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ORIGINAL ARTICLE

New chlorinated xanthone and anthraquinone produced by a mangrove-derived fungus *Penicillium citrinum* HL-5126

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Two new chlorinated metabolites 4-chloro-1-hydroxy-3-methoxy-6-methyl-8-methoxycarbonyl-xanthen-9-one (1) and 2′-acetoxy-7-chlorocitreorosein (2), together with three known compounds (3–5), were obtained from the EtOAc extract of the endophytic fungus *Penicillium citrinum* HL-5126 isolated from the mangrove *Bruguiera sexangula var. rhynchopetala* collected in the South China Sea. Their structures were elucidated by the detailed analysis of comprehensive spectroscopic data. All compounds were evaluated for their antibacterial and topoisomerase I inhibitory activities. Compound 2 exhibited antibacterial activity against *Vibrio parahaemolyticus* with an MIC value of 10 μM.
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INTRODUCTION

Marine organisms have attracted increasing attention from those searching for novel structural and biological diversity natural products in recent years. Especially, marine-derived natural halogenated compounds possess a variety of bioactivities such as cardiovascular, antibacterial, antifungal, cytotoxic, antiviral and insecticidal activities.[1] For example, the brominated testafuran A was used as lead for treatment of cardiovascular disorders, merochlorins A and B were both potent inhibitors of methicillin-resistant *Staphylococcus aureus* (MRSA) and dankastatin C exhibited potent growth inhibition of P388 cell lines.[2–5] To date, more than 5000 halogenated natural products have been discovered,[6,7] these results have drawn the attention of both pharmaceutical and natural product chemists. In our search for new bioactive natural products from marine fungi in the South China Sea, we have found several bioactive compounds, including anthraquinone derivatives, cytochalasins, benzopyrans, stemphol sulfates and α-pyrone derivatives, from marine-derived fungus.[8–12] Our previous investigation on the endophytic fungi *Penicillium citrinum* has resulted in the discovery of one new benzopyran derivative (2*R**,4*R**)-3,4-dihydro-5-methoxy-2-methyl-2H-1-benzopyran-4-ol and three new dihydroisocoumarin penicimarins G – I.[10,13] Further chemical investigation of the fermentation broth of *P. citrinum* resulted in the isolation of one new chlorinated xanthone 4-chloro-1-hydroxy-3-methoxy-6-methyl-8-methoxycarbonyl-xanthen-9-one (**1**) and one new chlorinated anthraquinone 2′-acetoxy-7-chlorocitreorosein (**2**), together with three known compounds, chloroisosulochrin dehydrate (**3**), citreorosein (**4**) and MT-1 (**5**) (Figure 1). All isolated metabolites (**1–5**) were evaluated for their antibacterial and topoisomerase I inhibitory activities. Herein we report the isolation, structure elucidation and biological activities of these compounds.

RESULTS AND DISCUSSION

Isolation and identification of compounds

Compound **1** was isolated as a pale yellow powder, which has a molecular formula of $C_{17}H_{13}ClO_6$, as determined by the HRESIMS, requiring 11 degrees of unsaturation and containing one chlorine atom. The ^{1}H NMR spectrum of **1** (Table 1) was characterized by resonances consistent with one hydrogen-bonded phenol moiety at δ_H 12.66 (s, 1-OH), three aromatic methine protons at δ_H 7.45 (brs, H-5), 7.15 (brs, H-7) and 6.44 (s, H-2), two methoxy groups at δ_H 4.01 (s, 10-OMe) and 3.99 (s, 3-OMe), and a methyl group at δ_H 2.52 (s, 6-Me). The ^{13}C NMR spectrum (Table 2) revealed 17 carbon signals, including two carbonyls δ_C (180.5 and 163.1), three aromatic methine carbons and nine aromatic quaternary carbons, together with two methoxy carbons at δ_C (57.9 and 54.2), and one methyl carbon at δ_C 23.0. Its ^{1}H- and ^{13}C-NMR spectroscopic data were closely resembled those of **3**,[14] except for the small shifting of the chemical shifts at 12 aromatic carbons in **1**. In the HMBC spectrum, the correlations from the protons of 1-OH to C-1, C-1a and C-2, indicating that 1-OH was located at C-1, 3-OMe to C-3, 6-Me to C-5, C-6, C-7 and 10-OMe to C-10 indicating that the two methoxy groups and one methyl group were located at C-3, C-6 and C-10. Further HMBC correlations from H-2 to C-1a, C-3 and C-4, from H-5 to C-7, C-9a, C-10a and from H-7 to C-5, C-9a and C-10 indicated the position of the substituents in the structure. Chlorination of C-4 was found to be consistent with the C-4 chemical

