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A New Inhibitor of γ-aminobutric Acid Aminotransferase from *Streptomyces* sp. ZZ035 Isolated from a Folk Medicinal Soil in China

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Abstract: Strain ZZ035 was isolated from a folk medicinal soil in China. Polyphasic taxonomy procedure indicated that this strain belonged to the genus *Streptomyces* and was closest to *S. cinnamonensis* with high similarities (99.2%) of 16*S* rDNA sequence. **1** was isolated from the broth of this strain and identified as 5-amonimethyl-2-iminoimidazolidine-4-carboxylic acid. Its chemical structure was established by its spectroscopic data, and ^{1}H and ^{13}C signals were assigned by its ^{1}H, ^{13}C, Dept and HSQC spectra. Considering that **1** was a *γ*-aminobutric acid (GABA) derivative, its inhibitory activity against GABA-aminotransferase (GABA-AT) was assayed, and the result showed that **1** can inhibit GABA-AT activity with the 50% inhibitory concentration of approximately 60 μM. As **1** was a 2,3-disubstituted GABA derivative not published before, the approximate GABA-AT inhibitory activity of **1** compared to positive control vigabatrin showed that **1** were worthy of further research and developing.

Keywords: Actinomycete; *Streptomyces* sp. ZZ035; *γ*-Aminobutric acid derivative; *γ*-Aminobutric acid-aminotransferase; Inhibitor

Introduction

Around the reservoir for domestic water in Chinese rural areas, a folk medicinal soil is used to prevent infection and accelerating cure by being spread around the wound after dog bite. To reveal the anti-infection reasons of the soil, a soil sample was collected in Xianjing Countryside in Zhuzhou County, China (Geographic coordinates: 27˚30' N, 113˚17' E), sixty-one actinomycete strains ZZ01 to ZZ061 were selectively isolated from this sample [1]. After chemical analysis were performed for discovering strains producing a series of secondary metabolites, the bioactive evaluation showed that thirteen strains had antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli* and/or *Candida albicans*, and then the identification of targeted seven strains indicated that they likely belonged to the genus *Streptomyces*. Among them, strain ZZ035 [2] showed remarkable antimicrobial activity against *S. aureus*, *E. coli* and *C. albicans*, and thereby was selected for our research focus to discover bioactive metabolites. During the isolation of antimicrobial components from the broth of this strain, **1** (Fig. 1a) with no antimicrobial activity were also isolated and identified.

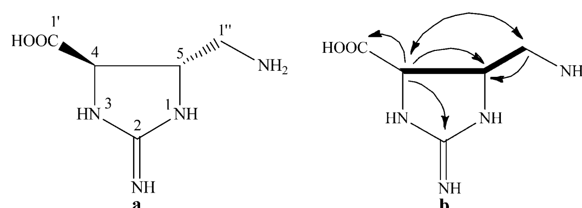

Fig. 1 The chemical structure of **1** (**a**) and its ^{1}H-^{1}H COSY and HMBC correlations (**b**). ^{1}H-^{1}H COSY correlations were indicated as bold bond; HMBC correlations were presented as arrows directed from ^{1}H to ^{13}C.

γ-Aminobutric acid (GABA) acts as an inhibitory neurotransmitter in the central nervous system, and it can block nerve impulses to cure anxiety disorders, epilepsy, Parkinson's syndrome, and so on. As GABA-AT is a key enzyme involved in the GABA metabolic pathways and GABA shunt [3], the inhibitors of this enzyme can increase the level of GABA, and have a potency to cure many diseases derived from the decrease of GABA. Considering that **1** was a *γ*-aminobutric acid (GABA) derivative, the inhibitory activity of **1** against GABA-aminotransferase (GABA-AT) was assayed.

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Materials and methods

Isolation and identification of Streptomyces sp. ZZ035

The methods and materials involved the isolation and identification of *Streptomyces* sp. ZZ035, together with the sample collection of the folk medicinal soil, can see in our previous work [1,2]. Briefly, *Streptomyces* sp. ZZ035 were selectively isolated with improved Gauss medium, and purified with ISP-2 medium. Its morphological characteristics were determined by macroscopic and microscopic methods [4] after incubation at 28˚C on Yeast Extract-Malt Extract Agar (ISP-2) medium. Compared its morphologies with those of actinomycete morphologies provided in Bergey's Manual, strain ZZ035 was presumptively identified [5]. Various tests, such as temperature tolerance, NaCl tolerance and pH tolerance, were carried out to determine its physiological-biochemical characteristics. Using mycelia obtained from the broth of *Streptomyces* sp. ZZ035 cultured in ISP-2 liquid medium at 28℃ for 7 d, the chemical characteristic analyses including cell-wall amino acids and sugars, and cell membrane phospholipids were performed with thin layer chromatography (TLC) technique [6,7].

