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Two New 5-Hydroxy-2-pyrone Derivatives Isolated from a Marine-derived Fungus *Aspergillus flavus*

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Abstract Two new compounds, 4-(hydroxymethyl)-5-hydroxy-2*H*-pyran-2-one (**1**) and (5-hydroxy-2-oxo-2*H*-pyran-4-yl) methyl acetate (**2**), have been isolated from a marine-derived fungus *Aspergillus flavus*. Their structures were determined by spectroscopic data. Compound **1** induced the production of cAMP on GPR12 transfected CHO and HEK293 cells in a dose-dependent manner, which indicated **1** might be a possible ligand for GPR12.

Keywords marine-derived fungus, *Aspergillus flavus*, 5-hydroxy-2-pyrone, cAMP, GPR12

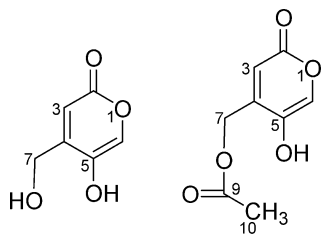
Introduction

2-Pyrone is a six-membered cyclic unsaturated ester, which is highly abundant in bacteria, microbial, plant, insect and animal systems and takes part in many different types of biological processes such as defence against other organisms, as key biosynthetic intermediates, and as metabolites [1]. A wide range of bioactivities such as plant growth-regulating [2, 3], antitumour [4, 5], antimicrobial and antifungal activities [6, 7] have demonstrated the medicinal importance of 2-pyrones. In our search for new bioactive compounds from the marine-derived fungus *Aspergillus flavus* c-f-3 separated from a marine algae sample collected in Putian Pinghai, China, two new 5-hydroxy-2-pyrone derivatives (**1**, **2**) were isolated. The biological activities of **1** and **2** were tested using cAMP assay in GPR12 transfected CHO and HEK293 cells. In this paper we described the isolation and structure elucidation of **1** and **2** (Fig. 1) as well as their bioactivities.

Materials and Methods

Microorganism

The *Aspergillus flavus* c-f-3 was separated from a marine algae *Enteromorpha tubulosa*, collected at Putian Pinghai, China, in August, 2005, using dilution-plate method [8] on PDA medium [9]. It was identified according to its morphological characteristic by Prof. Hong Kui (the Chinese Academy of Tropical Agricultural Sciences, Hainan, China). Working stocks were prepared on Potato Dextrose agar slants stored at 4°C.



1 **2**

Fig. 1 Structures of compounds 4-(hydroxymethyl)-5-hydroxy-2*H*-pyran-2-one (**1**) and (5-hydroxy-2-oxo-2*H*-pyran-4-yl) methyl acetate (**2**).

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Fermentation

A small spoon of spores growing on potato dextrose agar slant was inoculated into a 500 ml conical flask containing 150 ml of the liquid medium composed of glucose (1.0%), maltose (2.0%), and yeast extract (0.3%), mannitol (2.0%), monosodium glutamate (1.0%), KH_2PO_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.03%), corn plasm (0.1%) and artificial seawater after adjusting its pH to 6.5, and cultured at 28°C for three days on a rotary shaker at 165 rpm. Then 10 ml of the resultant seed culture was inoculated into 500 ml conical flask each containing 150 ml of the above culture medium and incubated at 28°C for seven days on a rotary shaker at 165 rpm.

Extraction and Isolation

The culture whole broth (30 liters) was filtered through cheesecloth to separate into supernatant and mycelia. The supernatant (26 liters) was extracted with ethyl acetate (3×30 liters), while the mycelia were extracted with 70% acetone-aqueous (3×4 liters). The acetone solution was concentrated under reduced pressure to afford an aqueous solution, which was extracted with ethyl acetate (3×3 liters). Both ethyl acetate solutions were combined and concentrated under reduced pressure to give a crude extract (30.2 g).

