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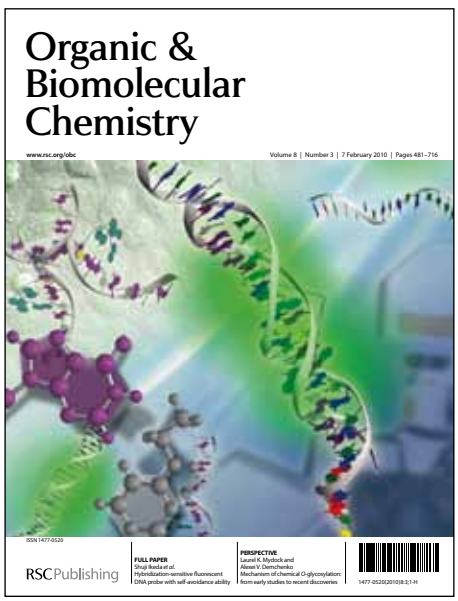

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ARTICLE TYPE

Diketopiperazine Alkaloids from a Mangrove Rhizosphere Soil Derived Fungus Aspergillus effuses H1-1

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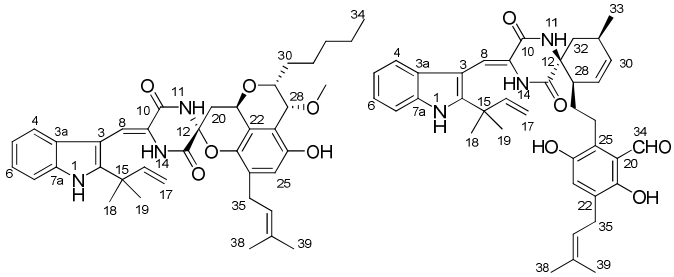
Effusin A (**1**), a spirobicyclic *N*,*O*-acetal derivative with an unprecedented 3',3a',5',6'-tetrahydrospiro[piperazine-2,2'-pyrano[2,3,4-de]chromene] ring system, and a spiro-polyketide-diketopiperazine hybrid dihydrocryptoechinulin D (**2**) were isolated from a mangrove rhizosphere soil derived fungus, *Aspergillus effuses* H1-1. Their structures were determined by detailed spectroscopic analysis. Effusin A (**1**) and dihydrocryptoechinulin D (**2**) occurred as racemates, the enantiomers of which were separated and characterized by online HPLC-ECD analysis and their absolute configurations were determined by the solution TDDFT ECD calculation approach. The cytotoxic effects of **1** and **2** were preliminarily evaluated and **2** showed potent activity on P388 cells with IC_{50} value of 1.83 μM. The target of racemic **2** were also investigated and the (12*R*, 28*S*, 31*S*)-**2** enantiomer showed selectivity against topoisomerase I.

Introduction

Fungi belonging to the genus *Aspergillus* are an attractive source of secondary metabolites with high structural diversities and interesting bioactivities, including pharmaceuticals such as the cholesterol-lowering drug lovastatin, psychoactive compounds such as xenovulene, and toxins such as aflatoxin B_1.[1] In our ongoing search for bioactive novel compounds from marine-derived fungi, a strain identified as *Aspergillus effuses* H1-1 was isolated from the mangrove rhizosphere soil collected in the coast of Fujian province, China. The chemical study led to the isolation of two new spiro-polyketide-diketopiperazine alkaloids, named effusin A (**1**) and dihydrocryptoechinulin D (**2**)[2] (Fig. 1). In this paper, the isolation, structural elucidation and bioactivities of compounds **1** and **2** are reported.



Effusin A (**1**)
1a: (12*R*, 21*R*, 28*R*, 29*R*) **-1**
1b: (12*S*, 21*S*, 28*S*, 29*S*) **-1**

Dihydrocryptoechinulin D (**2**)
2a: (12*R*, 28*S*, 31*S*) **-2**
2b: (12*S*, 28*R*, 31*R*) **-2**

Fig. 1. Structures of enantiomers **1**-**2**.

Results and Discussion

The fermented whole broth (70 L) gave a crude extract (76 g). The extract was separated by repeated silica gel column chromatography and finally semi-prep. ODS HPLC to yield compounds **1** (28 mg) and **2** (23 mg).

