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Short Communication

Beta-lactamase inhibitory component from the roots of *Fissistigma cavaleriei*

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ABSTRACT

Therapeutic control of β-lactamase-producing bacteria has been a major clinical problem. Development of drug combinations containing the β-lactamase inhibitors has given clinicians a novel approach to controlling resistant organisms. In our search for beta-lactamase inhibitors from natural resources, we found that the methanol extract of the roots of *Fissistigma cavaleriei* showed an inhibitory effect on beta-lactamase. Bioassay-guided isolation of the extract yielded an active compound that was identified as salicylsalicylic acid by physical and spectroscopic methods. The compound showed inhibitory effects on beta-lactamase in a dose-dependent manner with IC_{50} values of 71 μM. Salicylsalicylic acid is not as potent as the original inhibitors such as clavulanic acid, but it may be developed to be potent beta-lactamase inhibitor by chemical modification.

Introduction

The important mechanism of beta-lactam resistance in bacteria is production of beta-lactamase. The use of beta-lactamase inhibitors in combination with beta-lactam antibiotics is currently the most successful strategy to combat a specific resistance mechanism (Lee et al. 2003). A number of beta-lactamase inhibitors such as tazobactam, clavulanic acid, sulbactam (Miller et al. 2001) and succinic acids (Merck Research Laboratories 2001) have been isolated from natural products or synthesized for the development of medicines.

Many plant extracts, combined with antibiotics, have exhibited synergistic activity against the standard microorganism strains as well as drug resistant bacteria (Hemaiswarya et al. 2008; Wagner and Ulrich-Merzenich 2009). Therefore, in order to discovery beta-lactamase inhibitor with novel structural core, we turned our attention to Traditional Chinese Medicine (TCM). As part of our ongoing screening for beta-lactamase inhibitors from TCM, it was found that the methanol extract from the roots of *Fissistigma cavaleriei* significantly inhibited the activity of beta-lactamase produced by *Pseudomonas aeruginosa*. *Fissistigma cavaleriei* (Levl) Rehd (Annonaceae) is a perennial shrub, which is distributed mainly in Guizhou province of China. This plant has been used as a folklore medicine for anti-inflammatory and anti-arthritic effects, and also used as antituberculor agent by Miao people (Bao and Ran 1987).

In the present study, we identified a beta-lactamase inhibitor in the root of *F. cavaleriei* and characterized its inhibitory effects on a kind of beta-lactamase.

Materials and Methods

General procedures

Melting points were measured on a Büchi model B-535 without correction. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100.6 MHz) spectra were recorded using a Bruker DRX 400 MHz NMR spectrometers in $CDCl_3$. El-MS was recorded on Hewlett-Packard 1100.

Plant materials

The dried roots of *F. cavaleriei* were collected on April 19 2008 from the Dushan County, Guizhou province, China, and identified by authors. A voucher specimen (accession number GZ49) has been deposited at the Pharmacy Laboratory, Guizhou University, China.

Extraction and bioassay-guided isolation

Air-dried, powdered root (2 kg) of *F. cavaleriei* was extracted with MeOH three times in the reflux extraction unit at 85 °C to yield a dark-brown residue (380 g, IC_{50} for beta-lactamase inhibitory activity was 6 mg/ml). The methanol extract suspended in water, and then partitioned in turn with hexane, ethyl acetate, chloroform, and water. The IC_{50} for these four extracts were 5, 2,

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32, and 80 mg/ml, respectively. The most active ethyl acetate extract (32 g) was separated by silica gel column chromatography with hexane and ethyl acetate as the solvent system (hexane : ethyl acetate = 5:1)to give five fractions. Fr.3 (3.2 g) showed the most potent beta-lactamase inhibitory activities (IC_{50} = 400 μg/ml). Fr.3 was further separated by silica gel column chromatography to give one active compound (**1**) (140 mg).

