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EXPERIMENTAL RESEARCH

Peniciisoquinoline A: A new tetrahydroisoquinoline from mangrove-derived fungus *Penicillium* sp. DM27

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[Abstract] **Objective** This study aims to search for structurally novel, natural bioactive products from mangrove-derived fungi. **Methods** Compounds were isolated and purified by column chromatography on HP20 macroreticular resin, silica gel, Sephadex LH-20 gel, and high performance liquid chromatography. The structures of the isolates were analyzed using NMR and MS data, and the absolute configuration of **1** was determined by calculated ECD spectroscopic methods. The cytotoxicity of all the isolates was tested against HCT-116, HepG2, HEK 293t, and 5637 cancer cell lines using CCK-8 assay. **Results** A new tetrahydroisoquinoline named peniciisoquinoline A (**1**) was obtained from *Penicillium* sp. DM27 along with five known compounds. Peniciisoquinoline A (**1**) was inactive against the four cancer cell lines. **Conclusion** This study conducted chemical research on *Penicillium* sp. DM27 and tested the cytotoxic activity of the new alkaloid (**1**), which will assist in the further development and utilization of mangrove-derived fungi.

[Key words] *Penicillium* sp.; Tetrahydroisoquinoline; Cytotoxicity

1 Introduction

Mangroves have an active microbial community that adapts to frequent and extreme environmental changes and special ecological conditions. As a part of the microbial community, mangrove rhizosphere soil-derived fungi play an essential role as a rich source of structurally complex and diverse natural bioactive products[1-2]. A large number of unique structures with potent bioactivities have been isolated from mangrove-derived fungi[2]. *Penicillium* sp. belongs to filamentous fungi, which are important producers of structurally unique natural products with pharmaceutical potential[3]. Since the discovery of penicillin G from *Penicillium notatum*[4], fungi belonging to the genus have received considerable attention in chemical and biological research

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communities and proven to be abundant sources of natural bioactive products[5-7].

Following our previous work on natural bioactive products derived from marine fungal habitats[8-9], we investigated *Penicillium* sp. DM27, a mangrove-associated fungus isolated from rhizosphere soil of *Bruguiera gymnorrhiza* (L.) Poir. in Thailand, because of its potent antitumor properties observed during our preliminary bioassay screening. As a result, a new tetrahydroisoquinoline named peniciisoquinoline A (**1**), together with five known compounds, have been isolated and identified. Details of the isolation and purification, structure elucidation, and biological evaluation are described herein.

2 Experiments

2.1 General experimental procedures

Optical rotations were measured using a PerkinElmer Model 341 polarimeter. The ECD spectra were recorded on a Chirascan V100 spectropolarimeter, while the NMR spectra were acquired using the Bruker AVANCE 400 MHz and 600 MHz NMR instruments. The HR-ESIMS spectra were measured on an LTQ Orbitrap XL mass instrument (ThermoFisher). A super clean workbench (sw-cj-2fd), Sujing Aetna; Microscope (nib-100), Ningbo Yongxin Optical Co., Ltd.; Carbon dioxide cell incubator (MCO-18AC), Phcbi; electric constant-temperature water bath pot (HWS-24), Shanghai Yiheng Technology Co., Ltd.; multifunctional microplate reader (Multiskan MK3), Thermo; one-ten-thousandth balance (MS105DU), METTLER; centrifuge (TD4N), Changsha Yingtai Instrument Co., Ltd; mixer (SCI-VS), Selo Czech Republic, USA. Column chromatography (CC) was performed with silica gel (100–400 mesh, Anhui Liangchen Guiyuan Material Ltd., Anhui, China) and size-exclusion chromatography with Sephadex LH-20 (Pharmacia, Sweden).