Effusin A (**1**) was obtained as a colorless solid. High-resolution electrospray mass spectrometry (HRESIMS) revealed ion at *m/z* 652.3377 ([M-H]$^-$), indicating a molecular formula of $C_{39}H_{47}N_3O_6$ (calcd. for $C_{39}H_{46}N_3O_6$, 652.3387). Its IR absorptions suggested the presence of hydroxyl, secondary amine (3428, 3368, 3195 cm^{-1}) and secondary amide (1693 and 1624 cm^{-1}) groups. The UV-vis absorptions at λ_{max} 358, 285 and 225 nm were characteristic of a dehydrotryptophan moiety.[3]

In the NMR spectrum, the dehydrotryptophan moiety was confirmed by the HMBC correlations from H-8 (δ_H 7.12) to C-2 (δ_C 144.7), C-3a (δ_C 126.3), and C-10 (δ_C 162.8), and from NH-1

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(δ_H 11.13) to C-2 and C-7a (δ_C 135.1) (Fig. 2). The 1,1-dimethyl-2-propenyl group was attached to C-2 based on the HMBC correlations from H-18 (δ_H 1.47) and H-19 (δ_H 1.51) to C-2. A diketopiperazine ring was deduced based on the two amide carbonyl carbons (δ_C 162.8, C-1; 162.7, C-13) observed in ^{13}C NMR spectrum and the HMBC correlations from NH-11 (δ_H 9.16) to C-9 (δ_C 124.0) and C-13 and from NH-14 (δ_H 9.67) to C-10 and the oxidized C-12 (δ_C 84.7) (Fig. 2). The isochroman moiety with a pentyl side-chain was deduced by the ^1H-^1H COSY correlations (H-28/H-29/H-30/H-31/H-32/H-33/H-34) and the HMBC correlations from H-28 (δ_H 4.39) to C-22 (δ_C 124.9) and C-26 (δ_C 149.7) and C-27 (δ_C 117.5), from OH-26 (δ_H 9.30) to C-26 and C-27, and from H-35 (δ_H 3.16) to C-23 (δ_C 140.3), C-24 (δ_C 130.2) and C-25 (δ_C 114.1), as well as the NOE enhancement of H-20b (δ_H 1.96) when H-29 (δ_H 3.49) was irradiated (Fig. 2). The prenyl group (from C-35 to C-39) was also deduced by the ^1H-^1H COSY correlations (H-35/H-36) and HMBC correlations from H-38 (δ_H 1.54) and H-39 (δ_H 1.60) to C-36 (δ_C 121.9) and C-37 (δ_C 132.1) (Fig. 2). The methoxyl was attached to C-28 evidenced by the HMBC correlation (Fig. 2). Then the planar structure of **1** was established by connection between C-12 and C-21 *via* C-20 based on the ^1H-^1H COSY (H-20/H-21) and HMBC correlations from H-20 (δ_H 2.73, 1.96) to C-12 (δ_C 84.7), C-13 (δ_C 162.7) and C-22, together with the connection of C-12 and C-23 *via* an oxygen according to the molecular formula, and the compound was named as effusin A.

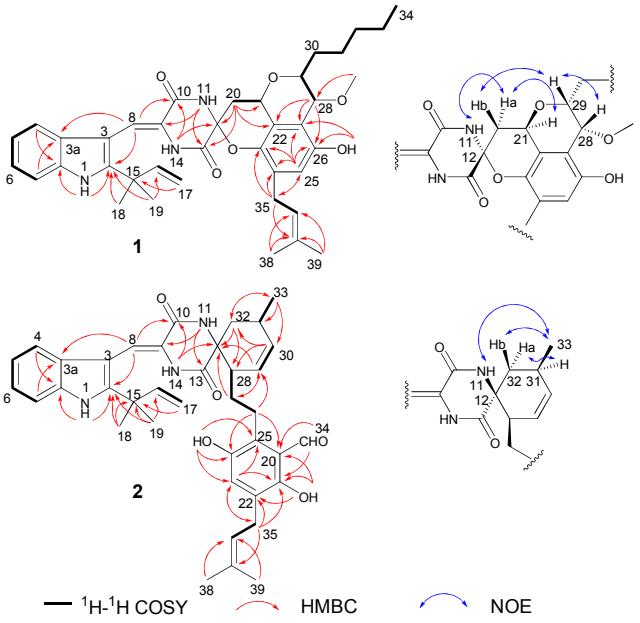


Fig. 2 Key ^1H-^1H COSY, HMBC and NOE correlations of **1** and **2**.

