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Tripartin, a Histone Demethylase Inhibitor from a Bacterium Associated with a Dung Beetle Larva

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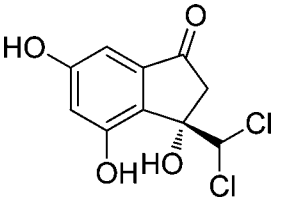
ABSTRACT



Tripartin (1), a new dichlorinated indanone, was isolated from the culture broth of the *Streptomyces* sp. associated with a larva of the dung beetle *Copris tripartitus* Waterhouse. The planar structure of tripartin (1) was identified by the spectroscopic analyses of NMR, mass, UV, and IR data. The structure was confirmed, and the absolute configuration of 1 was determined by X-ray crystallography. Tripartin displayed specific activity as an inhibitor of the histone H3 lysine 9 demethylase KDM4 in HeLa cells.

Investigation of the bacterial symbionts of eukaryotic hosts has become a powerful approach for the discovery of new chemical entities.[1] In particular, insects, the phylogenetic group with the most biodiversity on Earth, host numerous chemically prolific bacteria.[2] For example, the fungus-growing ant *Apterostigma dentigerum* utilizes dentigerumycin, a selective antifungal cyclic peptide produced by a *Pseudonocardia* symbiont, for the protection of a food fungus from a parasitic fungus.[3] The southern pine beetle *Dendroctonus frontalis* harbors a *Streptomyces* sp. in a specialized body structure, the mycangium, and controls the antagonistic fungus *Ophiostoma minus* to protect the larval food fungus *Entomocorticium* sp. with the bacterial compound mycangimycin.[4] Continuous research on identifying new bioactive compounds with novel structures from insect-associated bacteria has resulted in the discovery of a new macrocyclic lactam, sceliphrolactam, from a

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wasp system.[5] Additionally, a recent study on a rare actinomycete, *Amycolatopsis* sp., from a grasshopper resulted in the discovery of new tetrasaccharides, actinotetraoses, and a new angucycline, amycomycin A.[6] A termite-associated *Streptomyces* strain yielded cyclic depsipeptides with modified amino acids, microtermolides A and B.[7]

We have investigated the bacteria associated with the dung beetle *Copris tripartitus* Waterhouse in the search for new chemotypes because its system comprises extensive microbial communities that possibly originated from the feces of herbivores, which the beetle utilizes for feeding and making brood balls.[8] Our chemical investigation of the actinomycetes isolated from dung beetle brood balls led to the discovery of tripartilactam, a novel macrocyclic lactam with a cyclobutane ring with Na^+/K^+ ATPase inhibition activity,[9] and the coprismycins, which are new neuroprotective phenylpyridines.[10] We continuously isolated bacteria associated with the beetle's larvae and chemically screened the bacterial cultures. During our LC/MS chemical analysis, we identified a *Streptomyces* sp. (strain #SNC023) that produced a compound displaying a typical dichlorinated mass spectral pattern. We scaled up the culture of SNC023 and further investigated the compound, isolating and identifying a structurally novel dichlorinated indanone, tripartin (**1**).

For biological evaluation of new chemotypes, we primarily focused on the histone methylation regulation system because histone methylation plays a role in biology by regulating transcription, maintaining genomic integrity, and contributing to epigenetic effects.[11] The dynamic methylation of lysine and arginine residues has diverse transcriptional outcomes, with different methylation sites and states being associated with activation or repression of transcription. The methylation of specific lysine residues is regulated by the competition of two enzymes, histone methyltransferases (HMTs) and histone demethylases (KDMs). The largest identified family of KDMs is the JmjC-domain-containing family and 2-oxoglutarate and Fe(II)-dependent oxygenase.[12] The human KDM subfamily has five members (KDM2/7, KDM3, KDM4, KDM5, and KDM6). Several KDMs are targets for the treatment of diseases such as leukemia, breast, and prostate cancers[13] and inflammation.[14] Despite extensive effort, very few KDM-selective inhibitors have been identified.[15] Among the KDMs, KDM4 (histone H3 lysine 9 demethylase)-specific inhibitors are least known. We evaluated the biological activity of tripartin as a histone H3 lysine 9 demethylase KDM4 inhibitor. Here, we report the discovery of tripartin (**1**) including the isolation and structure elucidation and its biological activity as the first natural specific inhibitor of the histone lysine demethylase KDM4.