The genomic DNA used for the PCR was extracted from the single colonies grown on ISP-2 medium at 28℃ for 3 days according to the protocol of SK8255 Kit (Sangon Biotech (Shanghai) Co., Ltd, China). Using Applied Biosystems® 2720 Thermal Cycler, the PCR amplifications with a primer pair 27F-1492R [8] carried out, and the amplification products were resolved using electrophoresis in 1.0% agarose gel and purified according to the protocol of SK8131 Kit (Sangon Biotech (Shanghai) Co., Ltd, China). The 16S rRNA gene sequence was determined using the automatic sequencer Applied Biosystems® 3730XL DNA Analyzer, and was aligned against sequences of reference strains using the BLAST program (http://www.ncbi.nlm.nih.gov/). the selected DNA multiple sequence was matched by means of software package Clustal X [9], evolution distances were calculated using Kimura2-Parameter model of MEGA version 4.0 [10]. The phylogenetic tree was constructed using the neighbor-joining algorithms [11]. Based on 1000 replicates, the confidence coefficient of the phylogenetic tree was evaluated using bootstrap analysis [12].

Agar slants contained *Streptomyces* sp. ZZ035 were inoculated at 28 °C until good growth was observed, and stored at 4°C until use. Twenty percent glycerol was used for long-time preservation at -20°C.

Fermentation and isolation

Streptomyces sp. ZZ035 from agar slants was inoculated with ISP2 liquid medium at 28°C for 3 d. Then, the cultures were inoculated to 2A medium consisting of 1.0% glucose, 3.5% soluble starch, 0.2% yeast, 0.4% casein, 4.6% 3-[N-morpholino]propanesulfonic acid and 1.8% NaCl (w/v) at 28˚C for 8 d on a rotary shaker at 190 rpm until 60 L of broth obtained.

The broth was centrifuged to remove mycelium, and then put onto the top of an AB-8 macroporous adsorption resin column. This column was successively washed with purified water and 30% methanol. The 30% methanol eluent was concentrated under decompression to remove methanol, and then was freeze-dried to obtain lyophilized powder (35.2 g). The powder was separated on a silica column using gradient elution of MeOH-H_2O (100:0, 80:20, 20:80, 0:100), and then the MeOH-H_2O (20:80) eluent (2.74 g) was purified with a C_{18} reversed-phase chromatography eluted with MeOH:H_2O (10:90) and next with Sephadex G-10 chromatography eluted with H_2O to give **1** (257 mg).

*Structural analysis of **1***

All NMR experiments were recorded on a Bruker AV-400 NMR spectrometer equipped with a 5-mm PABBO BB-probe head. The chemical shifts were referenced to tetramethylsilane (In capillary) at δ_H 0 ppm and δ_C 0 ppm in D_2O. The HRESIMS spectrum was acquired in negative mode on an AB Sciex triple TOF 5600 System. The IR spectrum was obtained with Thermo Nicolet 380 FTIR spectrometer. Thin layer chromatography on silica GF_{254} plates with iodine vapor and 0.5% ninhydrin/acetone used as chromogenic agents, and Waters E2695 high performance liquid chromatography equipped with a 2998 PDA detector were used for the analysis of secondary metabolites.

Assays of GABA-AT

According to the published procedure [13], GABA-AT (2.38 mg/mL) was isolated and purified from the brain of SD male white mouse (170-180 g). The SSADH (1.92 mg/mL) from the brains of SD mouse was isolated and purified according to previous methods [14]. GABA-AT activity was assayed using a published method with a little modification [15,16], and GABA-AT and SSADH were used for the catalytic conversion of GABA to succinic semialdehyde (SSA) and succinate. Experiments were designed as follows for enzyme assays (n = 5). Blank group contained 0.8 mM α-ketoglutarate, 0.2 M NAD^+, 400 µL SSADH and 40 µL GABA-AT. Control group contained 0.8 mM α-ketoglutarate, 0.2 M NAD^+, 400 µL SSADH, 40 µL GABA-AT and 6 mM GABA. Drug groups were the same as control group except contained different concentrations of **1** (0-320 µM) or positive control vigabatrin (0-320 µM) purchased from Medchemexpress Co., Ltd. (Shanghai, China). All groups were incubated in buffer contained 0.1 M K_2HPO_4/KH_2PO_4 (pH 8.4) for 30 min at 37˚C, and the production of NAD^+ was monitored by Beckman Coulter DU 800 ultraviolet spectrophotometer (Brea, USA) with the detection wavelength at 340 nm. The absorbance in the reaction system was used to calculate

the relative activity of GABA-AT according to the following formula: GABA-AT (%) = $(A_{Drug}-A_{Blank})/(A_{Control}-A_{Blank}) \times 100\%$.

