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Two New Antifungal Alkaloids Produced by *Streptoverticillium morookaense*

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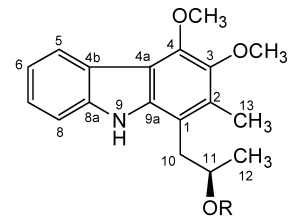
Abstract A new carbazole alkaloid, streptoverticillin, and a new 2-azetidinone, streptoverticillinone, along with three known cyclodipeptides were isolated from the mycelial solid culture of *Streptoverticillium morookaense*. Their structures were elucidated by analysis of 1D and 2D NMR, mass spectra and optical rotation data. Two new compounds exhibited antifungal activity against *Peronophythora litchii*.

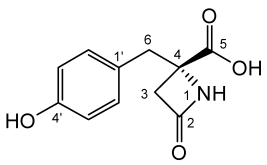
Keywords *Streptoverticillium*, alkaloids, streptoverticillin, streptoverticillinone, antifungal

Introduction

Litchi (*Litchi chinensis* Sonn.) is a tropical and subtropical fruit with luscious flavor and attractive appearance. One of the major limitations to its commercial value is the rapid rot after harvest, which is caused by phytopathogenic fungi. Although the currently used synthetic fungicides, such as carbendazim, prochloraz, and benomyl are effective in controlling postharvest rot [1], there is need for novel agents. The available fungicides aroused increasing public concern regarding contamination with residues and proliferation of antifungal resistance in the pathogen populations. Microorganisms have proved to be a rich source of bioactive secondary metabolites, and numerous compounds with potent biological activities and unique chemical structures have been isolated [2]. In screening for new antibacterial and antifungal metabolites produced by microorganisms isolated in South China [3, 4], an EtOH extract from a mycelial solid culture of *Streptoverticillium morookaense* was found to show antifungal activity against *Peronophythora litchii*, one of the main pathogens causing litchi fruit rot. We investigated the secondary metabolites of this actinomycete and isolated and characterized a new carbazole alkaloid, trivially named streptoverticillin (**1**), and a new 2-azetidinone, streptoverticillinone (**2**), with antifungal activity against *P. litchii*, along with three known cyclodipeptides. We herein report the isolation, structure elucidation, and antifungal activity of these two new compounds.



Streptoverticillin (**1**) R = H
1a R = (*R*)-MTPA
1b R = (*S*)-MTPA



Streptoverticillinone (**2**)

Experimental

General

Optical rotations were obtained on a Perkin-Elmer 341 polarimeter with MeOH as solvent. The UV spectra were recorded in MeOH on a Perkin-Elmer Lambda 25 UV-vis spectrophotometer. The ^{1}H (400 MHz), ^{13}C (100 MHz), and 2D NMR spectra were recorded on a Bruker DRX-400 instrument using TMS as an internal reference. HRTOFMS data were obtained on an API QSTAR TOF mass

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spectrometer in positive-ion mode. ESIMS were collected on MDS SCIEX API 2000 LC/MS/MS instrument. For column chromatography, silica gel 60 (100～200 mesh, Qingdao Marine Chemical Ltd., Qingdao, China), Develosil ODS (10 μm, Nomura Chemical Co. Ltd., Japan), and Sephadex LH-20 were used. Preparative TLC was performed on precoated silica gel plates (GF_{254}, Qingdao Marine Chemical Ltd., Qingdao, China, 0.25 mm thickness) with detection under fluorescent (λ=254 nm) light.

Producing Actinomycete

S. morookaense strain SC1169 was isolated from a soil sample (DH0814-5) collected in the pine (*Pinus massoniana*) forest at Dinghu Mountain Biosphere Reserve, Guangdong, China, in December, 2003. The actinomycete was authenticated by Prof. Songzhen Yang, Guangdong Institute of Microbiology, Guangzhou, China. The culture (SC1169) is deposited in the culture collection of South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China. For maintenance on agar slants and submerged cultures, the actinomycete was grown on PDA medium.