The crude extract was separated into eight fractions on a silica gel (300~400 mesh) column using a step gradient elution of $CHCl_3$ - MeOH (100% - 50%). The sixth fraction of 5.0% MeOH, was chromatographed on a Sephadex LH-20 column with 50% $CHCl_3$/MeOH. Every 100 ml eluent were collected and combined into four fractions based on TLC properties. The third fraction (1.7 g) was rechromatographed on a column of reverse silica gel (RP18, 40~60 μm, Merck) with H_2O/MeOH gradient system and the 100% H_2O eluate, containing the mixture of **1** and **2**, which showed red color when sprayed chromatoplate with $FeCl_3$ alcohol solution, was further purified by semipreparative HPLC using an ODS column [YMC-pack ODS (A), 10×250 mm, 5 μm, 4 ml/minute, 40% MeOH/H_2O] to give **1** (1.1 g) and **2** (9.7 mg).

Biological Assays

Plasmid Construction

Full-length cDNA of human GPR12 was amplified by PCR from pCMV6-XL4-GPR12 vector (a gift from Dr. Cecilia Jiang, Genomics Institute of Novartis Research Foundation, San Diego, USA) using the gene-specific primers 5′-GGGGTACCATGAATGAAGACCTGAAGG-TC-3′ and 5′-CCGCTCGAGCTACACATCACTGGGCG-AG-3′ for expression. The GPR12 PCR product was transferred into the KpnI-XhoI sites of the vector Myc-pcDNA3.1 (+) (also a gift from Dr. Cecilia Jiang) and sequenced to ensure correct insertion and sequence.

Cell Culture, Transfection and Subclone Selection

Chinese Hamster Ovary cells (CHO-K1, preserved in our lab) were cultured in RPMI Medium 1640 (with L-glutamine, without sodium bicarbonate, GIBCO) supplemented with 10% fetal bovine serum (FBS, PAA), penicillin/streptomycin (10,000 IU/ml - 10,000 ug/ml, PAA) and 400 ug/ml G418 (Calbiochem), pH 7.2, in a humidified 5.0% CO_2 incubator. Transfections were performed by using Lipofectamine^{TM} 2000 (invitrogen) as directed by the manufacturer for a 12-well plate, 2.0 μg of pcDNA3.1-myc -GPR12 vector and 3.0 μl lipofectamine^{TM} 2000 reagent were used. 48 hours after the transfection, 750 μg/ml G418 (Calbiochem) was added, and stable single clones were obtained by macrography selection using gradient dilution in 96-well plate. These clones were expanded in growth medium with G418 (500 μg/ml).

Cell Based cAMP Assay

PcDNA3.1-GPR12-transfected CHO (GPR12-CHO) subclone and PcDNA3.1-transfected CHO (vector-CHO) were cultured in 384-well plates for 24 hours. Both cells were treated with drugs for 30 minutes at room temperature and measured by Htrf cAMP dynamic bulk kit which is intended for direct quantitative determination of cyclic AMP. Fluorescence was detected at 620 and 665 nm using Analyst HT (Molecular Devices).

Data Evaluation and Statistic Analysis

The sigmaplot (Systat Software Inc, USA) was used for curve fitting; the *t* test was applied to evaluate statistical significance.

Results and Discussion

Physico-chemical Properties

Compound **1**: colorless crystal (MeOH); mp 138~140°C; HRESI-MS *m/z* 141.0183 for $[M-H]^-$ (calcd. for $C_6H_5O_4$, 141.0188); IR (KBr) ν_{max} 3258, 3176, 1615, 1460, 1283 cm^{-1}; UV (MeOH): λ_{max} nm (log ε) 213 (4.75), 273 (3.91); ^{1}H- and ^{13}C-NMR (DMSO-d_6, 600 MHz and 150 MHz) see Table 1.

Compound **2**: amber amorphous solid; mp 118~120°C; HRESI-MS *m/z* 207.0264 for $[M+Na]^+$ (calcd. for $C_8H_8O_5Na$, 207.0269); UV (MeOH): λ_{max} nm (log ε) 217 (4.63), 269 (4.35); ^{1}H- and ^{13}C-NMR (DMSO-d_6, 600 MHz and 150 MHz) see Table 1.

