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# Actinofuranone C, a New 3-Furanone-Bearing Polyketide from a Dung Beetle-Associated Bacterium

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**Abstract** – Actinofuranone C (**1**), a new 3-furanone-bearing polyketide, was isolated from an actinobacterium (*Amycolatopsis* sp.) associated with a female of the dung beetle, *Copris tripartitus* Waterhouse. The structure of actinofuranone C was elucidated by the spectroscopic interpretation of NMR, mass, UV, and IR data. The discovery of actinofuranone C indicates that chemical investigation of insect-associated microorganisms would be an effective strategy to explore natural chemical diversity.
**Keywords** – Dung beetle, Amycolatopsis, Polyketide

## Introduction

Insect-microorganism symbiotic systems are increasingly highlighted as sources of bioactive natural products (Bode, 2009). A famous story is the multipartite interactions in the fungus-growing ant (attine ant) symbiotic systems (Chapela *et al*., 1994). Fungus-growing ants (Attini: Formicidae), which have been studied since 1870s (Belt, 1874), cultivate a fungal food source by feeding plant material to the fungus. The ants' fungal gardens are preyed upon by specialized and virulent parasitic fungi, *Escovopsis* spp. (Chapela *et al*., 1994). How the ants protect their garden fungi and control parasitic fungi was a longstanding question. It was understood that such longtime successful mutualism is supported by another symbiont, which is an antibiotic-producing actinomycete, *Pseudonocardia* (Currie *et al*., 1999). The ants were found to support these mutualistic bacteria with coevolved crypts and exocrine glands (Currie *et al*., 2003). However, the molecules mediating this interesting symbiotic system had not been reported for long time. In 2009, a chemical study coupled with entomology revealed the chemical entity responsible for the selective antifungal activity as a new cyclic peptide (Oh *et al*., 2009a).

More insect-associated microbes are being investigated as potential sources of bioactive small molecules based on the clues from their ecology. The southern pine beetle *Dendroctonus frontalis* utilizes a bacterium belonging to the genus *Streptomyces* producing a new polyene peroxide with selective inhibitory activity against the antagonistic fungus (Scott *et al*., 2008; Oh *et al*., 2009b). Various classes of natural products including a new macrocyclic lactam were identified from mud dauber-associated bacteria (Poulsen *et al*., 2011; Oh *et al*., 2011). In addition, new immunosuppressive polyketides were reported from mantis-associated fungus (Zhang *et al*., 2011) and new macrolides were discovered from termite-associated *Streptomyces* sp. (Carr *et al*., 2012). Along these examples, the huge biological diversity of insects (~950,000 identified species; Berenbaum and Eisner, 2008) strongly supports the tremendous potential of these sources for drug discovery.

The dung beetle, *Copris tripartitus* Waterhouse, is an indigenous species in Korea. This interesting insect makes brood balls with herbivore's feces, which harbor larvae until they become adults. Because the dung beetle utilizes feces for their life, it possibly has a tremendous microbiome in its ecosystem, which can serve a great resource of new bioactive natural products. Our early works on microbial natural products from the microbial strains originated from the dung beetle ecosystem led to discover new neuroprotective phenylpyridines (Kim *et al*., 2011) and structurally-novel cyclobutane-bearing macrocyclic lactam (Park *et al*., 2012) from *Streptomyces* strains

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associated with brood balls. Our continuous investigation focused on the bacterial population found in adult dung beetles. Chemical analysis of the bacterial strains associated with an adult dung beetle specimen revealed that an actinobacterium (strain #AC43, *Amycolatopsis* sp.) produces a new polyketide compound, which we named actinofuranone C. Here we report the structure elucidation of actinofuranone C based on spectroscopic analysis.

## Experimental

**General experimental procedures** – UV spectra were recorded with a Perkin Elmer Lambda 35 UV-Vis spectrometer in a 1-cm cuvette. Optical rotation was measured on a JASCO P-1020 polarimeter with a 5-cm cell. IR spectra were recorded on a JASCO FT-IR-4200 spectrometer. Low-resolution LC-MS data were acquired on an Agilent Technologies 1200 series HPLC connected with an Agilent Technologies 6130 quadrupole mass spectrometer. High-resolution fast-atom bombardment (HR-FAB) mass spectrometry data were recorded with a Jeol JMS700 high-resolution mass spectrometer at the National Center for Inter-University Research Facilities in Seoul National University. NMR spectral data were obtained on a Bruker 400 MHz NMR spectrometer in Seoul National University.

**Biomaterial** – A pair of dung beetles was provided by a laboratory colony of *Copris tripartitus* which was raised in National Academy of Agricultural Science. The colony was originated from Jeju Island.