Results

Identification of Streptomyces sp. ZZ035

Strain ZZ035 exhibited good growth on medium ISP-2, and its cultural and morphological characteristics were respectively showed in Fig. 2. Its physiological-biochemical characteristics showed that it presented good and coral-like growth, and that the color of aerial and substrate mycelium presented white with a little grey red and yellowish brown, respectively. Together with trace *meso*-DAP, two major amino acids *LL*-DAP and glycine, and xylose were determined in the cell-wall component analyses. Moreover, only phosphatidylethanolamine was determined in the cell membrane phospholipids analyses. Its 16*S* rRNA gene sequence deposited at NCBI GenBank with accession numbers KJ995739 [2], together with its morphological, physiological-biochemical characteristics and chemical analyses, indicated this strain belonged to the genus *Streptomyces* [5-7]. The bootstrap value (56%) shown in Fig. 3 and the high 16*S* rDNA sequence similarities (99.2%) indicated *Streptomyces* sp. ZZ035 was closest to *S. cinnamonensis*.

Fig. 2 The cultural characteristic of *Streptomyces* sp. ZZ035.

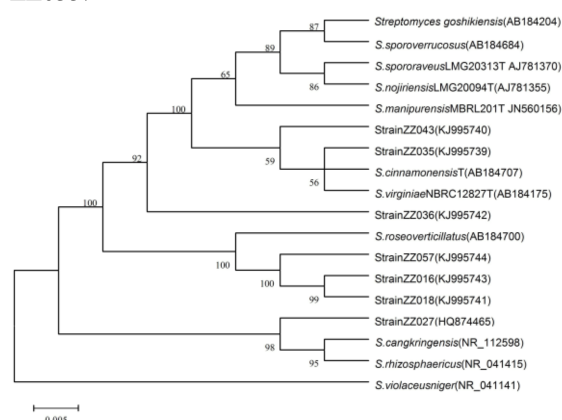

Fig. 3 Neighbour-joining tree based on nearly complete 16*S* rRNA gene sequences showing relationships between strain ZZ035 and closely related members of the genus *Streptomyces*. The numbers at the nodes indicate the level of bootstrap support (%) based on 1000 replicates; only values above 50% are shown. The scale bar corresponds to 0.005 nucleotide substitution per site. Strains ZZ035, ZZ016, ZZ018, ZZ027, ZZ36, ZZ43 and ZZ57 were isolated from the same soil sample.

*Structural elucidation of **1***

5-Amonimethyl-2-iminoimidazolidine-4-carboxylic acid (**1**, Fig. 1a) was obtained as a white amorphous powder. IR υ^{KBr}_{max} (cm^{-1}): 3423, 2936, 2853, 1636, 1560, 1411, 1209; ^{1}H NMR (400 MHz, D_2O) δ_H: 3.98 (1H, d, J = 2.40 Hz, H-4), 4.47 (1H, ddd, J = 2.40, 4.85, 5.62 Hz, H-5), 3.24 (1H, dd, J = 12.07, 4.85 Hz, 5-CH_aNH_2), 3.37 (1H, dd, J = 12.07, 5.62 Hz, 5-CH_bNH_2); ^{13}C NMR (100 MHz, D_2O) δ_C: 160.56 (C-2), 59.47 (C-5), 58.86 (C-4), 174.35 (C-1'), 42.64 (5-CH_2NH_2); ^{1}H and ^{13}C NMR signals were assigned by its ^{13}C, ^{1}H NMR, HSQC, HMBC and ^{1}H-^{1}H COSY spectra (Fig. 1b); HRESIMS *m/z* 157.0728 $[M-H]^-$ (calculated for $C_5H_9N_4O_2$, 157.0726).