Table 1 ^{1}H- and ^{13}C-NMR , HMBC data for 4-(hydroxymethyl)-5-hydroxy-2*H*-pyran-2-one (**1**) and (5-hydroxy-2-oxo-2*H*-pyran-4-yl) methyl acetate (**2**) (DMSO-d_6, 600 and 150 MHz, TMS, δ in ppm)

No.	**1**			**2**		
	δ_H (*J* in Hz)	δ_C	HMBC	δ_H (*J* in Hz)	δ_C	HMBC
2		173.9 s			173.7 s	
3	6.34 (1H, s)	109.8 d	2, 4, 5, 7	6.47 (1H, s)	112.5 d	2, 4, 5, 7
4		145.7 s			146.0 s	
5		168.1 s			161.6 s	
6	8.03 (1H, s)	139.2 d	2, 4, 5	8.09 (1H, s)	139.9 d	2, 4, 5
7	4.29 (2H, s)	59.5 t	3, 5	4.93 (2H, s)	61.3 t	3, 5, 9
9					169.8 s	
10				2.11 (3H, s)	20.4 q	9
5-OH	9.07 (1H, s)		6	9.26 (1H, s)		
7-OH	5.67 (1H, s)					

Structure Determination

Compound **1** was obtained as a colorless crystal. High-resolution ESI-MS revealed a molecular ion peak at *m*/*z* 141.0183 for $[M-H]^-$ (calcd. 141.0188) corresponding with the molecular formula $C_6H_6O_4$ and four degrees of unsaturation. The IR spectrum absorptions at 1615 and 3176 cm^{-1} indicated the presence of a carbonyl group and a hydroxyl functionality. The ^{13}C- and DEPT NMR spectra revealed the presence of one oxygenated aliphatic methylene carbon (δ_C 59.5), two olefinic methine carbons (δ_C 109.8, 139.2), one enolic carbon (δ_C 168.1), one quaternary olefinic carbon (δ_C 145.7), and a carbonyl carbon (δ_C 173.9). The above data indicated that **1** possessed a hydroxy-substituted 2-pyrone ring [10]. ^{1}H- and ^{13}C-NMR data of **1** were similar to those of 5-hydroxy-α-pyrone (except for the increase of one hydroxymethyl) [11]. The presence of the 4-hydroxymethyl of **1** was indicated by the ^{1}H-NMR [4.29 (2H, s), 5.67 (1H, s)]. Additionally, HMBC correlations (Fig. 2) from δ_H 6.34 (1H, s, H-3) to C-2, C-4, C-5 and C-7, from δ_H 8.03 (1H, s, H-6) to C-2, C-4 and C-5, from δ_H 4.29 (2H, s, H-7) to C-3, C-5 established the structure of **1** as 4-(hydroxymethyl)-5-hydroxy-2*H*-pyran-2-one.

Compound **2** was obtained as an amber amorphous solid. High-resolution ESI-MS supported the molecular formula $C_8H_8O_5$ (*m*/*z* 207.0264 for $[M+Na]^+$, calcd. 207.0269) with five degrees of unsaturation. Comparison of the NMR spectra of **1** and **2** indicated their close similarities. The differences of them were the replacement of 7-OH (δ 5.67) in **1** by an acetyl group (δ_H 2.11, δ_C 169.8, 20.4) in **2**. The HMBC correlations (Fig. 2) from δ_H 2.11 (3H, s, H-10) to C-9 (δ 169.8, C), from δ_H 4.93 (2H, s, H-7) to C-3, C-5 (δ 161.6), C-9 indicated an upfield shift of C-5 in **2** at 161.6 ppm compared to 168.1 ppm in **1** and the new acetyl carbonyl in **2** at 169.8 ppm. These data established the structure of **2** as (5-hydroxy-2-oxo-2*H*-pyran-4-yl) methyl acetate.

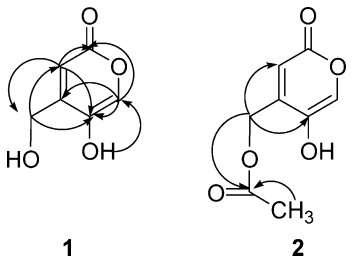

Fig. 2 Key HMBC correlations for **1** and **2**.

Biological Activities

Compounds **1** and **2** were tested by cAMP assay [12] on GPR12-transfected cells and their vector-transfected controls, including Chinese hamster ovary cells (CHO) and human embryonic kidney cells (HEK293). Our results (Fig. 3) showed **1** could promote a small but significant cAMP increase in a dose-dependent manner on both GPR12-CHO cells and GPR12-HEK293 cells, while no significant difference on their vector controls, however, 2 didn't show any activity at the concentration of 100 μM.

The cytotoxicities of **1** and **2** were evaluated on HL-60 and A-549 cell lines by the MTT method [13], but neither of them registered any cytotoxicity at the concentration of 100 μM.