Compound (**1**)

White powder; mp 145 °C; EI-MS *m/z* 258; ^{1}H-NMR(400 MHz, $CDCl_3$)δ: 10.27(1H,s), 8.12(1H, dd, *J* = 7.6 Hz), 8.05(1H, dd, *J* = 7.6 Hz), 7.67(1H, m, *J* = 8 Hz), 7.52(1H, m, *J* = 8.4 Hz), 7.40(1H, m, *J* = 7.6 Hz), 7.26(1H, m, *J* = 8 Hz), 7.03(1H, m, *J* = 8 Hz), 6.92(1H, m, *J* = 7.6 Hz); ^{13}C-NMR(100.6 MHz, $CDCl_3$)δ: 111.8(C-1′), 117.6(C-3′), 119.5(C-5′), 122.3(C-1), 124.0(C-3), 126.6(C-5), 130.6(C-6), 132.7(C-6′), 134.8(C-4), 136.4(C-4′), 150.3(C-2), 161.9(C-2′), 168.7(C-7′), 169.2(C-7).

Beta-latamase preparation and beta-latamase inhibition assays

Pseudomonas aeruginosa G19, a clinical strain obtained from the Department of Surgery of Guiyang Medicinal College Hospital, Guiyang, China, was used. Beta-lactamase production was detected with nitrocefin (Fig. 2) by the method of Tunér (Tunér et al. 1985). Fresh overnight cultures of bacteria were inoculated into broth and grown for 2 h at 35 °C in a rotary shaker. Inducer (penicillin G 400 μg/ml) was added, and incubation was continued for an additional 4 h. The cell pellets were collected by centrifugation, resuspended, and washed with potassium phosphate buffer (0.05 M, pH 7.0) at 4 °C. The bacteria were recentrifuged and subsequently resuspended in the same buffer, 10-fold concentrated. The bacteria were disrupted by sonic treatment for 5 min in an ice bath. Cellular debris was removed by centrifugation at 40,000 rpm for 2 h at 4 °C. The resulting supernatant was purified to homogeneity using a gel filtration column chromatography (Sephadex G-75) as described previously (Cantu et al. 1996).

Inhibition assays were based on a modified method as described previously (Huang et al. 2003). Briefly, various concentrations of compound (**1**) were incubated with beta-lactamase (0.9 nM) for 15 min in 50 mM phosphate buffer (pH 7.0) containing 1 mg/ml BSA. Following the incubation, nitrocefin was added at a concentration of 1 mM. After incubation at 25 °C for 10 min, the reaction was stopped by addition of $HgCl_2$ solution (2 mM). Hydrolysis of nitrocefin was monitored by measuring the increase in OD_{486} value. The concentration of compound (**1**) that reduced enzyme velocity by half was the IC_{50} value.

Results and Discussion

An active compound that can inhibit the beta-lactamase to hydrolyze nitrocefin was isolated from the methanol extract of *F. cavaleriei* roots by bioassay-guided isolation. The compound (Fig. 1) is identified as salicylsalicylic acid by physical and spectroscopic methods (mp, MS, ^{1}H- and^{13}C-NMR) and by comparing the data with the published values (Ramos et al. 2004). To our knowledge, it is a first report that isolation of salicylsalicylic acid from *F. cavaleriei* roots.

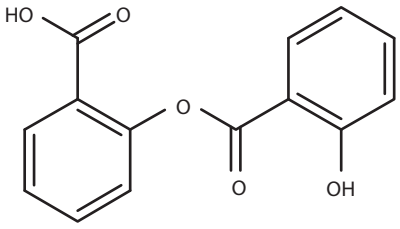

Fig. 1. Structure of compound (**1**) (salicylsalicylic acid).

Beta-lactamase was extracted from a clinical strain of *Pseudomonas aeruginosa* G19. Beta-latamase activity was measured using nitrocefin as substrate (Fig. 2). Salicylsalicylic acid showed inhibitory effects on the beta-lactamase in a dose- dependent manner (Table 1). The IC_{50} value was 71 ± 7 μM. The data indicated that the salicylsalicylic acid is not as potent as the original inhibitors such as clavulanic acid, sulbactam, and tazobactam.

