observations and the HMBC cross-peaks from the aromatic proton 10-H (δ_H 7.15) suggest the existence of the naphthalene system, which is composed of carbon atoms 5, 6, 7, 8, 9, 10, 4a, 8a, 9a, and 10a and substituted with a methoxy group at C-8. The naphthalene moiety is attached to cyclohexenone ring at C-4a and C-9a. This cyclohexenone ring is composed of carbon atoms 1, 2, 3, 4, 4a, and 9a and is substituted with a methyl group at C-3. The chemical shift of the 9-OH (δ_H 14.59) suggests that this proton forms a hydrogen bond (Figure 2). Finally, a side chain containing an acetoxy moiety (carbon atoms 11, 12, 13, and C-11-CH_3) is connected to C-4 to form a planar-like structure in **1**. Owing to this structure, compound **1** is a new member of the Julichrome family. Although several derivatives of this group have been identified previously [6–8], **1** is unique in its structural novelty.

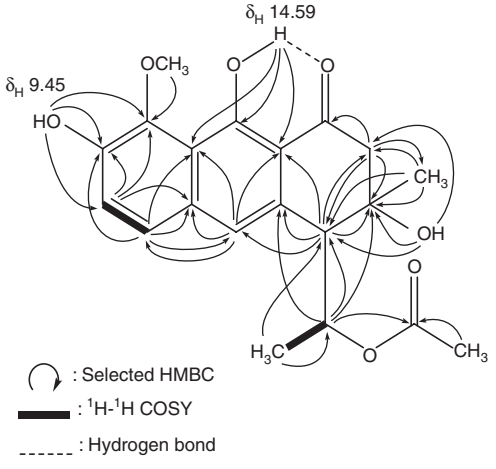

Figure 2 The structure of **1** derived by NMR methods.

The relative stereochemistry of **1** was elucidated on the basis of the coupling constants in the ^{1}H NMR spectrum and differential NOE experiments. Thus, the NOE for 2-H (δ_H 2.58)/3-CH_3 and 3-CH_3/4-H indicate that these protons are arranged on the upper side in our model. In addition, the small coupling constant (J = 1.5 Hz) between 4-H and 11-H in the ^{1}H NMR spectrum indicates that these two protons form a gauche arrangement. We propose the relative stereochemistry of **1** as shown in Figure 3. This

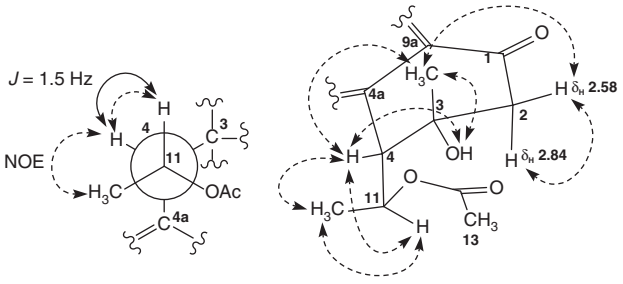

Figure 3 The relative stereochemistry of **1** obtained by NMR methods.

stereochemistry is consistent with the structures of previously identified Julichrome-type compounds (Figure 4). The suggested absolute stereochemistry of **1** is shown in Figure 1. Almost all of the Julichrome family derivatives reported previously have the absolute stereochemistry shown in Figure 4. Consequently, considering the biosynthetic nature of these compounds, **1** should have the same stereochemistry as these known Julichromes. More specifically, the stereochemistry of **1** (Figure 1) is proposed to be identical to that of known Julimycins or Julichromes. Almost all such compounds have a dimeric structure (Figure 4). Shaaban et al. [8] have reported Julichrome Q6 glucuronide as a first monomeric Julichrome-type compound with cytotoxic activity against tumor cells. In this paper, we report 12T061C (**1**) as a second monomeric Julichrome-type compound known to date. The results of our DPPH radical assay indicate that **1** shows radical scavenging activity with an ED_{50} of 18 μM. Two known antioxidants, namely α-tocopherol (ED_{50} 16 μM) and quercetin (ED_{50} 3 μM), were used as positive controls in this assay. During the course of this study, we found several radical scavenging compounds in the culture extract of *Streptomyces* sp. 12T061. Derivative 12T061C (**1**) is one such compound, and we are currently investigating other monomeric and dimeric Julichromes.