**Bacterial isolation** – A female dung beetle was washed with sterilized distilled water and then drenched in 97% EtOH to remove exterior bacteria for one minute. The dung beetle was severed into three main body parts - the head, the thorax and the abdomen- with a blade. The abdomen part was pressed down and vortexed in 40 mL of sterilized water. 500 μL of the suspension was spread on several different agar media with 100 mg/L of cyclohexamide and AC43 was isolated from the actinomycete isolation medium after 15 days. Sequencing analysis data obtained from COSMO co, Ltd revealed that AC43 is most closely related to a partial sequence of *Amycolatopsis* sp. (98% identity), identifying the strain as *Amycolatopsis* sp.

**Cultivation and extraction** – The AC43 strain was cultured in liquid YEME medium (4 g yeast extract, 10 g of malt extract and 4 g of glucose per 1 L of sterilized distilled water) in a 100 mL Erlenmeyer flask at 30 ºC for 3 days. The seed culture was then transferred to liquid YEME medium in a 500 mL Erlenmeyer flask containing 200 mL of medium. After incubation at 30 ºC with shaking at 200 rpm for 3 days, 10 mL of the culture was used to inoculate each 2.8 L Fernbach flask containing 1 L of YEME medium. The culture was cultivated for 5 days at 30 ºC with shaking at 200 rpm. The whole culture (18 L) was extracted with 30 L of distilled EtOAc by using a separation funnel. After adding anhydrous sodium sulfate to remove residual water, the crude extract was concentrated *in vacuo* to yield 5.0 g of extract.

**Isolation of actinofuranone C (1)** – Half of the dried crude extract was resuspended with celite in MeOH and dried *in vacuo* to generate celite-adsorbed extract. The celite-adsorbed extract was loaded onto 2.5 g of pre-packed $C_{18}$ Sepak resin. The extract was fractionated by elution with a step gradient composed of MeOH and $H_2O$. Actinofuranone C (**1**) eluted in the 80% MeOH fraction. To obtain pure actinofuranone C (**1**), the 80% MeOH fraction was subjected to semi-preparative reversed-phase HPLC (Phenomenex Luna 5 μm $C_{18}$ (2) 10 × 250 mm column, 68% aqueous MeOH isocratic solvent system, flow rate: 2 mL/min, detection: UV at 254 nm). Actinofuranone C (**1**) eluted at 45 min. The two-column procedure was repeated with the remaining half of the crude extract to yield 2.2 mg of actinofuranone C (**1**).

**Actinofuranone C** (**1**) – White powder, $[\alpha]_D^{20} = -10.5$ (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 231 (3.9), 282 (3.7) nm; IR (neat) $\nu_{max}$ 3383, 2924, 1601, 1357, 970 $cm^{-1}$. For $^1$H-NMR (400 MHz) and $^{13}$C (100 MHz) NMR data, see Table 1, HR-FAB mass spectrometry observed $[M - H]^-$ at *m/z* 363.2162 (calculated for $C_{21}H_{31}O_5$ $[M - H]^-$ 363.2171).

## Results and Discussion

The strain AC43 was isolated from a female specimen of the dung beetle *Copris tripartitus* (Fig. 1) using the actinomycete isolation medium. Cultivation of the strain in YEME (4 g yeast extract, 10 g of malt extract and 4 g of glucose per 1 L of sterilized distilled water) liquid medium and screening of its chemical profile by LC/MS led to observe the production of a distinct secondary metabolite (actinofuranone C) displaying UV absorption at 231 nm and a molecular ion $[M + Na]^+$ at *m/z* 387 in the positive ESI (electrospray ionization) mode. Our dereplication process utilizing UV and mass spectra indicated that the detected compound could be unknown, which prompted us to culture the bacterial strain in a large scale (18 L) and to purify this particular compound, actinofuranone C (**1**).

