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Citrofulvicin, an Antiosteoporotic Polyketide from *Penicillium velutinum*

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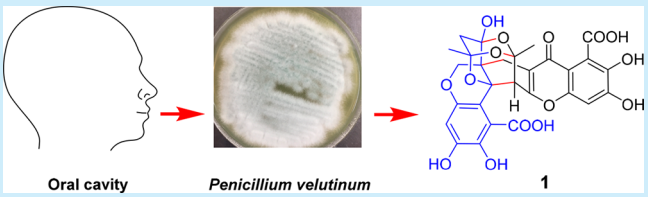
Supporting Information

ABSTRACT: Citrofulvicin (**1**), along with its early shunt product fulvionol (**2**), was characterized as a skeletally unprecedented antiosteoporotic agent from a human sputum-derived fungus *Penicillium velutinum*. The unique citrofulvicin framework is likely formed by a nonenzymatic intermolecular Diels−Alder cycloaddition between heptaketide-based intermediates. Citrofulvicin and fulvionol were demonstrated to be osteogenic at 0.1 μM in the prednisolone-induced osteoporotic zebrafish.



Our health depends on, or at least has multiple association with, the human microbiota capable of biosynthesizing structurally intriguing metabolites with various bioactivities. However, the human microbiome-derived natural products described so far are largely limited to the secondary metabolites of gut bacteria.[1,2] As a matter of fact, human bodies also harbor highly diverse, as yet undefined communities of fungi whose cells are typically more than 100-fold larger than those of bacteria.[3] Fungi have been found to exist in nearly all mucosal surfaces,[4] although the human body temperature was suspected to be unsuitable for fungal growth.[5] Like the cross-generation transmission of bacterial microbiota,[6] the early mycobiota (viz., fungal microbiota) is succeeded via the mother−offspring transfer in a host−phenotype dependent manner; however, the population of human commensal fungi is influenced by fungal strains in the diet and environment.[7]

The cross-feeding and/or -talking mechanisms have been suggested for the microbial interaction among/between different bacterial and fungal species in microbiota.[8,9] Moreover, the lateral gene transfers across microbiota members make the eukaryotic metabolism more chimeric to (help) survive the selection pressure applied to the commensal microbes.[10] In the particular host niches, the antagonism or competition occurs between fungi and bacteria for the metabolic substrate and mutual tolerance.[11] Therefore, fungi keep evolving to produce "special compounds" that antagonize bacterial growth and vice versa.

Fungi belonging to the *Penicillium* genus are a rich source of antibacterial natural products such as penicillin.[12] *Penicillium* species widely occur in the human bronchus[13] and gut[14] and in some processed foods such as blue cheese.[15,16] We intuited that diverse fungi may reside in the lungs and the oral cavity and might be gathered in the mucus sputum. To combat the companion bacteria, some of these fungi might be obligated/evolved to generate antibacterial molecules. Here, *Penicillium velutinum* CGMCC 3.7984, a mucus sputum-derived fungus, was shown to produce citrofulvicin (**1**) as a skeletally unprecedented polyketide with pronounced antiosteoporotic but negligible antibacterial activities.

P. velutinum was cultured on the malt extract (ME) medium, followed by extraction with ethyl acetate. The extract obtained was fractionated mainly by a combination of column chromatography and HPLC refinement as detailed in the Supporting Information. Citrofulvicin (**1**) was afforded as yellowish crystals with its molecular formula evidenced to be $C_{28}H_{22}O_{15}$ from its protonated molecular ion at m/z 599.1032 in its high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) ($C_{28}H_{23}O_{15}$ requires 599.1031). In line with the 18 double bond equivalents of the molecular formula, the ^{1}H and ^{13}C NMR spectra of **1** highlighted that its molecule has more quaternary carbons. This assumption was substantiated by the follow-up DEPT experiments, indicating its possession of twenty quaternary, three methine, three methylene, and two methyl carbons. The DEPT spectra of **1**, along with its molecular formula, highlighted the presence of a total of 15 carbon-connected protons in the molecule, thereby implying its possession of seven hydroxyl groups (alcoholic/phenolic/