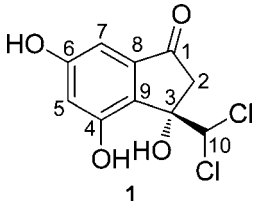

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Table 1. NMR Data for Tripartin (**1**) in DMSO-d_6

C/H	δ_H[a]	mult (*J* in Hz)	δ_C[b]		HMBC
1			201.8	C	
2a	3.10	d (18.9)	47.2	CH_2	1, 3, 8, 9, 10
2b	2.62	d (18.9)			1, 3, 8, 9, 10
3			80.2	C	
4			155.7	C	
5	6.57	d (1.8)	109.2	CH	3, 4, 6, 7, 9
6			160.4	C	
7	6.41	d (1.8)	98.5	CH	1, 5, 6, 9
8			139.2	C	
9			131.1	C	
10	6.66	s	76.7	CH	2, 3, 9

[a] 500 MHz. [b] 125 MHz.

The *Streptomyces* strain SNC023 was isolated from a larva of the dung beetle *C. tripartitus*.[16] The bacterium was cultivated in YPM (mannitol 4 g, yeast 2 g, and peptone 2 g)

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(16) SNC023 was isolated from chitin based agar (chitin 4 g, K_2HPO_4 0.75 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KH_2PO_4 3.5 g, $FeSO_4 \cdot 7H_2O$ 10 mg, $MnCl_2 \cdot 4H_2O$ 10 mg, $ZnSO_4 \cdot 7H_2O$ 10 mg, agar 15 g in distilled 1 L of water) with cycloheximide. 1 g of the pulverized larvae was suspended in 5 mL of distilled water. The suspension was heated at 65 °C for 5 min. From the suspension, 200 μL were spread on chitin based agar plates. From the 16S rDNA analysis, SNC023 was determined to be the most closely related to *Streptomyces diastaticus* (99% identity), identifying the strain as *Streptomyces* sp.

(17) White powder, $[\alpha]^{20}_D$ −109 (c 0.05, MeOH), UV (MeOH) λ_{max} (log ε) 217 (3.38) nm, 268 (2.77) nm, 330 (2.42) nm, IR (neat) ν_{max} 3360, 3195, 2924, 2853, 1659 cm^{-1}. For ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1, HR-CI mass spectrometry obsd $[M + H]^+$ at *m/z* 262.9873 (calcd for $C_{10}H_9Cl_2O_4$ $[M + H]^+$ 262.9878).

liquid medium, and the entire culture (20 L) was extracted with ethyl acetate. A dry extract was prepared after evaporating the organic solvent *in vacuo*. Tripartin (**1**)[17] was isolated as a white powder from the extract by repetitive reversed-phase chromatography using a flash column and HPLC. The molecular formula of **1** was deduced as $C_{10}H_8Cl_2O_4$ based on the high-resolution chemical ionization mass spectrum (obsd $[M + H]^+$ at m/z 262.9873, calcd $[M + H]^+$ 262.9878) and ^1H and ^{13}C NMR data. Analysis of the IR spectrum indicated the presence of hydroxy and carbonyl functionalities with IR absorption at 3360 and 1659 cm^{-1}, respectively.