Actinofuranone C (**1**) was purified as a white powder

**Fig. 1.** A female of the dung beetle *Copris tripartitus*.

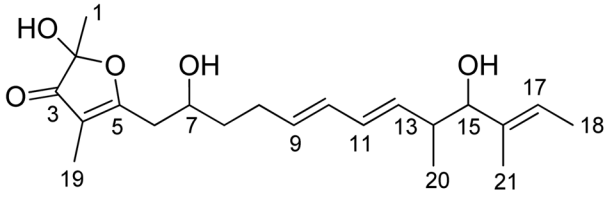


**Fig. 2.** The structure of actinofuranone C (**1**).

through reversed-phase column and HPLC (Fig. 2). Its molecular formula was deduced as $C_{21}H_{32}O_5$ (six double bond equivalents) based on the FAB high resolution mass spectrometry (observed $[M-H]^-$ at *m/z* 363.2162, calculated at *m/z* 363.2171) and $^1H$ and $^{13}C$ NMR data (Table 1). The $^{13}C$ and HSQC spectrum showed five methyl groups at 21.7, 17.1, 13.5, 10.8, and 5.2 ppm, three methylenes at 36.9, 36.1, and 27.9 ppm, eight methines at 135.5, 131.2, 130.6, 129.0, 119.4, 80.2, 67.1, and 39.5, and five quaternary carbons at 201.7, 184.1, 138.0, 107.9, and 102.3. The analysis of the COSY NMR spectrum constructed a chain structure from C-6 to C-15 including C-20 methyl group. Additionally, the C-18 methyl group was directly bonded to the C-17 double bond carbon by the COSY correlation between $H_3$-18 and H-17. These two substructures were connected by the HMBC correlations from $H_3$-21 to C-15, C-16, and C-17. The last part of the molecule was elucidated mainly by long-range heteronuclear couplings from the two methyl groups (C-1 and C-19). The methyl protons $H_3$-1 displayed two strong HMBC correlations to C-2 ($\delta_C$ 102.3) and C-3 ($\delta_C$ 201.7).

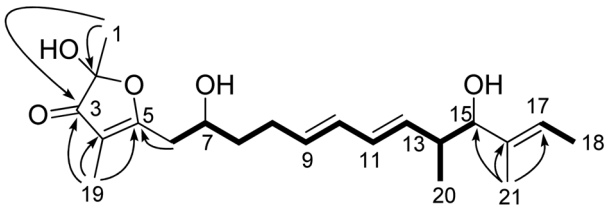


**Fig. 3.** Key COSY and HMBC correlations of actinofuranone C (**1**).

The downfield $^{13}C$ signal at 201.7 ppm was assigned to be a ketone as supported by IR absorption at 1601 $cm^{-1}$. Because the methyl group has only two HMBC correlations, not three or four, C-2 should bear at least one hetero atom such as oxygen in this case. Two oxygen-bearing carbons (C-7 and C-15) were assigned in the chain part by $^{13}C$ and $^1H$ NMR chemical shifts and one oxygen atom was located at C-3 as a ketone. Then two oxygen atoms were left because the molecular formula has five oxygen atoms. The $^{13}C$ NMR chemical shift of C-2 ($\delta_C$ 102.3) indicated that this carbon has two oxygen atoms. $H_3$-19 showed HMBC couplings to C-3, C-4 ($\delta_C$ 107.9), and C-5 ($\delta_C$ 184.1). These correlations enabled to elucidate a partial structure from C-1 to C-5, bearing an α, β-unsaturated ketone. The feature of the highly polarized double bond C-4-C-5 indicated that the olefinic carbon C-5 directly bonds with an oxygen atom. Three double bonds in the chain part and one double bond and a carbonyl in an α, β-unsaturated ketone moiety accounted for five unsaturation equivalents out of six, suggesting that this molecule possesses one ring. The ring was turned out to be 2,4-dimethylfuran-4-en-3-one, bearing a hemiacetal functionality at C-2 based on the HMBC correlations (Fig. 3).

This ring structure was lastly connected to the chain part by the C-5-C-6 bonding, supported by the HMBC correlation from $H_2$-6 to C-5 (Fig 3). Therefore the structure of actinofuranone C (**1**) was elucidated as a new member of 3-furanone-bearing polyketide such as actinofuranone A and B from a marine-derived *Streptomyces* (Cho *et al*., 2006). The absolute configuration of **1** was not determined due to the low yield of the compound.

Actinofuranone C (**1**) is most similar to actinofuranone A except for the absence of one allylic methyl group (Fig.