The relative stereochemistry of **1** was deduced as (12*R**,21*R**,28*R**,29*R**) on the basis of selective NOE difference experiments (Fig. 2). When H-29 (δ_H 3.49) was irradiated, the signals of H-28 (δ_H 4.39) and H-20b (δ 1.96) were enhanced, which revealed that these three hydrogens located at the same side. The enhancement of H-21 (δ_H 5.02) and NH-11 (δ_H 9.16) upon irradiation of H-20a (δ 2.73) indicated the *cis* relative configuration of H-20a, H-21 and the proximity of NH-11. Additionally, the *Z*-geometry of the Δ^8 double bond was deduced from the downfield shift of H-8 (δ_H 7.12) attributed to the deshielding effect of the carbonyl group on β-vinyl protons.[3] This was also in agreement with the lack of NOE effect between H-8 and H-14.

Table 1. The ^1H (600 MHz), and ^{13}C NMR (150 MHz) data of **1** and **2** in [D6]DMSO.

Atom no.	**1** δ_C	**1** δ_H mult.(*J* in Hz)	**2** δ_C	**2** δ_H mult.(*J* in Hz)
1		11.13, s		11.05, s
2	144.7		144.2	
3	104.0		103.6	
3a	126.3		127.1	
4	119.4	7.34, d (7.8)	118.8	7.07, m
5	119.4	6.96, dd (7.8, 7.3)	119.4	7.00, dd (7.8, 6.9)
6	120.8	7.07, dd (7.8, 7.3)	120.7	7.07, m
7	111.5	7.42, d (7.8)	111.6	7.41, d (8.2)
7a	135.1		135.1	
8	113.7	7.12, s	110.7	
9	124.0		124.4	
10	162.8		168.3	
11		9.16, s		7.71, s
12	84.7		59.7	
13	162.7		161.3	
14		9.67, s		8.86, s
15	39.7		39.2	
16	145.1	6.09, dd (17.2, 10.8)	145.1	6.05, dd (17.2, 10.3)
17	111.8	5.09, bd (10.8) 5.07, bd (17.2)	111.7	5.01, d (17.2) 5.03, d (10.3)
18	27.9	1.47, s	27.5	1.45, s
19	27.4	1.51, s	27.6	1.47, s
20	35.1	a:2.73, dd (12.4, 6.3) b:1.96, dd (12.4, 11.0)	117.2	
21	64.7	5.02, dd (11.0, 6.3)	153.4	
22	124.9		127.1	
23	140.3		125.2	6.92, s
24	130.2		146.8	
25	114.1	6.61, s	128.0	
26	149.7		20.9	2.88, m 3.07, m
27	117.5		32.7	1.54, m
28	69.4	4.39, brs	39.7	2.74, m
29	73.5	3.49, t (6.6)	126.1	5.78, brd (10.3)
30	31.0	1.65, m	131.8	5.71, brd (10.3)
31	25.0	1.41, m	28.0	2.58, m
32	31.3	1.33, m	38.6	1.74, dd (13.7, 2.8) 2.08, dd (13.7, 7.3)
33	22.1	1.33, m	21.6	1.10, d (7.4)
34	14.0	0.88, t (6.9)	196.9	10.18, s
35	27.4	3.16, m	26.6	3.12, m
36	121.9	5.22, brt (7.3)	121.7	5.16, brt (7.3)
37	132.1		132.5	
38	17.5	1.54, s	17.6	1.62, s
39	25.4 C	1.60, s	25.5	1.67, s
OCH3	56.2	3.26, s		
OH-26		9.30, s		
OH-21				11.76, s
OH-24				9.05, s

Since effusin A (**1**) had zero specific rotation and a baseline ECD curve, **1** was supposed to be a racemate, which was confirmed by the baseline separation of its enantiomers by chiral

HPLC using Chiralpak IC column (Fig. 3B). The separated enantiomers were characterized by their online HPLC-ECD spectra, which had been found an efficient tool to study stereoisomeric mixtures of natural products.[4,5,6] The HPLC-ECD spectra of the enantiomers showed mirror image curves (Fig. 3C), and the first-eluting enantiomer (**1a**) had a broad negative Cotton effect (CE) at 349 nm, positive ones at 283 and 234 nm, and a negative one at 211 nm. The DFT reoptimization of the 30 (for computational procedure see Table S1, SI) initial MMFF conformers was carried out on the truncated model compound **1a′** (C-29 n-pentyl group in **1a** was replaced by methyl) at the B3LYP/6-31G(d) level, which afforded two major conformers with 64.8% (conf. A) and 30.6% population (conf. B) (Fig. 3A) above 3% population. The two conformers differed in the orientation of the C-2 and C-24 alkenyl substituents. The ECD spectra of the arbitrarily chosen (12*R*, 21*R*, 28*R*, 29*R*) enantiomer (**1a′**) were calculated with various functionals (B3LYP, BH&HLYP, PBE0) and 6-311G(d,p) basis set. All the Boltzmann-weighted ECD spectra reproduced well the experimental curve of the first-eluting enantiomer (**1a**) with PBE0 giving the best agreement (Fig. 3D), which allowed the determination of the absolute configuration of the first-eluting enantiomer (**1a**) as (12*R*, 21*R*, 28*R*, 29*R*). Consequently, the sencond-eluting enantiomer (**1b**) was also determined as (12*S*, 21*S*, 28*S*, 29*S*) by the ECD curve.