2.2 Fungal material

The mangrove-derived *Penicillium* sp. DM27 fungus was isolated from mangrove rhizosphere soil of *Bruguiera gymnorrhiza* (L.) Poir. on Sept 26th, 2013, at Tachalab subdistrict, Tamai district, Chantaburi Province, Thailand (Tachalab is located at 102°E 3.4′ longitude and 12°N 32′ latitude) and was identified according to the morphological characteristics and the ITS sequences. A voucher specimen (DM27) is available for inspection at the School of Pharmaceutical Sciences, Wuhan University.

2.3 Mass culture of *Penicillium* sp. DM27

Penicillium sp. DM27 was initially inoculated into potato dextrose agar media (6 g of potato extract, 20 g of glucose, 15 g of sea salt, 20 g of agar, and 1 000 mL of distilled water, adjusted to pH 6.5) and then incubated for 7 days at 28 ℃. It was subsequently transferred into a 300 mL potato dextrose aqueous medium held in a 1 L flask to ferment for 28 days at 28 ℃.

2.4 Extraction and isolation

The brown culture was filtered to separate the mycelia and liquid phase. The mycelia were extracted fully using ultrasound and 100% acetone (3 × 3 L). The combined acetone solution was concentrated using a rotary evaporator in vacuo to obtain crude extract. The crude extract combined with the liquid phase (30 L) was extracted with macroreticular resin HP20 eluted with MeOH/H_2O to produce four fractions (30%, 50%, 70%, and 90%). The 50% MeOH/H_2O eluted fraction (6.64 g) was subjected to silica gel CC and eluted with CH_2Cl_2/MeOH (40 : 1 to 1 : 1) into six fractions (F1–F6). Fraction F3 (306 mg) was separated using Sephadex LH-20 (MeOH-CH_2Cl_2/1 : 1) to obtain seven fractions (F3.1–F3.7). Fraction F3.7 (84 mg) then underwent separation on a Sepex-C18 column (250 × 10 mm, 5 μm) using a Shimodzu HPLC system with a 254 nm DAD detection and a flow

rate of 3.0 mL/min to obtain **1** (2 mg, t_R 27.48 min, 30%–60% MeOH-H_2O over 30 min with 0.1% formic acid). Fraction F5 (504 mg) was separated using Sephadex LH-20 (MeOH-$CHCl_3$/1 : 1) to produce twelve fractions (F5.1–F5.12), fraction F5.8 (57 mg) using HPLC to obtain **5** (3 mg, t_R 18.31 min, 40%–70% MeOH-H_2O with 0.1% formic acid) and **6** (2 mg, t_R 25.31 min, 40%–70% MeOH-H_2O with 0.1% formic acid), and fraction F5.9 (27 mg) using HPLC to obtain **4** (5 mg, t_R 25.51 min, 40%–55% MeOH-H_2O with 0.1% formic acid). The 70% MeOH/H_2O eluted fraction (1.42 g) was exposed to silica gel CC and eluted with CH_2Cl_2/MeOH (60 : 1 to 1 : 1) into thirteen fractions (F1′–F13′). Fraction F4′ (158 mg) then underwent separation on a Sepex-C18 column using a Shimodzu HPLC to obtain **2** (3 mg t_R 24.51 min, 30%-55% MeOH-H_2O with 0.1% formic acid) and **3** (4 mg t_R 27.51 min, 30%–55% MeOH-H_2O with 0.1% formic acid).

Peniciisoquinoline A (**1**): $[\alpha]_D^{20}$ −11.2 (*c* 0.33, MeOH); ECD {MeOH, λ [nm] ($\Delta\varepsilon$), $c = 0.79 \times 10^{-4}$ M} 222 (+0.65), 204 (−4.62); ^{1}H and ^{13}C NMR data (400 MHz, $CDCl_3$), see Table 1; HRESIMS *m/z* 252.123 1 $[M + H]^+$ (calcd. 252.123 6).