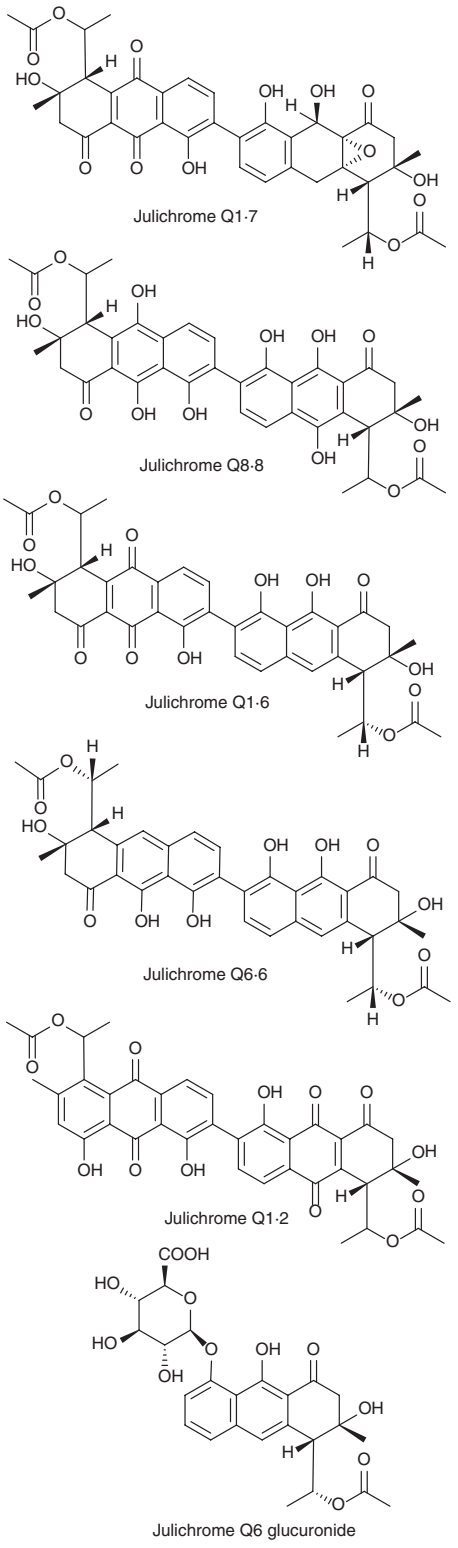

Figure 4 Previously identified Julichrome-type natural products.

In summary, we have successfully isolated and characterized 12T061C (**1**), a new Julichrome compound with potent DPPH radical scavenging activity, from a culture of *Streptomyces* sp. 12T061.

Experimental

DMSO-d_6 for NMR measurements was obtained from Cambridge Isotope Laboratories (Andover, MA, USA), the yeast extract was purchased from Kyokuto (Tokyo, Japan), and trimethylsilyldiazomethane diethyl ether solution was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Spectroscopic measurements were performed using the following instruments: NMR, Jeol (Tokyo, Japan) ECX-400P spectrometer; HR-FAB-MS, Jeol (Tokyo, Japan) JMS-700; UV-Vis spectra, Hitachi (Tokyo, Japan) U2900A spectrometer; melting point, AS ONE (Osaka, Japan) ATM-01; IR, Perkin Elmer (Perkin Elmer Japan, Yokohama, Japan) Spectrum One FT-IR; analytical HPLC, Hitachi (Tokyo, Japan) L-2130 (pump) and L-2400 (UV), Esa Coulochem III (ECD, electrochemical detector, 300 mV; Thermo Scientific, Yokohama, Japan); preparative HPLC, GL-Science (Tokyo, Japan) PU715 (pump) and UV702 (UV-Vis).

Cultivation of *Streptomyces* species 12T061

The 12T061 strain was inoculated into 800 mL of medium (glucose, 30 g/L; malt extract, 2 g/L; yeast extract, 2 g/L; pH 7.3) in a 2-L Erlenmeyer flask and cultivated at 30°C for 14 days on a rotary shaker (150 rpm).

Isolation of 12T061C (1)

The filtrate from the *Streptomyces* sp. 12T061 culture broth (9.6 L) was acidified to pH 3.0 with hydrochloric acid and extracted using an equal volume of EtOAc. After concentration under reduced pressure, the crude extract was purified by silica gel column chromatography. After washing with hexanes, the subsequent acetone eluent showed DPPH radical-scavenging activity. The acetone fraction was analyzed by TLC. After development using a $CHCl_3$/MeOH (95/15) system, the TLC plate was sprayed with a 2% DPPH/EtOH solution. The radical scavenger reduces the DPPH radical to the corresponding hydrazine, as seen by the color change from purple to colorless. The active fraction was then further purified by Sephadex LH-20 column chromatography (MeOH) and preparative HPLC (75% MeOH/0.1% TFA and 40% CH_3CN/0.1% TFA; Shiseido (Tokyo, Japan) Capcell Pak C18 MG II column, φ20×250 mm; UV, 280 nm) to yield 30 mg of **1**.