Fermentation

The mycelia of *S. morookaense* grown on PDA plates were used to inoculate twenty 100-ml Erlenmeyer flasks containing 30 ml of YMG medium (glucose 0.4%, malt extract 1.0%, yeast extract 0.4%, pH 5.5). The flasks were incubated on a rotary shaker for 5 days in the dark at 25°C with shaking at 150 rpm. Then the cultures were transferred into twenty 500-ml flasks containing 150 ml of YMG at the same incubation condition. At last the cultures were transferred into twenty 5.0-liter flasks containing 1000 ml of YMG medium and 550 g of wheat grains, and the cultivation was carried out in the stationary phase in the dark at 25°C for 40 days.

Extraction and Isolation

The solid cultures of *S. morookaense* were extracted with 95% EtOH three times at room temperature. The resultant EtOH solution, after concentration *in vacuo*, was suspended in H_2O, and this aqueous suspension was sequentially extracted three times each with petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH. The EtOAc-soluble extract (36.7 g) was subjected to passage over a silica gel column, eluted with petroleum ether - acetone mixtures of increasing polarity (100 : 0 to 3 : 2) and acetone to obtain ten fractions (I～X). Fraction V, obtained by elution of petroleum ether - acetone (9 : 1), was chromatographed over a Sephadex LH-20 column, eluted with MeOH, to afford **1** (7 mg). Fraction VII, obtained on elution with petroleum ether - acetone (4 : 1), was applied to a silica gel column with $CHCl_3$ - MeOH (49 : 1), to afford cyclo(leucylprolyl) [5] (15 mg) and the mixture of cyclo(isoleucylleucyl) [6] and cyclo(leucylvalyl) [7] (8 mg) in the rough ratio of 3 : 2 (assessed from the ^{1}H NMR signal intensity of H-11). Fraction VIII, obtained by elution with petroleum ether - acetone (3 : 2), was separated by silica gel column chromatography, eluted with $CHCl_3$ - MeOH mixtures of increasing polarity (95 : 5～85 : 15), to afford eight subfractions (VIII-1～VIII-8). Subfraction VIII-6 was subjected to a silica gel column with petroleum ether - acetone (3 : 2), to afford **2** (20 mg).

Streptoverticillin (**1**)

Yellowish amorphous powder, $[\alpha]_D^{20}$ +18.4° (*c* 0.179, MeOH); UV λ_{max} (MeOH) (log ε) 220 (4.41), 242 (4.48), 293 (4.12), 328 (3.61) nm; ^{1}H NMR (400 MHz, CD_3OD) δ 8.11 (1H, d, *J*=8.0 Hz, H-5), 7.41 (1H, d, *J*=8.0 Hz, H-8), 7.29 (1H, t, *J*=8.0 Hz, H-7), 7.08 (1H, t, *J*=8.0 Hz, H-6), 4.09 (1H, m, H-11), 4.03 (3H, s, OCH_3-4), 3.85 (3H, s, OCH_3-3), 3.07 (1H, dd, *J*=6.8, 14.0 Hz, H-10a), 3.01 (1H, dd, *J*=6.8, 14.0 Hz, H-10b), 2.39 (3H, s, H-13), 1.22 (3H, d, *J*=6.4 Hz, H-12); ^{13}C NMR (100 MHz, CD_3OD) δ 147.5 (C, C-4), 145.2 (C, C-3), 141.5 (C, C-8a), 138.8 (C, C-9a), 130.0 (C, C-2), 125.9 (CH, C-7), 123.4 (C, C-4b), 123.1 (CH, C-5), 119.6 (CH, C-6), 117.0 (C, C-1), 115.6 (C, C-4a), 111.5 (CH, C-8), 68.9 (CH, C-11), 61.3 (OCH_3-3), 60.8 (OCH_3-4), 38.9 (CH_2, C-10), 23.1 (CH_3, C-12), 13.0 (CH_3, C-13); ESIMS *m/z* 621 $[2M+Na]^+$, 599 $[2M+H]^+$, 338 $[M+K]^+$, 322 $[M+Na]^+$, 300 $[M+H]^+$, 282 $[M-OH]^+$, 254 $[M-CH(OH)CH_3]^+$; HRTOFMS *m/z* 322.1416 $[M+Na]^+$ (calcd for $C_{18}H_{21}NO_3Na$, 322.1414).