Meleagrin (**2**): Colorless crystalline; ^{1}H NMR (400 MHz, CD_3OD) δ: 8.32 (1H, s, H-15), 7.82 (1H, s, H-18), 7.62 (1H, d, J = 7.7 Hz, H-4), 7.38 (1H, s, H-20), 7.29 (1H, t, J = 7.7 Hz, H-6), 7.09 (1H, t, J = 7.7 Hz, H-5), 7.02 (1H, d, J = 7.7 Hz, H-7), 5.41 (1H, s, H-8), 5.06 (2H, m, H-23), 3.77 (3H, s, 1-OMe), 1.32 (3H, s, H-24), 1.32 (3H, s, H-25); ^{13}C NMR (100 MHz, CD_3OD) δ: 165.6 (C-13), 159.4 (C-10), 146.7 (C-7a), 142.9 (C-9), 136.5 (C-18), 128.0 (C-6), 127.0 (C-3a), 125.9 (C-16), 124.6 (C-4), 124.5 (C-12), 123.1 (C-5), 112.3 (C-23), 111.5 (C-7), 109.2 (C-8), 107.7 (C-15), 101.7 (C-2), 64.2 (*N*-OMe), 52.6 (C-3), 42.1 (C-21).

Table 1 ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data for 1 in $CDCl_3$

No.	δ_C	δ_H (mult., *J* in Hz)
1	50.0	4.07 (d, 16.3); 3.71 (d, 16.3)
3	62.1	3.52 (t, 6.0)
4	31.0	3.08 (dd, 6.0, 3.3)
4a	125.1	
5	119.1	6.64 (d, 8.3)
6	108.9	6.73 (d, 8.3)
7	144.2	
8	141.6	
8a	120.7	
9	173.2	
N-*Me*	42.9	2.58 (s)
7-*OMe*	56.2	3.88 (s)
9-CO*OMe*	51.8	3.74 (s)

Neoxaline (**3**): Colorless crystalline; ^{1}H NMR (400 MHz, $CDCl_3$): 8.32 (1H, s, H-15), 7.60 (1H, d, J = 7.8 Hz, H-4), 7.57 (1H, s, H-18), 7.35 (1H, t, J = 7.7 Hz, H-6), 7.31 (1H, s, H-20), 7.14 (1H, t, J = 7.7 Hz, H-5), 6.98 (1H, s, H-7), 6.12 (1H, m, H-7), 5.11 (2H, m, H-23), 4.60 (1H, m, H-9), 3.78 (1H, s, 1-OMe), 2.44 (2H, m, H-8), 1.31 (3H, S, H-25), 13.1, (3H, s, H-25); ^{13}C NMR (100 MHz, $CDCl_3$): 171.7 (C-10), 165.6 (C-13), 145.8 (C-7a), 144.5 (C-22), 137.1 (C-18), 134.9 (C-20), 129.2 (C-6), 128.2 (C-3a), 125.9 (C-16), 125.0 (C-4), 123.9 (C-5), 122.3 (C-3), 114.1 (C-23), 111.7 (C-7), 110.3 (C-15), 100.6 (C-2), 66.4 (C-9), 65.3 (1-OMe), 53.3 (C-3), 43.6 (C-21), 40.1 (C-8), 24.7 (C-24), 24.5 (C-25).

Brevianamide F (**4**): White powder; ^{1}H NMR (400 MHz, $CDCl_3$), δ_H 3.77 (1H, ddd, J = 15.0, 3.8, 1.0 Hz, H-2α), 3.00 (1H, dd, J = 15.1, 10.8 Hz, H-2β), 2.00-1.84 (2H, m, H-3), 2.08-1.97 (2H, m, H-4), 4.09 (1H, ddd, J = 8.8, 6.9, 1.7 Hz, H-5), 5.84 (1H, s, H-7), 4.39 (1H, ddd, J = 10.7, 3.9, 1.7 Hz, H-8), 3.73-3.55 (2H, m, H-10), 7.61 (1H, d, J = 7.9 Hz, H-12), 8.53 (1H, s, H-13), 7.10 (1H, d, J = 2.4 Hz, H-16), 7.16 (1H, ddd, J = 8.0, 7.0, 1.0 Hz, H-17), 7.25 (1H, ddd, J = 8.2, 7.0, 1.2 Hz, H-18), 7.41 (1H, d, J = 8.2 Hz, H-16). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 28.3 (C-2), 22.6 (C-3), 26.8 (C-4), 59.3 (C-5), 165.6 (C-6), 54.6 (C-7), 169.6 (C-9), 45.5 (C-10), 111.6 (C-11), 123.5 (C-12), 136.7 (C-14), 126.7 (C-15), 122.7 (C-16), 120.0 (C-17), 118.5 (C-18), 109.7 (C-19).