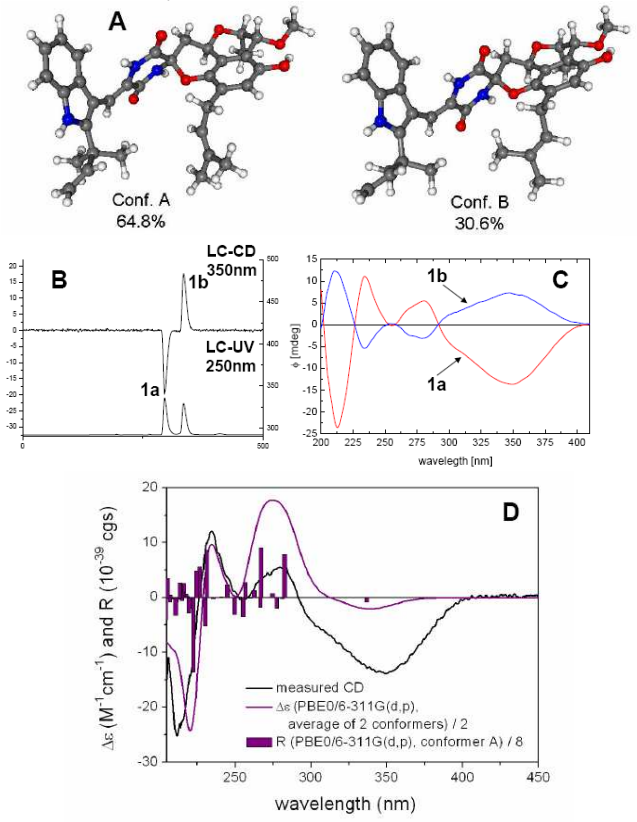


Fig. 3 DFT optimized conformational isomers of the truncated model compound of (12*R*, 21*R*, 28*R*, 29*R*)-**1** (**1a′**) above 3% and their Boltzmann populations obtained by B3LYP/6-31G(d) (**A**); HPLC spectrum of **1** on a chiral phase (**B**); HPLC-ECD spectra of the separated enantiomers of effusin A (**1**); first-eluting enantiomer (**1a**) (red curve) and second-eluting enantiomer (**1b**) (blue curve) (**C**) and solution ECD spectrum of **1a** (peak 1, black curve) compared with the PBE0/6-311G(d,p) computed ECD spectrum for the truncated model of **1a′** obtained as the Boltzmann-weighted average of the solution conformers. Bars represent the rotatory strengths of the lowest-energy conformer (**D**).

Dihydrocryptoechinuline D (**2**) was obtained as a yellow solid. Its molecular formula was determined as $C_{38}H_{43}N_3O_5$ according to the positive HRESIMS peak at *m/z* 622.3256 $[M+H]^+$ (calcd. 622.3281). It's IR and UV-Vis spectra were very similar to those of cryptoechinuline D,[7] which suggested the presence of dehydrotryptophan and diketopiperazine moieties.[3]

The ^1H and ^{13}C NMR spectra of **2** were very similar to cryptoechinuline D, except the appearance of two sp^3 methylene signals (CH_2-26: δ_H 2.88, 3.07; δ_C 20.9 and CH_2-27: δ_H 1.54, δ_C 32.7) instead of two sp^2-hybridized carbons in cryptoechinuline D. This suggested that **2** was the dihydro analogue of cryptoechinuline D. This assumption was confirmed by ^1H-^1H COSY and HMBC (Fig. 2) spectra of **2**, and it was named as dihydrocryptoechinuline D.