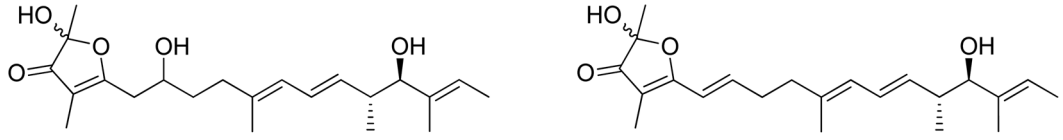


**Fig. 4.** The structures of actinofuranone A (left) and B (right).

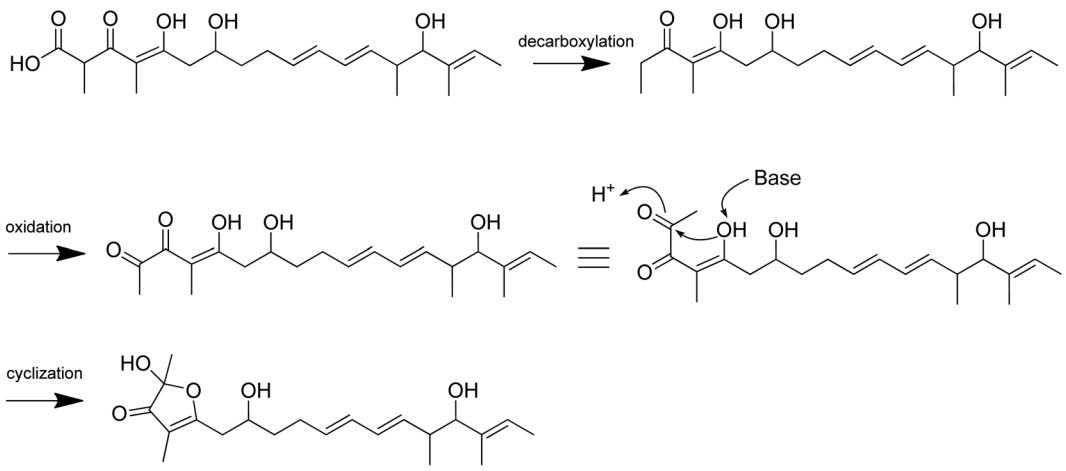


**Fig. 5.** Proposed biosynthesis of actinofuranone C (**1**).

**Table. 1.** NMR data for actinofuranone C (**1**) in DMSO-$d_6$

| C/H | $\delta_H$[a] | mult (*J*in Hz) | $\delta_C$[b] | | COSY | HMBC |
|---|---|---|---|---|---|---|
| 1 | 1.27 | s | 21.7 | $CH_3$ | | C-2, C-3 |
| 2 | | | 102.3 | C | | |
| 3 | | | 201.7 | C | | |
| 4 | | | 107.9 | C | | |
| 5 | | | 184.1 | C | | |
| 6a | 2.57 | m | 36.9 | $CH_2$ | 7 | |
| 6b | 2.49 | m | | | 7 | |
| 7 | 3.77 | m | 67.1 | CH | 6ab, 8ab | |
| 8a | 1.46 | m | 36.1 | $CH_2$ | 7, 9ab | C-9 |
| 8b | 1.41 | m | | | 7, 9ab | C-9 |
| 9a | 2.15 | m | 27.9 | $CH_2$ | 8ab, 10 | C-8 |
| 9b | 2.05 | m | | | 8ab, 10 | C-8 |
| 10 | 5.53 | m | 131.2 | CH | 9ab, 11 | C-9, C-11 |
| 11 | 5.91 | dd (15.0, 11.0) | 129.0 | CH | 10, 12 | C-9, C-12 |
| 12 | 5.93 | dd (15.0, 11.0) | 130.6 | CH | 11, 13 | C-10, C-13 |
| 13 | 5.58 | dd (15.0, 7.0) | 135.5 | CH | 12, 14 | C-12, C-14, C-15 |
| 14 | 2.19 | m | 39.5 | CH | 13, 15, 20 | C-13, C-15, C-20 |
| 15 | 3.50 | d (7.0) | 80.2 | CH | 14 | C-14, C-16, C-20, C-21 |
| 16 | | | 138.0 | C | | |
| 17 | 5.29 | br q (7.0) | 119.4 | CH | 18 | C-15, C-21 |
| 18 | 1.52 | d (7.0) | 13.5 | $CH_3$ | 17 | C-16, C-17 |
| 19 | 1.54 | s | 5.2 | $CH_3$ | | C-3, C-4, C-5 |
| 20 | 0.76 | d (7.0) | 17.1 | $CH_3$ | 14 | C-13, C-14, C-15 |
| 21 | 1.46 | s | 10.8 | $CH_3$ | | C-15, C-16, C-17 |

[a]400 MHz, [b]100 MHz

4). Based on the molecular structure, actinofuranone C (**1**) could be biosynthesized through a polyketide synthase type I pathway. However, the 3-furanone structure cannot be formed in a straightforward manner. We assume that a linear precursor is synthesized by a polyketide type I module and then postmodular decarboxylation and oxidation led a cyclization to yield the characteristic 3-furanone moiety (Fig. 5).

The discovery of actinofuranone C from a bacterium associated with a female of the dung beetle *Copris*

*tripartitus* provides additional evidence that the microbiome in this dung beetle ecosystem is a prolific source of new natural products. Furthermore, this finding indicates that chemical investigation of insect-associated microorganisms would be an effective strategy to disclose untapped natural chemical diversity.

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