Maculosin (**5**): White powder; ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 3.58 (1H, m, H-2α), 3.63 (1H, dd, J = 13.4, 5.4, H-2β), 1.98 (2H, m, H-3), 2.35 (2H, m, H-4), 4.09 (1H, t, J = 7.8 Hz, H-5), 5.66 (1H, s, H-7),

4.21 (1H, dd, J = 10.4, 3.7 Hz, H-8), 3.73-3.55 (2H, m, H-10), 7.07 (2H, d, J = 8.0 Hz, H-12/H-12′), 6.80 (2H, d, J = 8.0 Hz, H-13/H-13′), 2.73 (1H, s, H-14). ^{13}C NMR (100 MHz, $CDCl_3$) ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 170.7 (C-6), 166.9 (C-9), 157.6 (C-14), 132.1 (C-12/C-12′), 127.6 (C-11/C-11′), 116.2 (C-13), 60.0 (C-5), 57.9 (C-8), 45.9 (C-2), 37.6 (C-10), 29.4 (C-4), 22.7 (C-3).

Okaramines U (**6**): Yellow powder; ^{1}H NMR (600 MHz, CD_3OD) δ_H 4.65 (1H, dd, J = 11.4, 6.4 Hz, H-2), 2.42(1H, dd, J = 13.0, 6.5 Hz, H-3α), 1.82 (1H, dd, J = 13.0, 11.5 Hz, H-3β), 7.18 (1H, d, J = 7.3 Hz, H-4), 6.65 (1H, dd, J = 7.3, 7.2 Hz, H-5), 7.04 (1H, dd, J = 8.0, 7.3 Hz, H-6), 6.54 (1H, d, J = 8.0 Hz, H-7), 6.68 (1H, d, J = 4.1 Hz, H-8), 5.32 (1H, d, J = 4.1 Hz, H-8a), 6.03 (1H, br s, 3a-OH), 7.72 (1H, s, H-1'), 4.46 (1H, dd, J = 5.5, 5.0 Hz, H-2'), 3.35 (1H, m, H-3α), 3.05 (1H, dd, J = 15.6, 6.9 Hz, H-3β), 7.59 (1H, d, J = 7.8 Hz, H-4'), 6.99 (1H, dd, J = 7.8, 7.3 Hz, H-5'), 7.07 (1H, dd, J = 8.0, 7.3 Hz, H-6'), 7.33 (1H, d, J = 8.0 Hz, H-7'), 10.87 (1H, br s, H-8'), 7.25 (1H, br s). ^{13}C NMR (150 MHz, $CDCl_3$) δ_C 60.1 (C-2), 42.6 (C-3), 87.6 (C-3a), 131.7 (C-3b), 123.7 (C-4), 119.3 (C-5), 130.7 (C-6), 111.5 (C-7), 149.3 (C-7a), 86.1 (C-8a), 171.6 (C-9), 56.6 (C-2'), 27.4 (C-3'), 109.9 (C-3a'), 128.7 (C-3b'), 120.2 (C-4'), 120.0 (C-5'), 122.6 (C-6'), 112.4 (C-7'), 138.1 (C-7a'), 125.0 (C-8a'), 169.5 (C-9').