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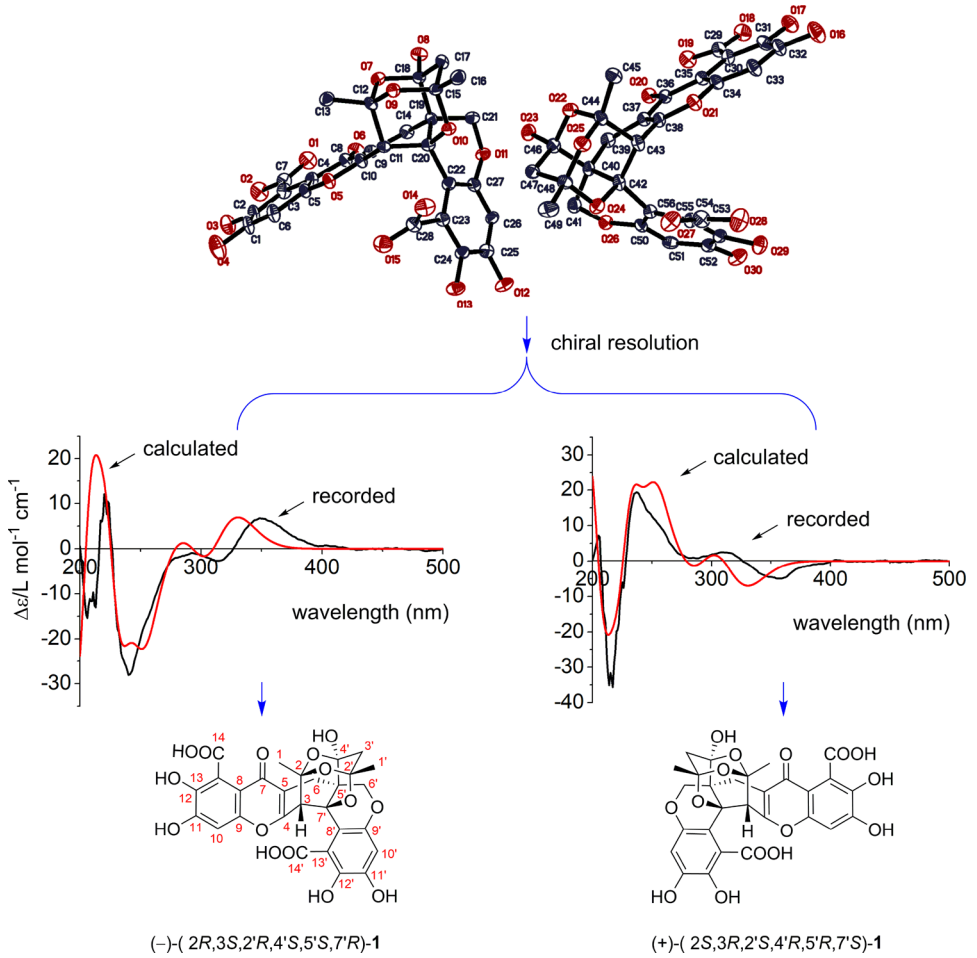

Figure 1. Absolute configuration assignments of citrofulvicin (**1**). Top: X-ray structure of (±)-**1**. Middle: comparison between the recorded and calculated ECD spectra. Bottom: absolute structures of (+)-**1** and (−)-**1**.

carboxylic). Such an unusual situation made the NMR methodology incapable of determining the exact structure of **1**, although some individual NMR signals could be readily ascribed to the isolated motifs such as two methyls (appearing as singlets at δ_H 1.24 and 1.32), two aromatic protons (corresponding to the singlets at δ_H 6.25 and 6.79), and three methylenes resonating as geminally coupled doublet pairs at δ_H 1.74 and 2.05 (J = 12.7 Hz), at δ_H 2.40 and 3.04 (J = 18.4 Hz), and at δ_H 3.81 and 4.23 (J = 10.6 Hz). Gratifyingly, this frustration was overcome by the single-crystal X-ray diffraction of **1** (Cu Kα), clarifying its structure and racemic nature (Figure 1). Finally, **1** was separated by chiral HPLC to yield (+)-**1** and (−)-**1**, which were demonstrated to have (2*S*,3*R*,2′*S*,4′*R*,5′*R*,7′*S*)- and (2*R*,3*S*,2′*R*,4′*S*,5′*S*,7′*R*)-configurations, respectively, by comparing the recorded electronic circular dichroism (ECD) curves with those calculated for all of the optional stereoisomers (Figure 1).

With **1** addressed stereochemically, we asked how it might be formed by the fungus. Scrutiny of the molecular framework of **1** suggested that its unusual carbon skeleton might result from the hybridization of appropriately structured precursors leading to the heptaketides citromycetin and/or fulvic acid.[17] To address the hypothesis, the mother liquors (leftovers upon the isolation of **1**) were combined and refractionated with an intention of hitting the heptaketide(s). As expected, two major heptaketides were afforded and identified to be citromycetin and fulvic acid (as a racemate).[17,18] A minor companion was also obtained as yellowish crystals and was shown to be a new heptaketide that we have named fulvionol (**2**). The molecular formula of **2** was indicated to be $C_{14}H_{14}O_8$ by its protonated molecular ion at m/z 311.0759 in its HR-ESI-MS (calcd for $C_{14}H_{15}O_8$, 311.0761). The structure of **2** was established by a correlative interpretation of its NMR spectra (^{1}H and ^{13}C NMR, DEPT, ^{1}H−^{1}H COSY, HSQC, and HMBC). The structure of **2** was confirmed by its single-crystal X-ray crystallographic analysis (Cu Kα) (Figure S21). However, we faced a challenge in addressing its absolute configuration, owing to the vibration of the 2-hydroxypropyl group during the X-ray diffraction analysis (Figure S21). Alternatively, the (2*R*)-configuration of **2** was established by comparing its optical rotation ($[\alpha]^{20}_D$ +28.0, *c* 0.09, MeOH) with those of chirally inverted analogues with a single chiral carbon (Figure S1), (2′*S*)-2-(propan-2′-ol)-5-methyl-7-hydroxy-benzopyran-4-one ($[\alpha]^{24}$ −9.1, *c* 0.23, MeOH)[19] and (2′*R*)-2-(propan-2′-ol)-5-hydroxybenzopyran-4-one ($[\alpha]^{25}_D$ +30.0, *c* 0.25, MeOH), whose structure is indicative of its (2′*R*)-configuration (not "2′*S*" as indicated incorrectly therein).[20] This was reinforced by the positive $[\alpha]_D$ nature discerned with (2*R*)-fulvionol peracetate (**2a**, formed with pure acetic anhydride) and computed for (2′*R*)-2-(propan-2′-ol)-5-hydroxybenzopyran-4-one and (2*R*)-fulvionol (**2**) (Tables S3 and S4).

The coidentification of heptaketides **2**, citromycetin, and fulvic acid inspired the postulation of the biosynthetic pathway of citrofulvicin (**1**). As illustrated in Scheme 1, the

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