J. Antibiot. 61(12): 756–758, 2008

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THE JOURNAL OF ANTIBIOTICS

JBIR-26, a Novel Natural Compound from *Streptomyces* sp. AK-AH76, Regulates Mammalian Circadian Clock

Miho Izumikawa, Hideki Ukai, Motoki Takagi, Hiroki R. Ueda, Kazuo Shin-ya

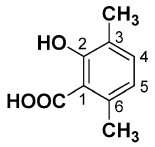
Received: September 24, 2008 / Accepted: November 26, 2008

Abstract In the course of our screening program for regulators of circadian clock system (circadian rhythms), we isolated a new natural compound JBIR-26 (**1**) from *Streptomyces* sp. AK-AH76. The structure was determined to be 2-hydroxy-3,6-dimethylbenzoic acid on the basis of spectroscopic data. Compound **1** lengthened the period-length of mammalian clocks for 1.5 hours at a concentration of 10 μg/ml.

Keywords 2-hydroxy-3,6-dimethylbenzoic acid, JBIR-26, *Streptomyces* sp., circadian clock, *PER2*

Daily rhythms play a prominent role in everything from sleep/wake cycles, body temperature, hormone levels, and even cognition, attention and mood. Disruptions in circadian rhythms associate with not only abnormal sleep/wake but also the pathogenesis of mood disorders such as bipolar disorder, major depression and seasonal affective disorder [1]. Therefore, controlling the clock systems to maintain accurate circadian rhythms will be one of therapeutic strategies for those disorders. However, because the mammalian circadian clock consists of complexly integrated transcriptional feed-back and feed-forward loops of a set of clock genes and regulated protein turnover to maintain 24-hour periodicity [2～5], it would be difficult to logically derive such a control method. In this situation, chemical screening using high-throughput assays is a powerful approach [6].

In mammals, the circadian clock system is composed of a central pacemaker in the suprachiasmatic nucleus and subsidiary oscillators in most peripheral tissues. Recent studies have also shown that even cultured fibroblasts contain a self-sustainable circadian clock [7]. A high-throughput method to monitor the dynamics of the clock of these fibroblasts has been developed [8]. Therefore, these fibroblasts are useful tool to verify the influence of perturbations on mammalian circadian clock [9～11]. In the course of our screening program for circadian clock regulators with a clock-controlled reporter system, we isolated a novel natural product designated as JBIR-26 (**1**) from *Streptomyces* sp. AK-AH76 (Fig. 1). We report herein



1 : 2-hydroxy-3,6-dimethylbenzoic acid

Fig. 1 Structure of JBIR-26 (**1**).

K. Shin-ya (Corresponding author)**:** Biomedicinal Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-42 Aomi, Koto-ku, Tokyo 135-0064, Japan, E-mail: k-shinya@aist.go.jp
M. Izumikawa, M. Takagi (Corresponding author)**:** Biomedicinal Information Research Center (BIRC), Japan Biological Informatics Consortium (JBIC), 2-42 Aomi, Koto-ku, Tokyo 135-0064, Japan, E-mail: motoki-takagi@aist.go.jp
H. Ukai, H. R. Ueda: Laboratory for Systems Biology and Functional Genomics Unit, Center for Developmental Biology (CDB), RIKEN, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan

Table 1 Physico-chemical properties of JBIR-26 (**1**)

Appearance	Colorless amorphous solid
Melting point[a]	98～101°C
Molecular formula	$C_9H_{10}O_3$
HR-ESI-MS (*m/z*)[b]	found: 165.0555 $[M-H]^-$
	calcd: 165.0552
UV λ_{max} (MeOH) nm (ε)[c]	311 (4,070), 249 (5,550)
IR ν_{max} (KBr) cm^{-1} [d]	1646, 1247

[a] Melting point was determined with a Yanagimoto micro melting point apparatus. [b] HR-ESI-MS data were recorded on a Waters LCT-Premier XE. [c] UV spectrum was measured on a BECKMAN COULTER DU730 UV/Vis spectrophotometer. [d] IR spectrum was obtained using a HORIBA FT-720 spectrophotometer.

Table 2 ^{13}C- (150 MHz) and ^{1}H- (600 MHz) NMR data for **1**

Position	δ_C	δ_H
1	113.5	
1-COOH	175.7	
2	162.4	
3	124.7	
3-Me	15.9	2.15 (s, 3H)
4	135.5	7.11 (d, 7.8)
5	122.8	6.60 (d, 7.8)
6	140.2	
6-Me	23.9	2.51 (s, 3H)

NMR spectra were recorded on a Varian NMR System 600 NB CL. The sample was measured in CHD_2OD, and the solvent peak was used as an internal standard (δ_H 3.30 and δ_C 49.0).

the fermentation, isolation, structure determination and brief biological activity of **1**.