Of the eight protons in the molecular formula, the ^1H NMR spectrum showed three downfield protons (δ_H 6.41, 6.57, and 6.66) and two protons (δ_H 2.62 and 3.10) in the aliphatic region, indicating that tripartin possesses three exchangeable protons (Table 1). Analysis of the ^{13}C NMR spectrum revealed one ketone carbon (δ_C 201.8), six sp^2 carbons (δ_C 160.4, 155.7, 139.2, 131.1, 109.2, and 98.5), and three sp^3 carbons (δ_C 80.2, 76.7, and 47.2). The $^1H-^{13}C$ one-bond correlations in the gHSQC spectrum showed that the protons at 6.57 and 6.41 ppm were directly connected to the double-bond carbons at 109.2 and 98.5 ppm, respectively. The singlet proton at 6.66 ppm correlated with the carbon at 76.7 ppm in the gHSQC spectrum, indicating that this carbon is possibly dichlorinated. The $^1H-^1H$ COSY spectrum provided a long-range coupling between the two protons at δ_H 6.57 and 6.41, with a small coupling constant of 1.8 Hz, a typical *meta* coupling in an aromatic ring system. Further analysis of the gHMBC correlations led to the construction of the dihydroxy aromatic ring based on the heteronuclear coupling from H-5 (δ_H 6.57) to C-3, C-4, C-6, C-7, and C-9 and from H-7 (δ_H 6.41) to C-1, C-5, C-6, and C-9 (Figure 1). The particularly large geminal coupling constant (18.9 Hz) between the two protons at C-2 indicated that this methylene belongs to a five-membered ring system.[18] In addition, the key HMBC correlations from H_2-2 to C-1, C-3, C-8, C-9, and C-10 connected the five-membered ring with a ketone to the aromatic ring, constructing an indanone skeleton. The dichlorinated C-10 was connected to C-3 by the HMBC couplings between H-10 and C-3, C-2, and C-9, completing the planar structure of the dichlorinated indanone tripartin (**1**) (Figure 1).

To confirm the planar structure of tripartin (**1**) and determine the absolute configuration of the only stereogenic center at C-3, we attempted to crystallize **1** for single-crystal X-ray diffraction analysis. Fortunately, we were successful in crystallizing **1** in diethyl ether/methanol to yield a single and platelet crystal, which diffracted as a triclinic system. Based on the crystallographic analysis, the absolute configuration of C-3 was determined to be 3*S* (Figure 2).

The indanone structure is often found in various natural sources. For example, afzeliindanone was isolated from

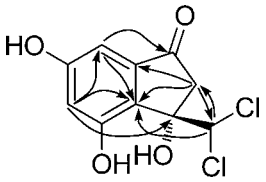

Figure 1. Key HMBC correlations of tripartin (**1**).

the roots of *Uvaria afzelii* plants, and pterosin class compounds were reported from *Pteris semipinnata*.[19] Nodulisporin C was reported to be a secondary metabolite of the fungus *Nodulisporium* sp., which is associated with *Juniperus cedre*.[20] In addition, an indanone (1-methoxy-6-methyl-3-oxo-2,3-dihydro-1*H*-indene-4-carbaldehyde) was discovered from the marine cyanobacterium *Lyngbya majuscula*.[21]

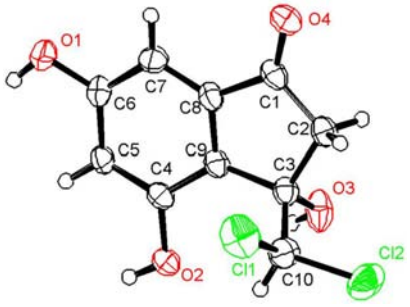

Figure 2. ORTEP diagram for tripartin (**1**).

However, to our best knowledge, tripartin is the first indanone compound isolated from actinomycete bacteria. The dichlorine functionality is unique; thus, tripartin is the first dichlorinated example of the indanone class of natural products. Tripartin (**1**) could be biosynthesized by adding and cyclizing acetate-derived precursors. The two chlorine atoms at C-10 are possibly introduced through a flavin-dependent halogenase, which was proposed to produce a dichlorinated acetate unit incorporated in chloramphenicol from *Streptomyces venezuleae* (Figure S6).[22] However, the biosynthetic chlorination mechanism should be further studied to exclude the alternative nonheme iron halogenation, which introduces chlorine atoms into the sp^3 carbon of leucine, as shown for barbamide biosynthesis.[23]

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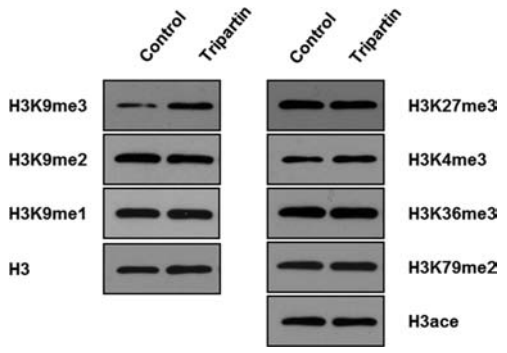