Streptoverticillinone (**2**)

White amorphous powder, $[\alpha]_D^{20}$ +62.8° (*c* 0.32, MeOH); UV λ_{max} (MeOH) (log ε) 225 (3.89), 278 (3.17) nm; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.06 (1H, br s, OH-5), 9.26 (1H, br s, OH-4′), 7.00 (2H, d, *J*=8.0 Hz, H-2′, 6′), 6.63 (2H, d, *J*=8.0 Hz, H-3′, 5′), 6.04 (1H, br s, H-1), 2.87 and 2.77 (each 1H, d, *J*=16.0 Hz, H_2-6), 2.73 and 2.34 (each 1H, d, *J*=16.0 Hz, H_2-3); ^{13}C NMR (400 MHz, DMSO-d_6) δ 181.0 (C, C-5), 175.6 (C, C-2), 156.2 (C, C-4′), 131.1 (CH, C-2′, 6′), 125.4 (C, C-1′), 115.0 (CH, C-3′, 5′), 76.3 (C, C-4), 41.5 (CH_2, C-3), 41.3 (CH_2, C-6); ESIMS *m/z* 465 $[2M+Na]^+$, 260 $[M+K]^+$, 222 [M+H], 205 $[M-OH]^+$; HRTOFMS *m/z* 244.0570 $[M+Na]^+$ (calcd for $C_{11}H_{11}NO_4Na$, 244.0580).

Preparation of (*R*)- and (*S*)-MTPA Esters of 1

A solution of the compound (1.2 mg, 0.004 mmol) in

CH_2Cl_2 (1.0 ml) was treated with (*R*)-α-methoxy-α-trifluoromethylphenylacetic acid [(*R*)-MTPA, 4.7 mg, 0.02 mmol] in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC-HCl, 3.8 mg, 0.02 mmol) and 4-dimethylaminopyridine (4-DMAP, 2.4 mg, 0.02 mmol), and the mixture was stirred at room temperature (20°C) for 24 hours. The reaction mixture was poured into ice-water and the whole was extracted with EtOAc. The EtOAc extract was successively washed with 5% aq HCl, aq sat $NaHCO_3$, and brine, then dried over $MgSO_4$ and filtered. Evaporation of the solvent from the filtrate under reduced pressure furnished a residue, which was purified by preparative TLC, developed with petroleum ether - EtOAc (9 : 1) to give (*R*)-MTPA ester (1.5 mg). Through a similar procedure, the (*S*)-MTPA ester (1.3 mg) was prepared from the compound (1.2 mg) by the use of (*S*)-MTPA (4.7 mg), EDC-HCl (3.8 mg), and 4-DMAP (2.4 mg).

(*R*)-MTPA Ester of **1** (**1a**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.412 (3H, d, J=6.2 Hz, H-12), 2.413 (3H, s, H-13), 3.133 (1H, dd, J=7.4, 14.4 Hz, H-10b), 3.282 (1H, dd, J=6.0, 14.4 Hz, H-10a), 3.282 (3H, s, OCH_3-MTPA), 3.889 (3H, s, OCH_3-3), 4.118 (3H, s, OCH_3-4), 5.332 (1H, m, H-11), 7.20～7.39 (8H, overlapping, H-Ar), 8.203 (1H, d, J=7.8 Hz, H-5), 8.676 (1H, br s, H-9).

(*S*)-MTPA Ester of **1** (**1b**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.408 (3H, d, J=6.3 Hz, H-12), 2.392 (3H, s, H-13), 3.053 (1H, dd, J=8.0, 14.0 Hz, H-10b), 3.303 (1H, dd, J=4.6, 14.0 Hz, H_2-10a), 3.538 (3H, s, OCH_3-MTPA), 3.879 (3H, s, OCH_3-3), 4.111 (3H, s, OCH_3-4), 5.369 (1H, m, H-11), 7.19～7.47 (8H, overlapping, H-Ar), 8.197 (d, J=8.0 Hz, H-5), 8.944 (1H, br s, H-9).

