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New citrinin analogues produced by coculture of the marine algal-derived endophytic fungal strains *Aspergillus sydowii* EN-534 and *Penicillium citrinum* EN-535

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ABSTRACT

Ten citrinin analogues (**1**–**10**), including a new citrinin dimer (*seco*-penicitrinol A, **2**) and a new citrinin monomer (penicitrinol L, **3**), were isolated from the EtOAc extracts of the coculture of two marine algal-derived endophytic fungal strains *Aspergillus sydowii* EN-534 and *Penicillium citrinum* EN-535. Their structures were elucidated by detailed analysis of their NMR spectra, ECDs, HRESIMS, optical rotation, and X-ray crystallographic data, and by comparison with literature data as well. The crystal structure of compound **1** was reported for the first time in this paper and resulted in unambiguous confirmation of its absolute configuration as 3*R*, 4*S*, 2′*R*, and 3′*S*, while the absolute configuration of compound **3** was determined by TDDFT calculations of its ECD spectrum. The antimicrobial and influenza neuraminidase inhibitory activities of compounds **1**–**10** were evaluated.

1. Introduction

Marine-derived microbes are revealed as a prolific source of structurally diverse and biologically active natural products (Marmann et al., 2014). However, the discovery of new compounds getting more difficult, while the re-isolating of known compounds becoming more frequently (Oh et al., 2007; Marmann et al., 2014). On the other hand, many microbial biosynthetic genes related to secondary metabolites are silent or in lower transcription under a standard/common laboratory fermentation conditions (Gross, 2009; Scherlach and Hertweck, 2009). Recently, diverse approaches to activate the silent biosynthetic genes have been developed, and among these approaches, coculture of two or more different microbial strains to accumulate “cryptic compounds” seems to be a useful strategy (Oh et al., 2007; Ola et al., 2013; Rateb et al., 2013; Marmann et al., 2014; Whitt et al., 2014; Meng et al., 2015; Stierle et al., 2017). *Aspergillus sydowii* EN-534 and *Penicillium citrinum* EN-535 are two endophytic fungi isolated from the same fresh tissue of the marine red alga *Laurencia okamurai*. When they are cocultured, more metabolites can be detected by high performance liquid chromatography (HPLC) compared to axenic cultivation (Fig. 1). We thus performed chemical investigations on the coculture of *A. sydowii* EN-534 and *P. citrinum* EN-535, and resulted in the isolation of a new citrinin dimer *seco*-penicitrinol A (**2**) and a new citrinin monomer penicitrinol L (**3**), together with eight known citrinin derivatives, penicitrinone A (**1**) (Clark et al., 2006; Wakana et al., 2006), penicitrinone F (**4**) (Chen et al., 2017), penicitrinol A (**5**) (Wakana et al., 2006), citrinin (**6**) (Rödel and Gerlach, 1995), dihydrocitrinone (**7**) (Curtis et al., 1968), decarboxydihydrocitrinone (**8**) (Curtis et al., 1968), phenol A acid (**9**) (Rödel and Gerlach, 1995), and phenol A (**10**) (Rödel and Gerlach, 1995) (Fig. 2). Herein, we report the isolation, structure elucidation, and bioactivities of these compounds.

2. Results and discussion

The coculture of *A. sydowii* EN-534 and *P. citrinum* EN-535 was performed on solid rice medium, and the EtOAc extract was purified by a combination of column chromatographies (CC) including Si gel, Sephadex LH-20, and Lobar LiChroprep RP-18, as well as preparative TLC, to yield compounds **1**–**10**.

Compound **1**, initially obtained as a red amorphous powder, was previously reported in 2006 by two groups independently and was named as penicitrinone A (Wakana et al., 2006) and dicitrinin A (Clark et al., 2006). Wakana and co-workers confirmed its relative configuration by NOESY correlations while Clark and co-workers indicated that this compound was likely a dimerized artefact of citrinin (**6**), and assigned its absolute configuration by comparison with compound **6**. As

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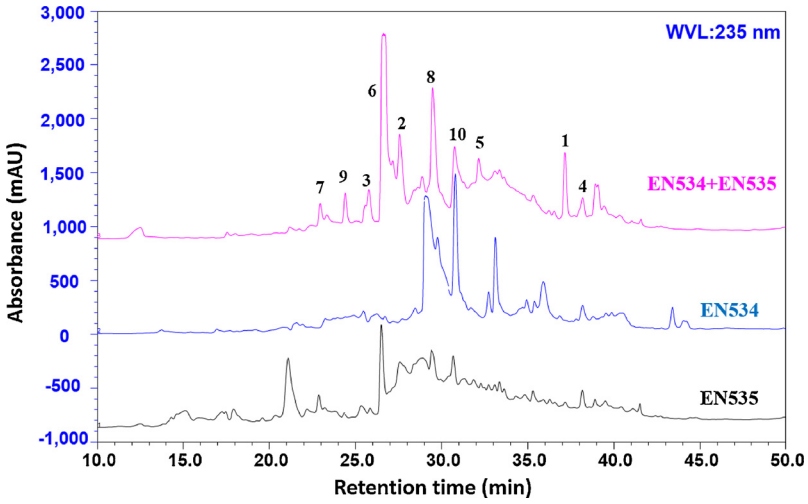

Fig. 1. HPLC analysis of extracts from *A. sydowii* EN-534, *P. citrinum* EN-535, and their coculture.