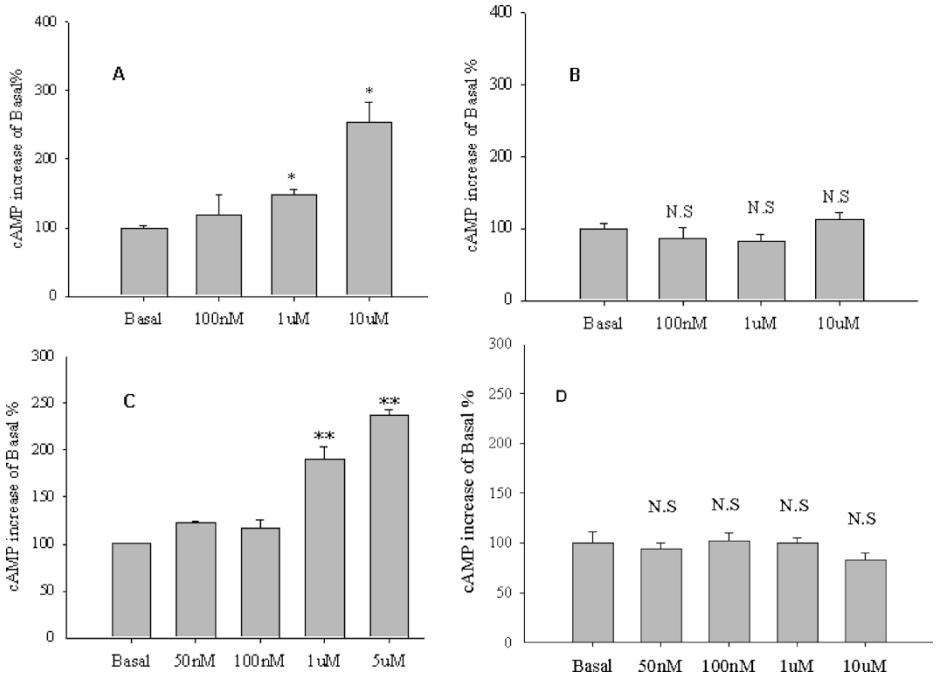

Fig. 3 Characterization of compound **1** using cAMP assay on GPR12 transfected cells, including CHO and HEK293.

1 promoted cAMP increase on GRR12-CHO cells (A); NS on vector-CHO cells (B); on GPR12-HEK293 cells (C); on vector-HEK293 cells (D); The Sigmaplot (Systat Software Inc, USA) was used for curve fitting; the *t* test was applied to evaluate statistical significance. Statistical significant was indicated: * $P<0.05$, ** $P<0.005$, N.S refers to no significant difference.

Discussion

G protein-coupled receptors (GPCRs) have been a productive and useful target for the pharmaceutical industry and recently some of these receptors seem to be potent targets of an estimated 45% of marketed drugs [14]. Therefore, the uncharacterized orphan GPCRs can be expected to represent possible novel therapeutic targets in the future [15]. The GPR12 homologue was initially cloned from rat pituitary gland [16] and human GPR12 was cloned in 1995 [17]. The latest studies show that GPR12 together with GPR3 and GPR6 are highly expressed in the central nervous system and their expression in neurons results in constitutive stimulation of cAMP production, stimulates neurite outgrowth, and counteracts myelin inhibition, indicating that GPR12 can be significant molecular target for treating a variety of neurological disorders, including brain and spinal cord injuries, stroke, and neurodegenerative disorders [18].

Early in 2002, Uhlenbrock *et al.* reported sphingosine-1-phosphate (S1p) was possibly a ligand for GPR12 [12]. And several months later, Ignatov *et al.* reported sphingosyl-phosphorylcholine (SPC) was a much higher-affinity ligand than S1p for GPR12 [19]. We for the first time reported the 2-pyrone derivative might be a possible ligand for GPR12. The much more inductive effect of **1** than **2** indicates that 7-OH moiety plays an important role in the structure-activity relationship. Further pharmacological analysis should be performed to determine whether the compound could be specific agonist for GPR12.