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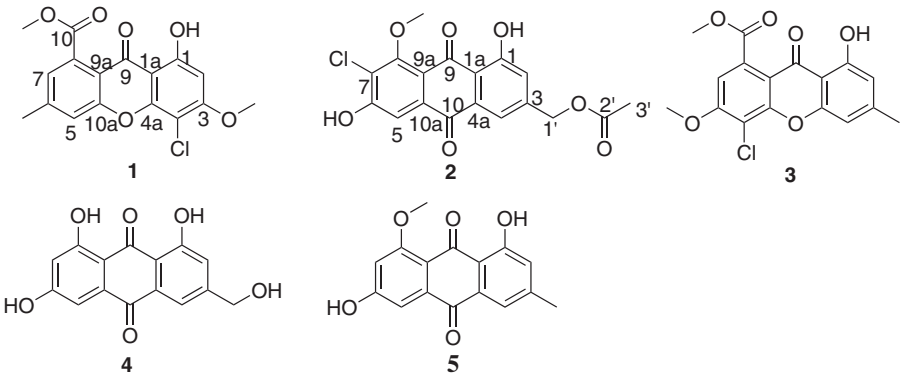

Figure 1 The structures of compounds **1**–**5**.

Table 1 ^{1}H NMR and ^{13}C NMR data for compounds 1 and 2 (1 in $CDCl_3$; 2 in DMSO-d_6)

	1		*2*	
Position	*^{1}H (J in Hz)*	*^{13}C*	*^{1}H (J in Hz)*	*^{13}C*
1		163.1, C		161.6, C
1a		105.0, C		115.9, C
2	6.44, s	96.3, CH	7.31 (d,1.2)	122.3, CH
3		163.1, C		145.0, C
4		100.5, C	7.59 (d,1.2)	116.9, CH
4a		153.1, C		132.4, C
5	7.45, brs	120.4, CH	7.56, s	110.6, CH
6		148.1, C		160.4, C
7	7.15, brs	125.6, CH		122.6, C
8		134.4, C		158.7, C
9		180.5, C		186.0, C
9a		116.0, C		117.8, C
10		170.6, C		181.3, C
10a		157.0, C		133.8, C
1'			5.18, s	64.2, CH_2
2'				170.1, C
3'			2.14, s	20.6, CH_3
1-OH	12.66, s		12.93, s	
3-OMe	3.99, s	57.9, CH_3		
2-Me	2.52, s	23.0, CH_3		
10-OMe	4.01, s	54.2, CH_3	3.88, s	60.9, CH_3

shift at δ_C 100.5. The ^{1}H,^{1}H-COSY, HMQC and HMBC spectra allowed the complete assignment for **1** (Figure 2). Thus, the structure of **1** was identified shown in Figure 1. We named compound **1** as 4-chloro-1-hydroxy-3-methoxy-6-methyl-8-methoxycarbonyl-xanthen-9-one.

Compound **2** was isolated as a yellow powder, and it has a molecular formula of $C_{18}H_{13}ClO_7$, as determined by the HRESIMS, requiring 12 degrees of unsaturation and containing one chlorine atom. The ^{1}H NMR spectrum of **2** (Table 1) displayed one hydrogen-bonded phenol moiety at δ_H 12.93 (s, 1-OH), three aromatic methine protons at δ_H 7.59 (d, J = 1.2 Hz, H-4), 7.31 (d, J = 1.2 Hz, H-2) and 7.56 (s, H-5), one oxygen methylene group at δ_H 5.18 (s, H-1′), one methoxy group at δ_H 3.88 (s, 8-OMe) and a methyl group at δ_H 2.14 (s, H-3′). The ^{13}C NMR spectrum (Table 1) revealed 18 carbon signals, including two carbonyls (δ_C 186.0 and 181.3, C-9 and C-10), three aromatic methine carbons, nine aromatic quaternary carbons. These spectroscopic features and the chemical shifts indicated that **2** has a highly substituted anthraquinone scaffold, and together with one methoxy carbon at δ_C 60.9, one oxygen methylene carbon at δ_C 64.2, and an acetyl group at δ_C 20.6 for methyl carbon and δ_C 170.1 for carbonyl carbon. In the HMBC spectrum, both H-4 and H-5 showed correlations to the carbonyl moiety at δ_C 181.3 (Figure 1), indicating the connectivity of these protons to C-4 and C-5, the correlations from 1-OH to C-1/1a/2 indicated the OH was linked to C-1, the correlation from 8-OMe to C-8 revealed that the methoxy group was located at C-8, the correlations from H-2 and H-4 to C-1′ indicated the oxygen methylene group was linked to C-3, and the correlations from H-1′ and 3′-Me to C-2′ indicated the acetyl group was linked at C-1′. Chlorination of C-7 was found to be consistent with the C-7 chemical shift at δ_C 122.6. The ^{1}H,^{1}H-COSY, HMQC and HMBC spectra allowed the complete assignment for **2** (Figure 2). Ultimately, the structure of new compound **2** was elucidated as 2′-acetoxy-7-chlorocitreorosein.