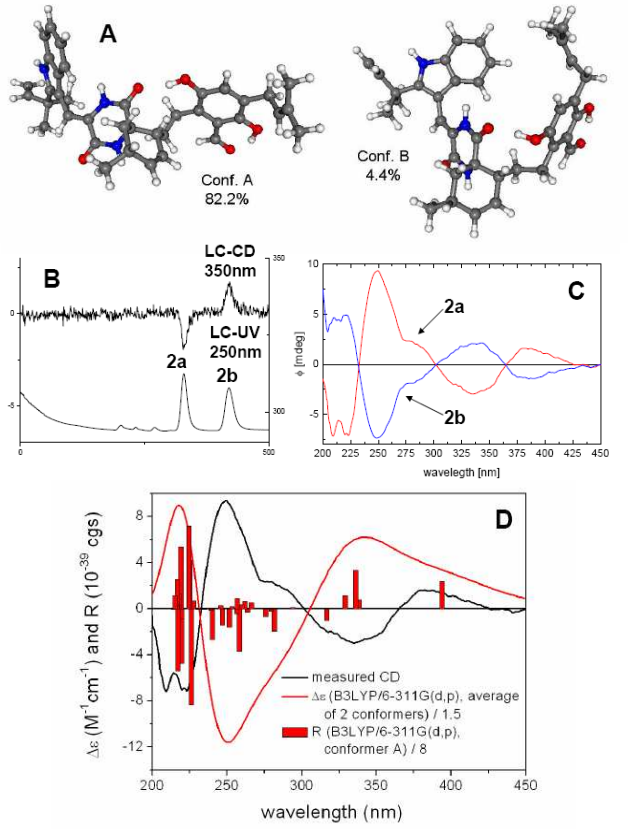


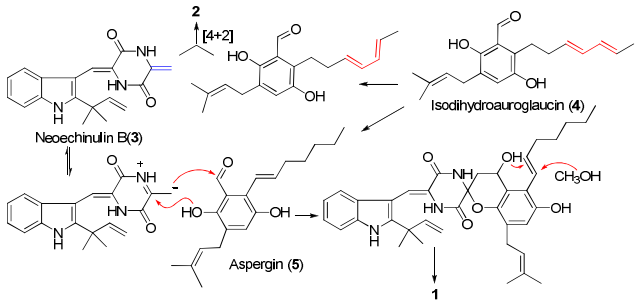
Fig. 4 Conformational isomers of (12*S*, 28*R*, 31*R*)-**2** (**2b**) above 3% and their Boltzmann populations obtained by B3LYP/6-31G(d) reoptimizations (**A**); HPLC spectrum of **2** on a chiral phase (**B**); the ECD spectra of the seprated enantiomers of **2**, first-eluting enantiomer (**2a**) (red curve), second-eluting enantiomer (**2b**) (blue curve) (**C**); and the comparison of the ECD spectrum of **2a** (black curve) to the calculated one of (12*S*,28*R*,31*R*)-**2** (red curve) (**D**).

In the selective NOE experiments (Fig. 2), when H-33 (δ_H 1.10) was irradiated, the signals of H-32b (δ_H 1.74) and NH-11 (δ_H 7.71) were enhanced, indicating the β orientation of these protons. The

enhancement of H-32a (δ_H 2.08) upon irradiation of H-31 (δ_H 2.58) indicated that they were at the same side. Although no enhancement of hydrogen signals was observed when H-28 was irradiated, the relative stereochemistry of C-28 could be deduced the same as cryptoechinulin D,[8] which was also isolated from this strain, from the biosyntheses opinions.

Similarly to effusin A (**1**), dihydrocryptoechinuline D (**2**) was also a racemic mixture as justified by the zero specific rotation and baseline ECD curve. The enantiomers were separated on a Chiralpak IC column and mirror image HPLC-ECD curves were recorded (Fig. 4B, 4C). Since the enantiomers of dihydrocryptoechinuline D (**2**) exhibited quite different ECD curves from those of effusin A (**1**), TDDFT ECD calculation was used again to determine the absolute configuration. The initial MMFF conformational search provided 72 conformers, the DFT reoptimization of which reduced the number of conformers to two above 3% population. The two conformers had 82.2% and 4.4% population (Fig. 4A), and they were used for the calculation of the ECD spectrum. The Boltzmann-weighted TDDFT ECD spectra of (12*S*, 28*R*, 31*R*)-**2** were mirror image of the experimental HPLC-ECD of the first eluting enantiomer except for the lowest-energy transition, which suggested that the first eluting enantiomer (**2a**) has (12*R*, 28*S*, 31*S*) absolute configuration (Fig. 4D). A patent application for dihydrocryptoechinuline D (**2**) had been submitted in China describing the new structure and its cytotoxicities against P388, HL-60, BEL-7402 and A-549 cell lines[2] but the separation, determination of absolute configuration and the molecular target of the two enantiomers were not mentioned.