Antifungal Activity Test

Antifungal activity was evaluated by the well plate diffusion method [8]. The test microorganism was *P. litchii*, obtained from Key Laboratory of Pesticides and Chemical Biology, Ministry of Education, South China Agriculture University, Guangzhou, China. Compounds **1**, **2** and carbendazim (positive control) were individually dissolved and diluted with DMSO to obtain serial concentrations of 2000, 1000, 500, 250, and 125 μg/ml. Three 6 mm wide wells were opened in the PDA medium inoculated test microorganism in each Petri dish using a sterile steel borer. The compound solution (100 μl) of a specific concentration was added to each of the wells. DMSO was used as a negative control. The plates were then incubated at 25°C for 3 days. The inhibition zones around the holes were measured and MIC which was defined as the lowest concentration able to inhibit any visible fungal growth was recorded. The assays were performed three times for each compound in order to guarantee reproducibility of results.

Results and Discussion

The actinomycete, *S. morookaense* strain SC1169, was isolated from a soil sample collected from Dinghu Mountain Biosphere Reserve, Guangdong, China. The mycelia were grown on a solid culture for 40 days at 25°C in the dark. The EtOH extract of the mycelial culture was sequentially fractionated with petroleum ether, $CHCl_3$, and EtOAc. The EtOAc-soluble extract, by separation with silica gel and Sephadex LH-20 column chromatography, yielded compounds **1** and **2** along with cyclo(leucylprolyl) [5] and a mixture of cyclo(isoleucylleucyl) [6] and cyclo(leucylvalyl) [7], which were identified by interpretation of their spectroscopic (ESIMS, ^{1}H NMR, and ^{13}C NMR) data as well as by comparison of their physical and spectroscopic data with those in the indicated literatures.

1 had a molecular formula of $C_{18}H_{21}NO_3$ by analysis of its HRTOFMS, ^{13}C NMR and DEPT data. The ^{1}H NMR spectrum (see Experimental section) in combination with the COSY spectrum (Fig. 1) showed the presence of an aromatic methyl group at δ 2.39 (3H, s, H-13), two aromatic methoxyl groups at δ 3.85 (3H, s, OCH_3-3) and 4.03 (3H, s, OCH_3-4), a 2-hydroxypropyl group [δ 1.22 (3H, d, J=6.4 Hz, H-12), 4.09 (1H, m, H-11), 3.01 (1H, dd, J=6.8, 14.0 Hz, H-10b), and 3.07 (1H, dd, J=6.8, 14.0 Hz, H-10a)], and a 1,2-disubstituted benzene ring [δ 7.08 (1H, t, J=8.0 Hz, H-6), 7.29 (1H, t, J=8.0 Hz, H-7), 7.41 (1H, d, J=8.0 Hz, H-8), and 8.11 (1H, d, J=8.0 Hz, H-5)]. These groups were also indicated by the ^{13}C NMR and DEPT spectra. Besides, the ^{13}C NMR (see Experimental section) spectrum exhibited the signals at δ 117.0 (C-1), 130.0 (C-2), 145.2 (C-3), 147.5 (C-4), 115.6 (C-4a), and 138.8 (C-9a) for a fully substituted benzene ring. The connectivities among these groups were readily derived from the HMBC correlations as shown in Fig. 1. On the basis of the above

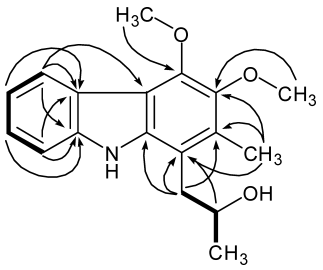

Fig. 1 ^{1}H-^{1}H COSY (bold line) and key HMBC correlations (arrow) of **1**.

evidence, the basic structure of **1** was determined as 1-(2-hydroxypropyl)-3,4-dimethoxy-2-methyl-9*H*-carbazole.