The structure of known **3–5** was identified by comparison of their ^{1}H/^{13}C NMR spectra with those in the literature.[15–17] Compounds **1** and **3** were xanthones, and compounds **2**, **4** and **5** were anthraquinones. The xanthones may be derived from the corresponding anthraquinones. The proposed biosynthesis pathway of the isolated compounds **1–5** is shown in Scheme 1.

Biological properties of compounds 1–5

The antibacterial activities of all the compounds were determined against four terrestrial pathogenic bacteria *Escherichia coli* (ATCC 25922), *S. aureus* (ATCC 25923), methicillin-resistant *S. aureus* (ATCC 33591), *Bacillus cereus* (ATCC 11778) and two marine pathogenic bacteria *Vibrio parahaemolyticus* (ATCC17802) and *Vibrio alginolyticus* (ATCC17749). Compounds **2** and **4** exhibited antibacterial activity against *S. aureus* with the same MIC values of 22.8 μM, respectively (Table 2). Compound **2** exhibited antibacterial activity against *V. parahaemolyticus* with the MIC value of 10 μM (Table 2). All compounds were also tested for their topoisomerase I inhibitory activities, however, these compounds showed no topoisomerase I inhibitory activity at the concentration of 500 μM.

METHODS

Fungal materials

The fungal strain *P. citrinum* HL-5126 was isolated from the mangrove *Bruguiera sexangula var. rhynchopetala* collected in the South China Sea in August 2012. The strain was deposited in the Key Laboratory of Tropical Medicinal Plant Chemistry of Ministry of Education, College of Chemistry and Chemical Engineering, Hainan Normal University, Haikou, China, with the

Table 2 Antibacterial activity for compounds 1–5

	MIC (μM)					
Compound	*MRSA*	*S. aureus*	*E. coli*	*B. cereus*	*V. alginolyticus*	*V. parahaemolyticus*
1	>50	>50	>50	>50	>50	>50
2	>50	22.8	>50	>50	>50	10
3	50	50	50	50	50	50
4	>50	22.8	50	50	50	50
5	50	50	50	50	50	50
Ciprofloxacin[a]	1.25	0.31	0.62	0.62	1.25	1.25

Abbreviations: *B. cereus*, *Bacillus cereus*; *E. coli*, *Escherichia coli*; MRSA, methicillin-resistant *Staphylococcus aureus*; *S. aureus*, *Staphylococcus aureus*; *V. alginolyticus*, *Vibrio alginolyticus*; *V. parahaemolyticus*, *Vibrio parahaemolyticus*.
[a]Ciprofloxacin was used as a positive control.

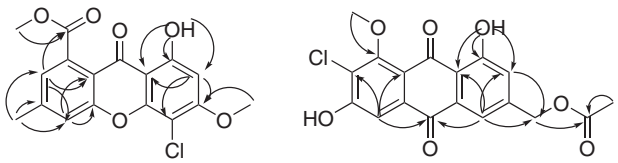

Figure 2 Key HMBC correlations of compounds **1** and **2**.

access code HL-5126. The fungal strain was cultivated in 30 l potato glucose liquid medium, 1 l Erlenmeyer flasks each containing 300 ml of medium for 100 Erlenmeyer flasks at 25 °C without shaking for 28 days. The potato glucose liquid medium contained 15 g of glucose and 30 g of sea salt in 1 l of potato infusion (potato infusion: 200 g slice potatoes thin were added to 1 l water, and boiled until soft for ~30 min. They were filtered through cheese cloth, and then the filtrate was hold to 1 l with distilled water).

Identification of fungus

The strain was identified as *P. citrinum* according to morphologic traits and molecular identification. Its 497 base pair ITS sequence had 99% sequence identity to that of *P. citrinum* (LP54362). The sequence data have been submitted to GenBank with the accession number KJ466981.