Figure 3. Effect of tripartin (**1**) on various histone H3 lysine methylation and acetylation. HeLa cells were treated with 0.1% DMSO (control) or 10 μM of **1**, as indicated. After 24 h, histones extracted from HeLa cells were resolved on a 15% SDS-polyacrylamide gel. Immunoblotting was performed with antipan histone H3, anti-H3K9me3, anti-H3K9me2, anti-H3K3me1, anti-H3K27me3, anti-H3K4me3, anti-H3K36me3, anti-H3K79me2, and anti-H3ace.

To test whether tripartin (**1**) is a potent and highly selective KDM4 inhibitor, we examined the ability of tripartin to inhibit KDM4 in HeLa cells by immunoblot analysis of extracted histone using antibodies specific for methylated H3K9. Treatment of HeLa cells with tripartin (10 μM) led to a substantial increase in global H3K9me3 levels, which signifies the accumulation of methylated lysine by inhibition of the demethylation process of KDM4 (Figure 3). KDM3 specifically demethylates H3K9me2/1,[24]the levels of which were not increased by tripartin. Thus tripartin is not an inhibitor of KDM3. Tripartin did not influence the methylation of other lysines such as Lys 27, Lys 4, or Lys 36. Because KDM6, KDM5, and KDM2/7 demethylate histone H3 Lys 27, Lys 4, and Lys 36 respectively, this result suggests that tripartin does not inhibit KDM6, KDM5, or KDM2/7. Even though a demethylation enzyme of histone H3 Lys 79 has not been identified yet, we measured the methylation level of H3K79me2 for a further specificity test. In this assay, tripartin did not change the methylation level of H3K79me2. Additionally, tripartin did not affect histone H3 acetylation (H3ace in Figure 3). These results strongly suggest that tripartin (**1**) can specifically inhibit histone H3 lysine 9 demethylase (KDM4).

Inhibitors of KDMs, which are the most recently discovered families of histone demethylases, are extremely rare.[24] Only a few KDM-selective inhibitors such as *N*-oxalylglycine, disulfiram, ebselen, and *N*-oxalyl-D-tyrosine derivatives have been identified from synthetic libraries.[13] Screening of small compound libraries provided quercetin as a KDM inhibitor.[25] However, quercetin, a natural flavonoid, was determined to be a promiscuous inhibitor. Tripartin (**1**) is the first natural product that exhibits specific inhibitory activity toward KDM4 as one of the rare KDM inhibitors.

We also investigated the cytotoxicity of **1** against various cancer cell lines such as HeLa, SNU638, A549, HCT116, K562, MDA-MB231, and SK-HEP-1. However it displayed no significant activity. We also evaluated tripartin's bioactivity using *in vitro* antibacterial assays but could not find significant inhibitory activity against the pathogenic bacteria *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Proteus vulgaris*, *Salmonella typhimurium*, and *Escherichia coli*. Using *in vitro* antifungal assays, tripartin did not inhibit *Candida albicans*, *Aspergillus fumigatus*, *Trichophyton rubrum*, and *Trichophyton mentagrophytes*. Additionally, we tested the inhibitory activity of tripartin against amyloid-β42 aggregation related to Alzheimer disease[26] but found no significant activity.

In conclusion, we identified a structurally novel dichlorinated indanone, tripartin, as a specific histone H3 lysine 9 demethylase (KDM4) inhibitor, from the culture broth of a *Streptomyces* sp. associated with a larva of the dung beetle *Copris tripartitus*. The discovery of this new chemotype with interesting biological activity highlights that microorganisms in insect symbiotic systems are a promising niche for exploring undiscovered natural chemical diversity.

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Supporting Information Available. Experimental methods, ^{1}H, ^{13}C, and 2D NMR spectra, X-ray crystallography data of **1**, and phylogenetic analysis of the bacterium SNC023. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare the following competing financial interest(s): We are preparing a patent application on tripartin.