Characterization of 12T061C (1)

This compound was obtained as a yellowish amorphous powder; mp 76–85°C; $[\alpha]_D$ +146° (*c* = 0.660, MeOH); UV-Vis: λ_{max} in MeOH, (ε) 223 (530,000), 256 (520,000), 410 nm (130,000); IR (ATR) ν_{max} 3360, 2971, 1605, 1376, 1237 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ_H 14.59 (s, 1H, 9-OH), 9.45 (s, 1H, 7-OH), 7.48 (d, 1H, *J* = 9.2 Hz, 5-H), 7.34 (d, 1H, *J* = 9.2 Hz, 6-H), 7.15 (s, 1H, 10-H), 5.61 (qd, 1H, *J* = 6.1 and 1.5 Hz, 11-H), 5.26 (br.s, 1H, 3-OH), 3.80 (s, 3H, 8-OCH_3), 2.84 (d, 1H, *J* = 18.3 Hz, 2-H), 2.58 (dd, 1H, *J* = 18.3 and 1.6 Hz, 2-H), 2.82 (br.s, 1H, 4-H), 1.69 (s, 3H, *J* = 9.2 Hz, 13-H), 1.20 (d, 3H, *J* = 6.1 Hz, 11-CH_3), 1.16 (s, 3H, 3-CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ_C 204.5 (s, C-1), 169.1 (s, C-12), 162.2 (s, C-9), 147.9 (s, C-7), 143.3 (s, C-8), 132.2 (s, C-10a), 131.8 (s, C-4a), 123.9 (d, C-5), 123.0 (d, C-6), 119.8 (d, C-10), 119.1 (s, C-8a), 111.1 (s, C-9a), 68.9 (s, C-3), 67.8 (d, C-11), 61.0 (q, C-8-OCH_3), 54.6 (d, C-4), 48.8 (t, C-2), 30.6 (q, C-3-CH_3), 20.8 (q, C-13), 19.2 (q, C-11-CH_3). HR-FAB-MS. Calcd for $C_{20}H_{22}O_7$ $[M+H]^+$: *m/z* 375.1444. Found: *m/z* 375.1440.

Measurement of DPPH radical scavenging activity

This assay was performed using a previously reported spectroscopic method [9, 10]. Briefly, a MeOH solution (2 mL) of each sample was mixed with a 0.5-mM solution of DPPH in MeOH (1 mL) in 0.1 M acetate buffer (pH 5.5, 2 mL). The solution was then allowed to stand for 30 min, and the absorbance (517 nm) was measured. The absorbance of pure MeOH was measured in a blank test. The DPPH radical-scavenging activity of each sample was recorded against the blank and expressed in percentage. The ED_{50} values were taken to be the concentrations required for 50% DPPH radical scavenging activity.

Supplementary material (online edition only): Primary NMR data of compound **1**.

References

[1] Komoda, T.; Akasaka, K.; Hirota, A. Ansaetherone, a new radical scavenger from Streptomyces sp. *Biosci. Biotechnol. Biochem.* **2008**, *72*, 2392–2397.

[2] Komoda, T.; Ono, M.; Koseki, Y.; Nishikawa, M. Sesquiterpene lactones from Asparagus chicory. *J. Clin. Biochem. Nutr.* **2008**, *73*, 575–578.

[3] Komoda, T.; Tamiya, Y.; Nishikawa, M. 10T024A, a new phenazine derivative, as a radical scavenger and a prostaglandin, leukotriene release suppressor. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 2056–2058.

[4] Inoue, K.-i.; Takano, H.; Kaewamatawong, T.; Shimada, A.; Suzuki, J.; Yanagisawa, R.; Tasaka, S.; Ishizaka, A.; Satoh, M. Role of metallothionein in lung inflammation induced by ozone exposure in mice. *Free Radic. Biol. Med.* **2008**, *45*, 1714–1722.

[5] Musiek, E. S.; Brooks, J. D.; Joo, M.; Brunoldi, E.; Porta, A.; Zanoni, G.; Vidari, G.; Blackwell, T. S.; Montine, T. J.; Milne, G. L.; et al. Electrophilic cyclopentenone neuroprostanes are anti-inflammatory mediators formed from the peroxidation of the ω-3 polyunsaturated fatty acid docosahexaenoic acid. *J. Biol. Chem.* **2008**, *283*, 19927–19935.

[6] Tsuji, N.; Nagashima, K. Julimycins. VIII. Structures of julichromes Q1.7, Q8.8, Q3.8, Q3.3, and Q1.9. *Tetrahedron* **1970**, *26*, 5201–5213.

[7] Tsuji, N.; Nagashima, K. Julimycins. VII. Structures of julichromes Q1.6, Q6.6, and Q5.6. *Tetrahedron* **1971**, *27*, 1557–1563.

[8] Shaaban, K. A.; Shaaban, M.; Gruen-Wollny, I.; Maier, A.; Fiebig, H. H.; Laatsch, H. Julichrome Q6 glucuronide, a monomeric subunit of the julimycin B-I complex from a terrestrial Streptomyces sp. *J. Nat. Prod.* **2007**, *70*, 1545–1550.

[9] Chen, Y.-C.; Sugiyama, Y.; Abe, N.; Kuruto-Niwa, R.; Nozawa, R.; Hirota, A. DPPH radical-scavenging compounds from dou-chi, a soybean fermented food. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 999–1006.

[10] Washida, K.; Abe, N.; Sugiyama, Y.; Hirota, A. Novel DPPH radical scavengers, demethylbisorbibutenolide and trichopyrone, from a fungus. *Biosci. Biotechnol. Biochem.* **2007**, *71*, 1052–1057.

Supplemental Material: The online version of this article (DOI: 10.1515/hc-2014-0126) offers supplementary material, available to authorized users.