General experimental procedures

Octadecylsilyl silica gel (YMC, Kyoto, Japan; 12 nm–50 μm), silica gel (Qing Dao Hai Yang Chemical Group, Qing Dao, China; 200–300 mesh) and Sephadex LH-20 (GE) were used for column chromatography (CC). Precoated silica gel plates (Yan Tai Zi Fu Chemical Group, Yantai, China; G60, F-254) were used for TLC. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV spectrometer (Bruker, Zurich, Switzerland) at 400 and 100 MHz. Chemical shifts δ are reported in p.p.m., using tetramethylsilane as internal standard, and coupling constants (J) are in Hz. HRESIMS spectra were obtained from a Micromass Q-TOF spectrometer and Thermo Scientific LTQ Orbitrap XL spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). UV spectra were recorded on a Beckman DU 640 spectrophotometer (Brea, CA, USA). IR spectra were recorded on a Nicolet 6700 spectrophotometer (Thermo Fisher Scientific).

Extraction and isolation

The fungal cultures were filtered through cheese cloth, and the filtrate was extracted with EtOAc (3 × 30 l, 24 h each). The organic extracts were concentrated *in vacuo* to yield an oily residue (22.0 g), which was subjected to silica gel CC (petroleum ether, EtOAc *v/v*, gradient 100:0–0:100) to generate six fractions (Fr. 1 − Fr. 7), Fr. 3 (1.2 g) was isolated by CC on silica gel eluted with petroleum ether–EtOAc to obtain Fr. 3. 1 (390.0 mg), Fr. 3. 2 (410.0 mg) and Fr. 3.3 (200.0 mg). Fr. 3.3 was subjected to Sephadex LH-20 CC eluting with mixtures of Petroleum ether: $CHCl_3$:MeOH (2:1:1), and further purified by using HPLC on an ODS semi-preparative column (Agilent C18, AgilentTechnologies, Santa Clara, CA, USA; 9.4 × 250 mm, 7 μm, 2 ml min^{-1}) eluted with 85% MeOH/H_2O to obtain compounds **1** (10.0 mg) and **3** (8.0 mg). Fr. 4 was isolated by CC on silica gel eluted with petroleum ether–EtOAc, and then subjected to Sephadex LH-20 CC eluting with mixtures of $CHCl_3$–MeOH (1:1) to obtain Fr. 4. 1 (510.0 mg), Fr. 4. 2 (210.0 mg) and Fr. 4. 3 (340.0 mg). Fr. 4. 2 was subjected to repeated Sephadex LH-20 CC ($CHCl_3$/MeOH, *v/v*, 1:1) and further purified by using HPLC on an ODS semi-preparative column (Agilent C18, 9.4 × 250 mm, 7 μm, 2 ml min^{-1}) eluted with 80% MeOH/H_2O to obtain compound **2** (9.0 mg). Fr. 4. 3 was subjected to repeated Sephadex LH-20 CC ($CHCl_3$/MeOH, *v/v*, 1:1) and further purified by using HPLC on an ODS semi-preparative column (Agilent C18, 9.4 × 250 mm, 7 μm, 2 ml min^{-1}) eluted with 75% MeOH/H_2O to obtain compounds **4** (11.0 mg) and **5** (7.0 mg).

Physical properties of compounds 1 and 2

4-chloro-1-hydroxy-3-methoxy-6-methyl-8-methoxycarbonyl-xanthen-9-one (**1**): white amorphous powder. λ_{max} (log ε) 238 (3.42), 262 (3.36), 296 (2.89), 385 (1.72) nm; IR (KBr) ν_{max} 3440, 1740, 1610, 1280, 1240, 1210, 1112, 1082, 816 cm^{-1}; ^{1}H and ^{13}C NMR see Table 1; HRESIMS *m/z* 349.0475 [M + H]$^+$ (calcd for, $C_{17}H_{13}ClO_6$ 349.0479).

2′-acetoxy-7-chlorocitreorosein (**2**): yellow powder. λ_{max} (log ε) 220 (4.02), 262 (3.80), 314 (4.12), 470 (3.37) nm; IR (KBr) ν_{max} 3530, 2910, 1712, 1630, 1260, 1112, 1080 cm^{-1}; ^{1}H and ^{13}C NMR see Table 1; HRESIMS *m/z* 775.0687 [2M + Na]$^+$ (calcd for $C_{36}H_{26}Cl_2O_{14}Na$, 775.0597).