2.5 Computational analysis

Conformational analysis within an energy window of 3.0 kcal/mol was performed by using the OPLS3 molecular mechanics force field via the MacroModel panel of Maestro 10.2. The conformers obtained in this way were further optimized with the software package Gaussian 09 at the B3LYP/6-311G (2d, p) level. Then, the 60 lowest electronic transitions of each conformer were calculated using the TDDFT methods at the B3LYP/6-311G (2d, p) level. The rotational strengths of each electronic excitation were expressed using both dipole length and dipole velocity representations. ECD spectra of different conformers were simulated using a Gaussian function with a half-bandwidth of 0.16–0.30 eV. The overall theoretical ECD spectrum was then obtained according to the Boltzmann weighting of each conformer.

2.6 Cytotoxicity assay

The cytotoxicity of all the isolated compounds were tested against the human colon (HCT-116), hepatoblastoma (HepG2), HEK 293t, and human primary bladder carcinoma (5637) cancer cell lines using CCK-8 assay[18-19]. All cell lines were purchased from American type culture collection. Doxorubicin hydrochloride was used as a positive control (purity: 99%, Solarbio Life Sciences). The cells were combined into a single cell suspension with the culture medium containing 10% fetal bovine serum. The 96 well plates were inoculated with 90 μL of cell culture medium (adherent cell viewed 5×10^4/mL and suspension cell viewed 9×10^4/mL) per well and then cultured at 5% CO_2 and 37 °C for 24 h. 10 μL of sample solution was added to each well. One concentration was prepared for each sample during preliminary screening and three multiple holes for each concentration. Eight concentration gradients were prepared for each sample for IC_{50} determination and three multiple holes for each concentration. The experimental samples were divided into the blank group, control group, and drug group. The old culture medium and drug solution of adherent cells was sucked out, then 100 μL of CCK-8 solution (diluted ten times with the basic medium) was added, the suspension cells were directly added to 10 μL of CCK-8 stock solution, and cultured at 37 °C with 5% CO_2 for 1–4 h (dark operation, real-time observation). The absorbance was measured at 450 nm with an enzyme labeling instrument. The toxicity was expressed by cell inhibition, for which the calculation formula is as follow: Cell inhibition (%) = $(OD_{Control} - OD_{Drug})/(OD_{Control} - OD_{Blank}) \times 100\%$. The IC_{50} was calculated by GraphPad Prism 8 (version 8.0.2, GraphPad Software Inc.), and the experimental results were expressed as ± SD.

3 Results and discussion

Peniciisoquinoline A (**1**) was obtained as an optically active brown oil $[\alpha]_D^{20}$ −11.2 (*c* 0.33, MeOH). Its molecular formula was determined to be $C_{13}H_{17}NO_4$ by HRESIMS (252.123 1) $[M + H]^+$ (cald. 252.123 6) and NMR data, suggesting the presence of six degrees of unsaturation.

The ^{1}H NMR spectrum (Table 1) displayed resonances for three methyls [δ_H 3.88 (s, 7-OMe), 3.74 (s, 9-COOMe), and 2.58 (s, N-Me)], two aromatic protons [δ_H 6.73 (d, J = 8.3 Hz, H-6) and 6.64 (d, J = 8.3 Hz, H-5)], two methylenes [δ_H 4.07 (d, J = 16.3 Hz, H-1) and 3.71 (d, J = 16.3 Hz, H-1), and 3.08 (dd, J = 6.0, 3.3 Hz, H-4)], and one methine proton 3.52 (t, J = 6.0 Hz, H-3). The ^{13}C NMR spectrum combined with HSQC data of **1** displayed 13 carbon signals due to three methyls (two oxygenated and one nitrogenated), two sp^3 methylene carbons including a nitrogenated one, three methine carbons including two aromatic and one sp^3 nitrogenated carbon, four aromatic quaternary carbon signals [δ_C 125.1 (C-4a), 144.2 (C-7), 141.6 (C-8), and 120.7 (C-8a)], and one carbonyl (δ_C 173.2).