Effusin A (**1**) contains a spirobicyclic N,O-acetal moiety, which could be obtained by a domino ring-closure reaction between the substituted salicylaldehyde moiety in aspergin and the eneamide moiety of diketopiperazine unit in neoechinulin B. In contrast, an enzyme-catalyzed regiospecific [4+2] Diels-Alder reaction produces the spirobicycle of **2** (Scheme 1). Similar Diels-Alder biosynthetic reaction has already been suggested for a few recent examples such as yaoshanenolides and lanceolatins.[9,10,11] Notably, all the key intermediates including neoechinulin B (**3**),[3] isodihydroauroglaucin (**4**)[12] and aspergin (**5**)[13] were isolted from this fungus *Aspergillus effuses* H1-1, which supported the biosynthetic hypothesis.



Scheme 1. Plausible biosynthetic pathway to **1** and **2**

Spiro-polyketide-diketopiperazines are rare in nature. To the best of our knowledge, only few examples belonging to this kind of spirocyclic alkaloids, variecolortides A-C[14] and cryptoechinuline D,[7] were reported, and their absolute configurations have not been reported yet. The synthesis of racemic variecolortide A and B has been recently reported by exploiting a hetero Diels-Alder reaction of a 1,4-anthraquinone with a didehydrodiketopiperazine to form the central spiro-cyclic core.[15]

The cytotoxic effects of **1** and **2** were preliminarily evaluated on P388, HL-60, BEL-7402 and A-549 cell lines (Table 2). Compound **2** showed remarkable activities with IC_{50} values of 1.83 and 4.80 μM on P388 and HL-60 cells, respectively.[2]

Table 2. Cytotoxicities of compounds **1-2** on four tumor cell lines

Compound	IC_{50} (μM)			
	P388	HL-60	BEL-7402	A-549
1	>100	>100	85.2 ± 0.08	>100
2	1.83 ± 0.21	4.80 ± 0.34	>100	17.1 ± 0.16
Doxorubicin	0.33 ± 0.002	0.05± 0.001	0.244 ± 0.019	0.0799 ± 0.0058

The molecule targets of racemic **2** were investigated by using various models including topoisomerase I, topoisomerase II, and Hsp90. The inhibitory activity only was observed on topoisomerase I assayed by relaxation of supercoiled plasmid DNA at 100 μM. To investigate the influence of absolute configurations on the activity, the separated enantiomers (**2a** and **2b**) of **2** were tested on topoisomerase I. Interestingly, (12*R*, 28*S*, 31*S*)-**2** (**2a**) showed moderate activity against topoisomerase I than **2**, while **2b** was almost inactive (Fig. 5).

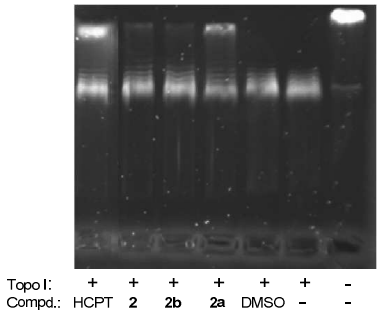


Fig. 5 Effects of **2**, (12*R*, 28*S*, 31*S*)-**2** (**2a**) and (12*S*, 28*R*, 31*R*)-**2** (**2b**) on Topo I-mediated supercoiled pBR322 relaxation

Experimental Section

General

Specific rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were taken on a Nicolet Nexus 470 spectrophotometer in KBr discs. NMR spectra were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as internal standard, and chemical shifts were recorded as δ values. ESIMS utilized on a Q-TOF Ultima Global GAA076 LC mass spectrometer. Semiprepartive HPLC was performed using an ODS column [HPLC :YMC-Pack ODS-A (5 μm, 10 × 250 mm, 4 mL/min)]. HPLC-ECD spectra of **1** and **2** were run on a

Chiralpak IC column (5 μm, 150×4.6 mm, hexane/isopropanol eluent, 1 mL/min flow rate) and were recorded in stopped-flow mode on a JASCO J-810 electronic circular dichroism spectropolarimeter equipped with a 10 mm HPLC flow cell. TLC and column chromatography (CC) were performed on plates precoated with silica gel GF254 (10–40 μm) and over silica gel (200–300 mesh, Qingdao Marine Chemical Factory). Vacuum-liquid chromatography (VLC) was carried out over silica gel H (Qingdao Marine Chemical Factory).

Fungal Material

The working strain *Aspergillus effuses* H1-1 was isolated from the mud under mangrove along the coast of Fujian province, China. It was identified by Prof. Li Tian, the First Institute of Oceanography, SOA, Qingdao, China. The voucher specimen is deposited in the Key Laboratory of Marine Drugs, Chinese Ministry of Education.