In order to establish the absolute configuration at C-11, the modified Mosher's method [9] was applied as the configuration of carquinostatin A, a carbozole structurally related to **1**, was successfully determined by using this method [10]. Treatment of **1** with (*R*)- and (*S*)-MTPA in the presence of EDC-HCl using catalytic DMAP afforded (*R*)- and (*S*)-MTPA esters (**1a** and **1b**, respectively) (see Experimental section). However, analysis of the proton chemical shift differences showed the irregular distribution of $\Delta\delta$ (δ_S–δ_R) signs [negative for H-10b (−0.080), H-12 (−0.004), and H-13 (−0.021), and positive for H-10a (+0.021) and H-11 (+0.037)], indicating that the method was inapplicable to this compound. Furthermore, the limited amount of compound sample did not allow us to try other methods. But then, the enantioselective synthesis of neocarazostatin B and its analogues, which are closely similar to **1** in both the basic structure and chiral center, were reported and these synthetic compounds, all with 11*R* configuration, showed negative optical rotation values. However, **1** demonstrated the positive optical rotation with value similar to that of neocarazostatin B in MeOH ($[\alpha]_D^{20}$ +18.4° against $[\alpha]_D^{25}$ −16° in neocarazostatin B) and should, therefore, have 11*S* absolute configuration.

2 had a molecular formula, $C_{11}H_{11}NO_4$, as determined by analysis of its HRTOFMS, ESIMS, and NMR (^{1}H, ^{13}C, and DEPT) data. The ^{1}H and ^{13}C NMR spectra of **2** (see Experimental section) revealed the presence of two isolate methylenes [δ_H 2.34 and 2.73 (each 1H, d, J=16.0 Hz, H_2-3), δ_C 41.5 (C-3); δ_H 2.77 and 2.87 (each 1H, d, J=16.0 Hz, H_2-6), δ_C 41.3 (C-6)], a carboxyl group [δ_H 11.06 (1H, br s, OH-5), δ_C 181.0 (C-5)], an acylamino group [δ_H 6.04 (1H, br s, H-1), δ_C 175.6 (C-2)], as well as a *p*-hydroxylphenyl group [δ_H 7.00 (2H, d, J=8.0 Hz, H-2′ and 6′), 6.63 (2H, d, J=8.0 Hz, H-3′ and 5′), and 9.26 (1H, br s, OH-4′), δ_C 125.4 (C-1′), 131.1 (C-2′ and 6′), 115.0 (C-3′ and 5′), and 156.2 (C-4′)]. The HMBC correlations (Fig. 2) from H-1 and H_2-3 to C-2, C-4, C-5, and C-6, from H_2-6 to C-1′, C-2′, C-6′, C-3, C-4, and C-5, and from H-1 to C-3 indicated a connectivity of N-1, C-2, C-3, and C-4 to create a 2-azetidinone ring with a carboxyl group and a *p*-hydroxylbenzyl group attached to C-4. The configuration at C-4 was tentatively determined as *S* by comparison of its optical rotation value with those of previously synthesized 4-benzyl-4-carboxy-2-azetidinone derivatives [14]. Thus, **2** was elucidated as (4*S*)-4-(*p*-hydroxybenzyl)-4-carboxy-2-azetidinone.

In an assessment of antifungal activity by the well plate diffusion method [8] using carbendazim, a commercial antifungal agent, as a positive control, both **1** and **2** exhibited potency against *P. litchii*. The MICs of **1** and **2** against *P. litchii* were 250 and 1000 μg/ml, respectively. The potency of **1** was equivalent to that of carbendazim.

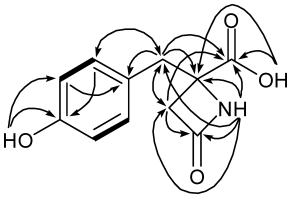

Fig. 2 ^{1}H-^{1}H COSY (bold line) and key HMBC correlations (arrow) of **2**.

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