A detailed analysis of the ^{1}H–^{1}H COSY and HSQC spectra revealed just two isolated spin systems corresponding to the C-3–C-4 and C-5–C-6 subunits of structure **1**, as well as an isolated methylene group at C-1 [δ_C 50.0; δ_H 4.07 (d, J = 16.3 Hz), 3.71 (d, J = 16.3 Hz)] (Fig. 2). The HMBC correlations from H-5 to C-7 and C-8a, and from H-6 to C-4a and C-8 indicated the presence of a 1,2,3,4-tetrasubstituted benzene ring. The HMBC correlations from 7-OMe to C-7, suggested methoxyl was attached to C-7. The HMBCs from H-5 to C-8a (δ_C 120.7) and C-4 (δ_C 31.0), from H-4 to C-5, C-8a and the carbonyl group at C-9, from H-3 to C-4a (δ_C 125.1), and the methoxyl (δ_H 3.74) to C-9 indicated that C-4 was connected to the benzene ring at C-4a and the C-9 was attached at C-3. The HMBC correlations from the N-Me (δ_H 2.58) to C-1 and C-3, and from the isolated protons at H-1 to C-4a, C-8a and C-8 showed that C-1 was attached to the benzene ring at C-8a, and C-1, C-3, and the nitrogenated methyl were connected through a nitrogen atom to form a tetrahydroisoquinoline skeleton. Based on the chemical shifts, carbons C-8 were considered as oxygenated. The above analysis produced the planar structure of **1** shown in Fig. 1.

To determine the absolute configuration of **1**, a stereoisomer **1a** (3*S*) was used as the model compound. The calculated weighted ECD spectrum of **1a** was obtained using the time-dependent density functional theory (TDDFT) calculations[10].

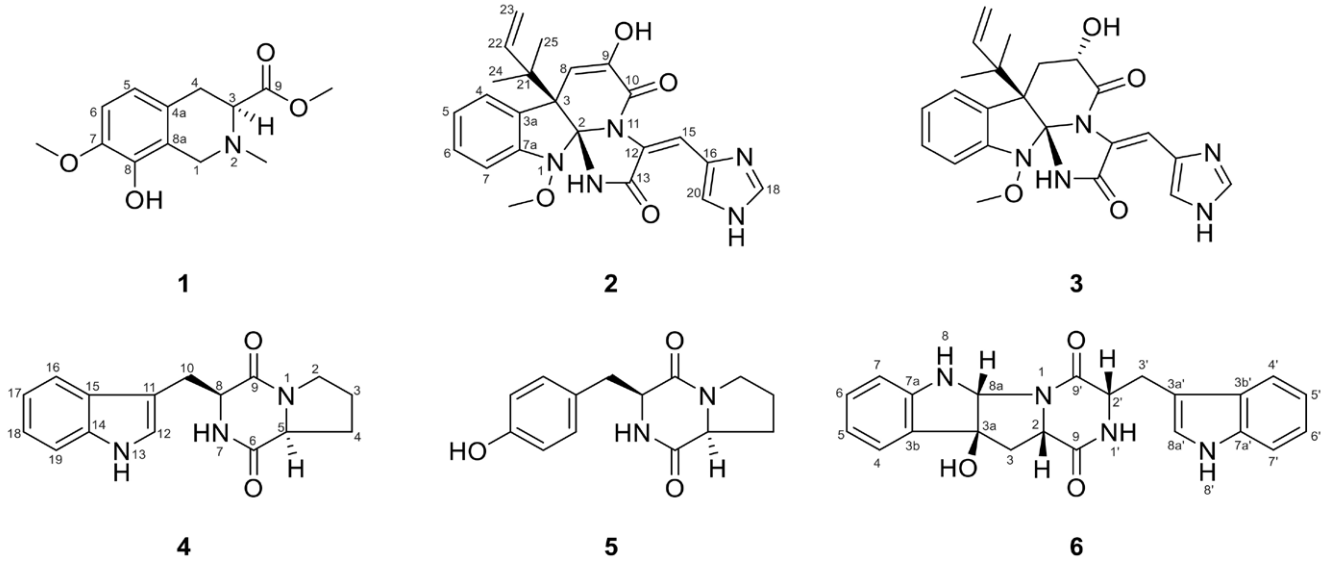

Fig. 1 Structures of 1–6.