Fermentation and Extraction

A small spoon of spore growing on potato dextrose agar slant was inoculated into 250 mL Erlenmeyer flask containing 75 mL culture medium consisting of glucose 2%, maltose 2%, monosodium glutamate 10%, beef extract 0.3%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.03% (in sea water) and cultured at 28˚C for two days on a rotary shaker at 160 rpm. Then 10 mL resultant seed culture was inoculated into 500 mL Erlenmeyer flask containing 150 mL the above culture medium and incubated at 28˚C for seven days on a rotary shaker at 160 rpm. The fermented whole broth (70 L) was filtrated through cheesecloth to separate into broth supernatant and mycelium. The former was extracted three times with ethyl acetate to give an ethyl acetate solution. The latter was extracted three times with methanol, which was evaporated under reduced pressure to remove methanol to afford an aqueous solution. The aqueous solution was extracted three times with ethyl acetate to give another ethyl acetate solution. Both the ethyl acetate solutions were combined and concentrated in vacuo to give a crude extract (76 g).

Purification

The crude extract (76 g) was subjected to silica gel (200~300 mesh) column packed in petroleum ether, and was separated into seven fractions (Fr.1 - Fr.7) using a step gradient elution of petroleum ether-chloroform and chloroform-methanol. The fraction (Fr.4), eluted with chloroform-methanol (100:1) solution from the silica gel column was further chromatographed gradiently on silica gel using chloroform-methanol (100:1~10:1) as elution and divided into 8 subfractions(Fr.4-1 - Fr.4-8). Subfraction Fr.4-1 yielded compound **1** (28 mg). Subfraction Fr.4-2 was separated by preparative HPLC on a ODS column using methanol-water (80:20) as eluting solvent to yield compound **2** (23 mg), and compound **2** (2.0 mg) were resolved to **2a** (0.8 mg) and **2b** (0.8 mg) by HPLC on a chiral phase. The fraction, Fr.3, eluted with chloroform-methanol (200:1) solution from the silica gel column was further chromatographed gradiently on silica gel using chloroform-methanol (200:1~50:1) as elution and divided into 3 subfractions(Fr.3-1 - Fr.3-3). Subfraction Fr.3-1 yielded compound **3** (150 mg). The fraction (Fr.1), eluted with petroleum ether-chloroform (1:1) solution from the silica gel column was further chromatographed gradiently on silica gel using petroleum ether-chloroform (2:1~1:1) as elution and divided into 4 subfractions (Fr.1 - 1-Fr.1-4). Subfraction Fr.4-1 yielded compound **4** (26 mg), and subfraction Fr.4-2 yielded compound **5** (98 mg).

Effusin A (1): colorless solid (actone); UV (MeOH) λ_{max} (log ε) 358 (3.62), 285 (3.60), 225 (4.12), 203 (4.38) nm; IR (KBr) ν_{max} 3428, 3368, 3195, 3079, 2961, 2869, 1693, 1624, 1442, 1377, 1079, 1036 cm^{-1}; ^1H NMR and ^{13}C NMR data, Table 1; HRESIMS *m/z* 652.3377 $[M-H]^-$ (calcd for $C_{39}H_{46}N_3O_6$ 652.3387).

(12*R*, 21*R*, 28*R*, 29*R*)-**1** (**1a**): retention time (t_R) 4.92 min (Chiralpak IC, hexane/isopropanol 85:15); ECD data were recorded as λ_{max} (ϕ) by stopping the flow of the eluent (hexane/isopropanol 85:15) at the maximum concentration: 349 (–13.87), 283 (5.75), 266sh (3.15), 245sh (3.38), 234 (12.09), 211 (–25.17).

(12*S*, 21*S*, 28*S*, 29*S*)-**1** (**1b**): retention time (t_R) 5.58 min (Chiralpak IC, hexane/isopropanol 85:15); ECD λ_{max} (ϕ) in hexane/isopropanol 85:15: 346 (7.49), 279 (–3.46), 267sh (–2.69), 241sh (–2.78), 211 (12.99).

Dihydrocryptoechinulin D (2): yellow solid (actone); UV (MeOH) λ_{max} (log ε) 348 (3.76), 275 (3.86), 224 (4.21) nm; IR (KBr) ν_{max} 3350, 3240, 3044, 2987, 2928, 1670, 1637, 1428, 1380, 1271, 1253, 747 cm^{-1}; ^1H NMR and ^{13}C NMR data, Table 1; HRESIMS *m/z* 622.3256 $[M+H]^+$ (calcd for $C_{38}H_{44}N_3O_5$ 622.3281).