Biological assays

Antibacterial assays. Antibacterial activity was determined by the conventional broth dilution assay.[18] Four terrestrial pathogenic bacteria *E. coli*, *S. aureus*, methicillin-resistant *S. aureus*, *B. cereus* and two marine pathogenic bacteria *V. parahaemolyticus* and *V. alginolyticus* were used, and ciprofloxacin was used as a positive control.

Topoisomerase I inhibitory assays. The enzyme topoisomerase I was from calf thymus. Relaxation activity of top I was performed as described by Bogurcu *et al.*,[19] with some modification, using 10-hydroxycamptothecin as a positive control.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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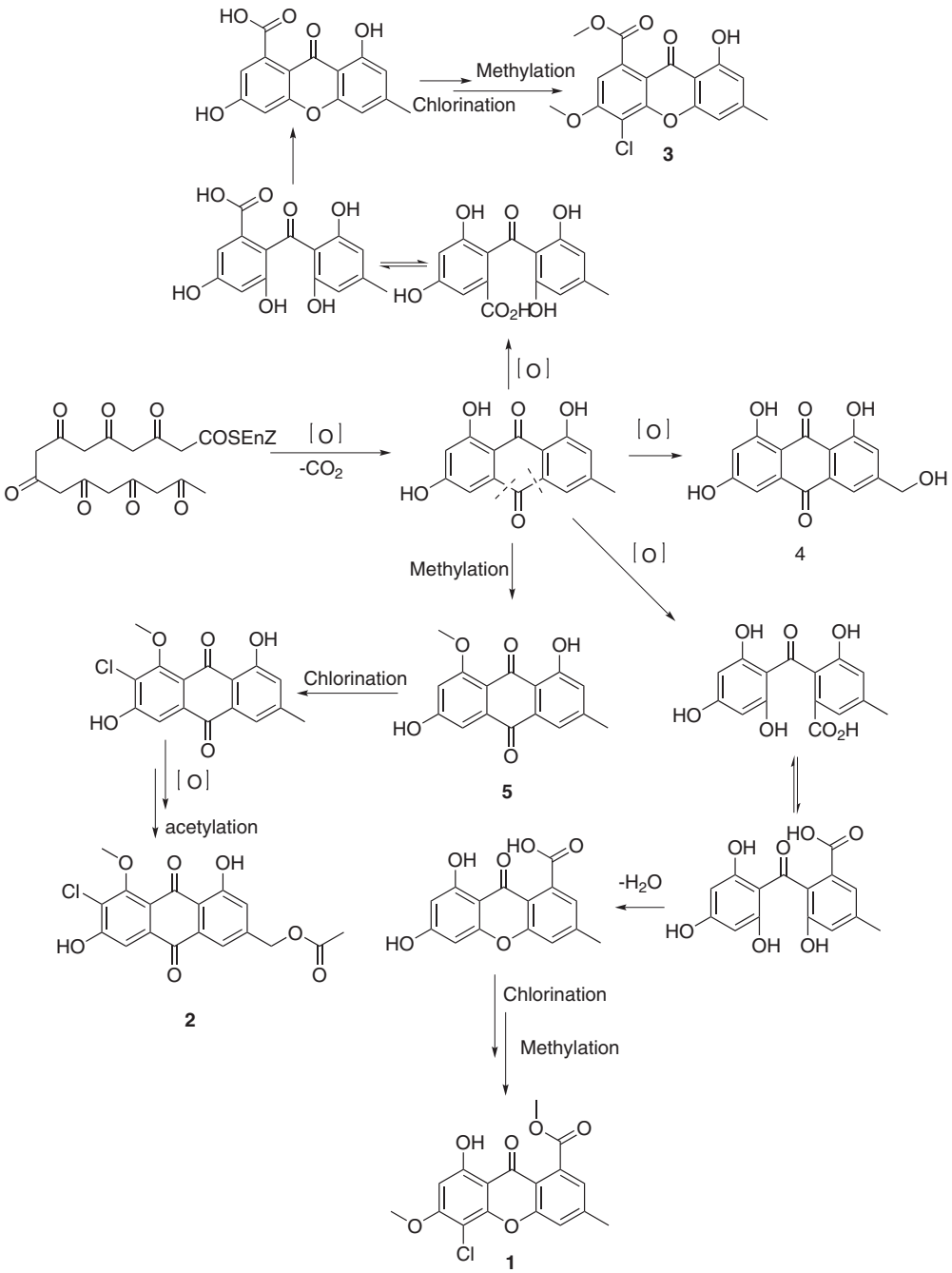

Scheme 1 Proposed biosynthesis pathway of the isolated compounds **1–5**.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)