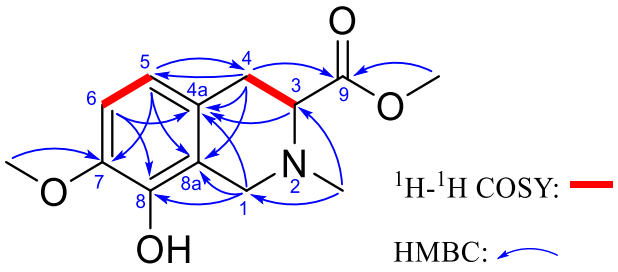

Fig. 2 Key HMBC and ^{1}H-^{1}H COSY correlations of 1.

A systematic conformational analysis was performed for **1** using the Merck Molecular Force Field model. The selected stereoisomer was reoptimized using TDDFT at the B3LYP/6-311G (2d, p) level to determine the lowest energy conformer. These were further filtered, assuming a Boltzmann distribution of the relative populations of different conformers, to obtain one significant conformer for each configuration. The ECD spectra calculated for the **1** (3*S*) diastereomer of the lowest energy conformer with various functionals (B3LYP, BH&HLYP, CAM-B3LYP) and TZVP basis set reproduced the experimental ECD spectrum well, with B3LYP/TZVP achieving the best agreement (Fig. 3). Thus, the absolute configuration of **1** was unambiguously determined as 3*S*.

The known compounds **2**–**6** were determined to be meleagrin (**2**)[11-12], neooxaline (**3**)[13], brevianamide F (**4**)[14-15], maculosin (**5**)[16], and okaramine U (**6**) [17] based on their NMR, ESI-MS, specific rotation data, and by comparison with data previously reported in the literature.

The new compound **1** isolated from *Penicillium* sp. DM27 was evaluated against the human colon (HCT-116), hepatoblastoma (HepG2), HEK 293t, and human primary bladder carcinoma (5637) cancer cell lines. The results indicated that compound **1** was inactive against the above cancer cells.

4 Conflicts of interest

The authors have no conflict of interest to declare.

5 Acknowledgments

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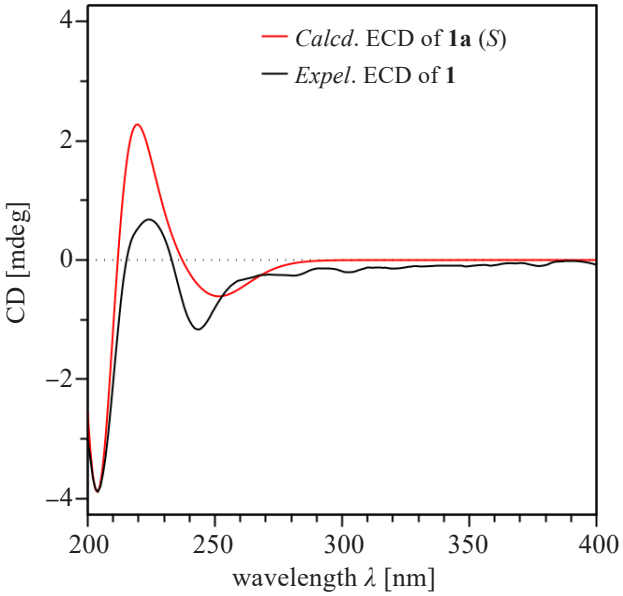

Fig. 3 Experimental ECD spectrum of 1 and calculated ECD spectrum of 1a.

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