Streptomyces sp. AK-AH76 isolated from soil sample collected in Tokyo, Japan, was cultured at 27°C for 5 days in 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of 2.5% starch, 1.5% soybean meal, 0.2% dry yeast and 0.4% $CaCO_3$ (pH 6.2 before sterilization) on a rotary shaker (180 rpm). The fermentation broth (2.0 liters) was centrifuged and the supernatant was extracted with EtOAc and evaporated to dryness. The organic layer (320 mg) was chromatographed on a silica gel flash column (Purif-Pack SI-60, Moritex) with a $CHCl_3$ - MeOH linear gradient system (0～100% MeOH), and fractions including active metabolites were collected. The 10～50% eluate (117 mg) was further purified by ODS flash column (Purif-Pack ODS, Moritex) with 40% aqueous MeOH to give an active fraction (16 mg). The fraction was further purified by reversed-phase preparative HPLC using PEGASIL ODS (Senshu Pak, 20 i.d.×150 mm) developed with 40% aqueous MeOH with 0.2% formic acid to yield JBIR-26 (**1**, Rt=26.0 minutes, 1.8 mg).

The physico-chemical properties of **1** are summarized in Table 1. Compound **1** was isolated as a colorless amorphous solid, and the molecular formula of **1** was established as $C_9H_{10}O_3$ on the basis of HR-ESI-MS analysis (found: 165.0555 $[M-H]^-$, calcd 165.0552). The ^{13}C- and ^{1}H-NMR spectral data for **1** are shown in Table 2. The direct connectivity of protons and carbons was established by the HSQC spectrum.

The structure of **1** was elucidated by NMR spectral analysis such as ^{1}H-^{1}H DQF-COSY and HMBC spectra. The ^{1}H-^{1}H *ortho*-coupling between 4-H (δ_H 7.11) and 5-H (δ_H 6.60) indicated the presence of a 1,2,3,4-tetrasubstituted benzene ring moiety. ^{1}H-^{13}C *m*-couplings in the HMBC spectrum from 4-H to aromatic quaternary

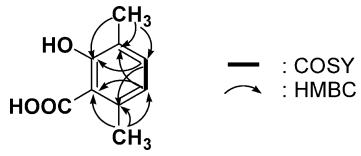


Fig. 2 Key correlations in 2D NMR of **1**.

carbons C-2 (δ_C 162.4) and C-6 (δ_C 140.2), and from 5-H to aromatic quaternary carbons C-1 (δ_C 113.5) and C-3 (δ_C 124.7) revealed assignments of these carbons. The ^{1}H-^{13}C long-range couplings were observed from singlet methyl group 3-Me (δ_H 2.15) to C-2, C-3, and C-4 (δ_C 135.5), and another singlet methyl group 6-Me (δ_H 2.51) to C-1, C-5 (δ_C 122.8), and C-6. The remaining units were a carbonyl carbon which should be assigned to carboxylic carbonyl carbon and a hydroxyl group from the molecular formula. By taking into consideration the ^{13}C chemical shifts of C-1 and C-2, the carboxylic acid and the hydroxyl residue should be substituted at the positions of C-1 and C-2, respectively. Therefore, the structure of **1** was determined as 2-hydroxy-3,6-dimethylbenzoic acid (Fig. 1). Although this compound has been already reported as a degradation product of polyketomycin from *Streptomyces* sp. MK277-AF1 [12], this is the first report of its isolation as a natural product.

To evaluate the effect on the state of circadian clocks by **1**, NIH3T3 cells were stably transfected with luciferase gene driven by the promoter of *PER2* clock gene and stimulated with forskolin to synchronize circadian rhythmicity [9～11]. After addition of **1**, we monitored multicell-level *PER2*-luciferase reporter activity in real time. The period-length of oscillation of *PER2*-luciferase

reporter activity in non-treated cells was 20.8±0.3 hours, but the period-length was lengthened to 22.3±0.8 hours by treatment with 10 μg/ml of **1**. Thus, **1** exhibited a 1.5 hours period-lengthening in transcriptional cycles of clock gene reporter compared with the control. Studies on detailed biological activity of **1** are now underway.

Acknowledgement This work was supported in part by the New Energy and Industrial Technology Development Organization of Japan (NEDO) and a Grant-in-Aid for Scientific Research, The Ministry of Education, Science, Sports and Culture, Japan.

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