Due to the frequent use of clavulanate-containing formulations in hospitals in general practice, the bacterial strains producing inhibitor-resistant enzymes have been emerged in recent years (Chaibi et al. 1999). There is a need for novel beta-lactamase inhibitors, not based on a beta-lactam core structure. Non-beta-lactamase inhibitors would not be hydrolyzed by beta-lactamase (Tondi et al. 2005). SB-202742 (Fig. 3), also known as an anacardi acid, is an 6-alk(en)ylsalcylic acid possessing beta-lactamase inhibitory activity (Coates et al. 1994). The epigallocatechin gallate (EGCg) (Fig. 4) could inhibit penicillinase activity, thus restoring the antibacterial activity of penicillin against

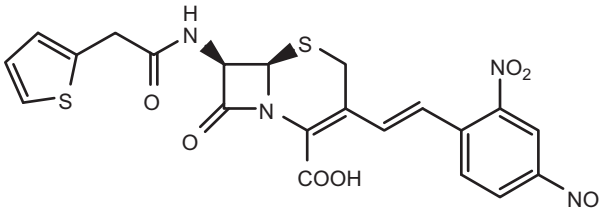

Fig. 2. Struture of nitrocefin. Nitrocefin is a useful chromogenic beta-lactamase substrate that undergoes distinctive color change from yellow to red as the amide bond in the beta-lactam ring is hydrolyzed by all known lactamases produced by Gram-positive and Gram-negative bacteria.

Table 1
Inhibitory effects of compound (**1**) on beta-lactamase.

	Concentration (μM)	Beta-lactamase activity (expressed in the OD_{486} value of product formed) (% of control)	IC_{50} (μM)
Control		0.76 ± 0.02 (100)	
Clavulanic acid*	10	0.15 ± 0.01 (22)	
	20	0.70 ± 0.01 (92)**	
	34	0.54 ± 0.01 (71)**	
Compound (**1**)	58	0.43 ± 0.02 (57)**	71 ± 7
	98	0.32 ± 0.02 (42)**	
	166	0.21 ± 0.01 (28)**	

The data represent the mean ± S.E.M. of experiments performed in triplicate; *Clavulanic acid used as reference drug; **$p < 0.001$, (versus control value) (Student's *t*-test).

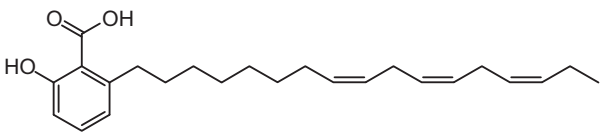

Fig. 3. Structure of SB-202742.

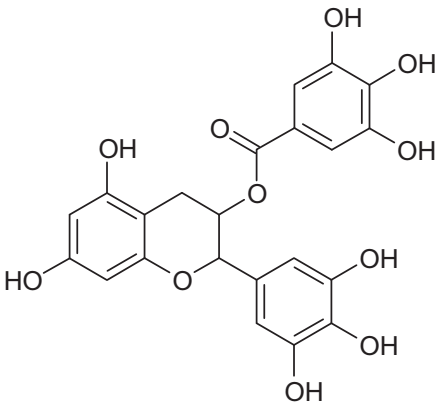

Fig. 4. Structure of epigallocatechin gallate (EGCg).

penicillinase-producing *S. aureus* (Zhao et al. 2002). Salicylsalicylic acid, SB-202742, and EGCg all are belong to phenolic acid. It suggested that phenolic acid could be a core structure of beta-lactamase inhibitor. Salicylsalicylic acid has been used as anti-inflammatory agent safely (Estes and Kaplan 1980). However, salicylsalicylic acid is only a slightly potent beta-lactamase inhibitor. Attempt is under way to modify the structure of salicylsalicylic acid to improve its inhibitory effects on beta-lactamase.

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