(12*R*, 28*S*, 31*S*)-**2** (**2a**): $[\alpha]^{20}_D$ +204.8 (*c* 0.15, MeOH); retention time (t_R) 5.48 min (Chiralpak IC, hexane/isopropanol 70:30); ECD λ_{max} (ϕ) in hexane/isopropanol 70:30: 402sh (1.07), 382 (1.60), 335 (–2.96), 280sh (2.30), 249 (9.37), 223 (–7.09), 209 (–7.16).

(12*S*, 28*R*, 31*R*)-**2** (**2b**): $[\alpha]^{20}_D$ –192.8 (*c* 0.15, MeOH); retention time (t_R) 7.00 min (Chiralpak IC, hexane/isopropanol 70:30); ECD λ_{max} (ϕ) in hexane/isopropanol 70:30: 408sh (–0.83), 385 (–1.43), 342 (2.14), 282sh (–1.86), 248 (–7.35), 221 (4.91), 210 (4.65).

Bioassys

Cell proliferation/viability assays: The maintance of HL-60, P388, A-549, and BEL-740 cells, compound treatment, and MTT (HL-60 or P388)[16] and SRB (A-549 or BEL-7402)[17] assays for cell proliferation/viability were the same as those described previously.

Topoisomerase I mediated DNA cleavage assay. Topoisomerases I were assayed by relaxation of supercoiled plasmid DNA. Relaxation of 250 ng of supercoiled by

topoisomerase I (2 U) was performed in 20 μL of topoisomerase I relaxation buffer [10mM Tris-HCl, pH 7.9, 1 mM EDTA, 150 mM NaCl, 0.1% (w/v) BSA, 0.1 mM spermidine, 5% (v/v) glycerol] in the presence and absence of varying amounts of the test compounds dissolved in dimethyl sulfoxide (5% (v/v) final concentration). Reactions were started by addition of DNA. Control groups were either DNA alone or DNA treated with topoisomerase. After 30 min at 37°C, the reaction was terminated by addition of 1% (w/v) SDS and digested with 50 mg/mL proteinase K at 55°C for 30 min. DNA was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and separated on 1% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, pH 8.0, and 2 mM EDTA) at 2 V/cm for 3.5 h. Gels were stained with 5 mg/mL ethidium bromide, destained, and photographed using Polaroid 665 film or a gel-imaging system for numerical quantification by densitometry scanning (Herolab, Wiesloch,Germany).

The effect of racemic **2,** (12*R*,28*S*,31*S*)**-2** (**2a**) and (12*S*, 28*R*, 31*R*)**-2** (**2b**) on topoisomerases was investigated using a conventional plasmid DNA relaxation assay. HCPT, a well-known Topo I inhibitor, was employed as a positive control. (12*R*, 28*S*, 31*S*)-**2** (**2a**) leads to the observed moderate inhibited the DNA relaxation activity of Topo I at the concentration of 100 μM.

Computational section

Conformational searches were carried out by means of the Macromodel 9.7.211[18] software using Merck Molecular Force Field (MMFF) with implicit solvent model for octanol. In order to decrease the number of conformers (246 and 405 for the truncated model of **1** and compound **2**, respectively, within 21 kJ/mol energy window), the MMFF geometries were reclustered for all heavy atoms except for carbons 16-19 and 36-39. Geometry reoptimizations at B3LYP/6-31G(d) level of theory followed by TD-DFT calculations using various functionals (B3LYP, BH&HLYP, PBE0) and 6-311G(d,p) basis set were performed by the Gaussian 03[19] package. Boltzmann distributions were estimated from the ZPVE corrected B3LYP/6-31G(d) energies. ECD spectra were generated as the sum of Gaussians[20] with 1800 and 3000 cm^{-1} half-height width (corresponding to ca. 19 and 32 nm at 325 nm, respectively), using dipole-velocity computed rotational strengths for conformers above 3%. The MOLEKEL[21] software package was used for visualization of the results.

Conclusions

Effusin A (**1**) and dihydrocryptoechinuline D (**2**), two new spiro-polyketide-diketopiperazines were isolated from the fungus *Aspergillus effuses* H1-1. Effusin A (**1**) possesed an unprecedented 3',3a',5',6'-tetrahydrospiro[piperazine-2,2'-pyrano[2,3,4-de]chromene] skeleton, and **2a** showed antitumor activity targeted to the topoisomerase I.

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