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References

1. McGlacken GP, Fairlamb IJS. 2-Pyrone natural products and mimetics: isolation, characterisation and biological activity. Nat Prod Rep 22: 369–385 (2005)
2. Kobayashi S, Tsuchiya K, Kurokawa T, Nakagawa T, Shimada N, Iitaka Y. Pironetin, a novel plant growth regulator produced by *Streptomyces* sp. NK10958. II. Structural elucidation. J Antibiot 47: 703–707 (1994)
3. Tsuchiya K, Kobayashi S, Nishikiori T, Nakagawa T, Tatsuta

K. Epopromycins, novel cell wall synthesis inhibitors of plant protoplast produced by *Streptomyces* sp. NK04000. J Antibiot 50: 261–263 (1997)

4. Suzuki K, Kuwahara A, Yoshida H, Fujita S, Nishikiori T, Nishikiori T. NF00659A1, A2, A3, B1 and B2, novel antitumor antibiotics produced by *Aspergillus* sp. NF 00659. I. Taxonomy, fermentation, isolation and biological activities. J Antibiot 50: 314–317 (1997)
5. Kondoh M, Usui T, Kobayashi S, Tsuchiya K, Nishikawa K, Nishikiori T, Mayumi T, Osada H. Cell cycle arrest and antitumor activity of pironetin and its derivatives. Cancer Lett 126: 29–32 (1998)
6. Barrero AF, Oltra JE, Herrador MM, Cabrera E, Sanchez JF, Quílez JF, Rojas F, Reyes JF. Gibepyrones: α-pyrones from Gibberella fujikuroi. Tetrahedron 49: 141–150 (1993)
7. Parker SR, Culter HG, Jacyno JM, Hillf RA. Biological activity of 6-pentyl-2*H*-pyran-2-one and its analogs. J Agric Food Chem 45: 2774–2776 (1997)
8. Christensen CM. Influence of small differences in moisture content upon the invasion of hard red winter wheat by *Aspergillus restrictus* and *A. repens*. Cereal Chem 40: 385–390 (1963)
9. Nielsen P, Sørensen J. Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. FEMS Microbiol Ecol 22: 183–192 (1997)
10. Hirota A, Nemoto A, Tsuchiya Y, Hojo H, Abe N. Isolation of a 2-pyrone compound as an antioxidant from a fungus and its new reaction product with 1,1-diphenyl-2-picrylhydrazyl radical. Biosci Biotechnol Biochem 63: 418–420 (1999)
11. Yasuyuki H. Isolation of 5-hydroxy-α-pyrone and its β-D-glucoside from Erigeron and their use as aging inhibitors for plants and glycosidase inhibitors. JP 04198177, July 17 (1992)
12. Uhlenbrock K, Gassenhuber H, Kostenis E. Sphingosine 1-phosphate is a ligand of the human GPR3, GPR6 and GPR12 family of constitutively active G protein-coupled receptors. Cell Signal 14: 941–953 (2002)
13. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63 (1983)
14. Drews J. Drug discovery: a historical perspective. Science 287: 1960–1964 (2000)
15. Bjursell M, Gerdin AK, Jönsson M, Surve VV, Svensson L, Huang XF, Törnell J, Bohlooly-YM. G protein-coupled receptor 12 deficiency results in dyslipidemia and obesity in mice. Biochem Biophys Res Commun 348: 359–366 (2006)
16. Eidne KA, Zabavnik J, Peters T, Yoshida S, Anderson L, Taylor PL. Cloning, sequencing and tissue distribution of a candidate G protein-coupled receptor from rat pituitary gland. FEBS 292: 243–248 (1991)
17. Song ZH, Modi W, Bommer TI. Molecular cloning and chromosomal localization of human genes encoding three closely related G protein-coupled receptors. Genomics 28: 347–349 (1995)
18. Tanaka S, Ishii K, Kasai K, Yoon SO, Saeki Y. Neural expression of G protein-coupled receptors GPR3, GPR6, and GPR12 up-regulates cyclic AMP levels and promotes neurite outgrowth. J Biol Chem 282: 10506–10515 (2007)
19. Ignatov A, Lintzel J, Hermans-Borgmeyer I, Kreienkamp HJ, Joost P, Thomsen S, Methner A, Schaller HC. Role of the G-protein-coupled receptor GPR12 as high-affinity receptor for sphingosylphosphorylcholine and its expression and function in brain development. J Neurosci 23: 907–914 (2003)