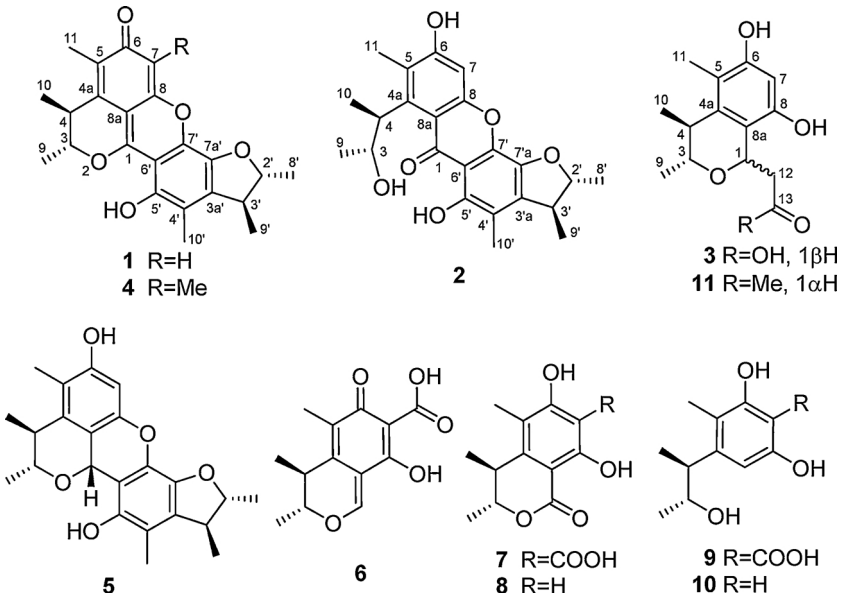

Fig. 2. Structures of the isolated compounds **1–10** and the reference compound penicitrinol C (**11**).

no literature has been reported to confirm its absolute stereochemistry directly, we performed X-ray crystallographic analysis of compound **1**. After evaporation of a mixed solvent of methanol-acetone with 0.5% acetic acid, quality crystals of **1** were obtained and were submitted to single-crystal X-ray diffraction experiment using Cu Kα radiation (See Fig. S1, Supplementary data). The resulting Flack parameter 0.0(2) allowed for the unambiguous establishment of the absolute configuration of **1** as 3*R*, 4*S*, 2′*R*, and 3′*S*.

Compound **2** was obtained as a red amorphous powder and its molecular formula was assigned as $C_{23}H_{26}O_6$ by positive ion HRESIMS at *m/z* 399.1805 $[M+H]^+$ (calcd 399.1802), requiring 11 ° of unsaturation. The ^{1}H NMR data of **2** (Table 1) indicated the presence of six methyls, five methines (with two oxygenated and one aromatic), and three exchangeable protons. The ^{13}C NMR and DEPT spectra revealed the presence of 23 carbons that were assigned as six methyls, five methines (with two oxygenated and one aromatic), and 12 non-protonated carbons. The NMR data of compound **2** were similar to those of penicitrinol A (**5**), indicating that they may share the same skeleton. Detailed analysis of the 1D NMR data for compounds **2** and **5** revealed that the resonance of C-3 shifted to upfield from δ_C 79.2 in **5** to 72.1 in **2**, while the resonance of C-1 shifted to downfield from δ_C 66.9 in **5** to 184.5 in **2** and C-4 from δ_C 37.6 in **5** to 41.9 in **2** (Wakana et al., 2006). In addition, differences in the chemical shifts of C-8, C-8a, and C-5′ to C-7′ in compounds **2** and **5** were also observed. These spectroscopic features suggested that the pyran ring in **5** was disclosed in **2**, which was supported by the COSY and HMBC data (Fig. 3). H-3 and H-4 were assigned as *trans* orientation by NOESY correlation from H_3-9 to H-4 and from the biogenetic consideration of citrinin dimers as well. In addition, H-2′ and H-3′ in the benzofuran moiety were also assigned as *trans* by the observed NOESY correlation from H_3-9′ to H-2′ (Fig. 4). The absolute configuration of penicitrinol A (**5**) was not assigned so it is not applicable to assign the absolute configuration of compound **2** by comparing with that of **5**. But, from a biosynthetic point of view, compound **2** might share the same biogenetic pathway with **1**, and the absolute configuration of **2** was thus tentatively assigned as 3*R*, 4*S*, 2′*R*, and 3′*S* and given the trivial name *seco*-penicitrinol A.

Compound **3** was isolated as a pale yellow gum. Its molecular formula was assigned as $C_{14}H_{18}O_5$ by positive ion HRESIMS at *m/z* 267.1232 $[M+H]^+$ (calcd 267.1227), requiring six degrees of unsaturation. The ^{1}H NMR data of **3** (Table 1) indicated the presence of three methyls, one methylene, and four methines (with one aromatic and two oxygenated). The ^{13}C NMR and DEPT spectra indicated the