Ninhydrin reaction showed purple red on TLC plates, which indicated its structure likely contained primary amino group. Its molecular formula $C_5H_{10}N_4O_2$ was established by the LC-HRESIMS spectrometric data at *m/z* 157.0728 $[M-H]^-$ (*calcd*. for $C_5H_9N_4O_2$, 157.0726). The ^{13}C NMR, Dept 90° and 135° spectra of **1** presented two quaternary carbon signals at 174.35 (C-1') and 160.56 ppm (C-2), two methine carbon signals at 59.47 (C-5) and 58.86 ppm (C-4), and a methylene signal at 42.64 ppm (5-CH_2NH_2). Together with ^{1}H-^{1}H COSY correlations between H-4 and H-5, and H-5 and H-1'', ^{1}H-^{13}C long range correlation (Fig. 1b) between H-4 and C-1' indicated the presence of fragment γ-amino butanoic acid, and which was also confirmed from the positive ninhydrin reaction on TLC plates and the strong absorbance at 1636 cm^{-1} in IR spectrum. The residue element composition CH_3N_3 and carbon signal at 160.56 ppm deduced the presence of guanidino group, which was also conformed from the ^{1}H-^{13}C long-range correlation (Fig. 1b) between H-4 and C-2, and C-4 signal at lower field. According to the molecular formula $C_5H_{10}N_4O_2$, the unsaturated degree of **1** was 3. One residue unsaturated degree deduced that another terminal of guanidine likely linked with C-5 (a methine carbon) through itself amino group to form a five-membered ring. So, **1** was identified as 5-amonimethyl-2-iminoimidazolidine-4-carboxylic acid. Further, the couple constant of 2.40 Hz between H-4 and H-5 deduced that the relative configuration at C-4/C-5 was *anti* [17], and which was also confirmed by the long-range correlation between $H_{1''b}$ and H-4 observed in the NOESY spectrum of **1**.

Inhibitory activity of GABA-AT

Considering that **1** was a *γ*-aminobutric acid derivative, the inhibitory activity of **1** against GABA-AT was assayed. According to the above method [13], a coupled assay system consisting of two purified enzymes, i.e. GABA-AT and SSADH, was used to study the catalytic conversion of 4-aminobutyrate to SSA. The first experiments were designed to determine the relative efficacy of **1** (200 μM) to inhibit GABA-AT activity, and the result (n = 5) showed that **1** had significantly inhibitory activity to GABA-AT (16.5±2.8%) at 200 μM concentration in incubation system. Next, A series of concentrations (0, 5, 10, 20, 40, 80, 160 and 320 μg/mL) of **1** and vigabatrin were further designed to determine the IC_{50} of **1** against GABA-AT (Fig. 4). The results showed the IC_{50} of **1** against GABA-AT was approximately 60 μM, and that of vigabatrin was about 36 μM.

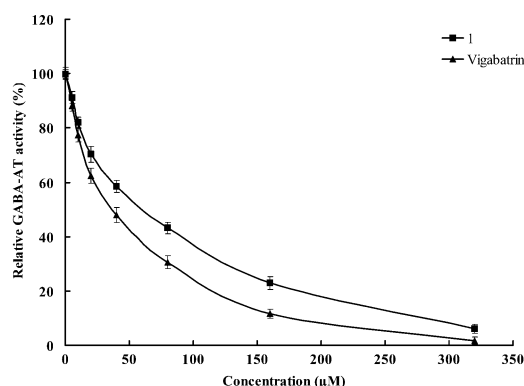

Fig. 4 Inhibition curves of **1** (5-amonimethyl-2-iminoimidazolidine-4-carboxylic acid) and vigabatrin to GABA-AT. Different concentrations of **1** or vigabatrin were incubated in 0.1 M K_2HPO_4/KH_2PO_4 (pH 8.4) buffer contained 0.8 mM *α*-ketoglutarate, 0.2 M NAD^+, 400 μL crude SSADH, 40 μL GABA-T, 6 mM GABA for 30 min at 37°C. The GABA-AT activities relative to control group were calculated from their absorbances in above enzyme reaction system, and the results (n = 5) were expressed as the mean ±C.V..

Discussion and conclusion

GABA acts as an inhibitory neurotransmitter in the central nervous system, and it can block nerve impulses to cure anxiety disorders, epilepsy, Parkinson's syndrome, and so on [3]. As GABA-AT is a key enzyme involved in the GABA metabolic pathways and GABA shunt, the inhibitors of this enzyme can increase the level of GABA, and have a potency to cure some diseases derived from the decrease of GABA. After vigabatrin was used as an irreversible GABA-AT inhibitor to cure epilepsy in clinic [18], many other GABA analogs were synthesized for the inhibitory evaluation of GABA-AT [16,19-27]. Moreover, gabaculine, a naturally occurring neurotoxin produced by the bacteria *Streptomyces toyocaensis* No. 1039, also presented remarkable inhibitory activity to GABA-AT [28]. Considering that **1** was a *γ*-aminobutric acid analog, we deduced that it probably had potency to inhibit GABA-AT. Thereby, the inhibitory activity of **1** against GABA-AT were assayed.

In the GABA metabolic pathway [3], GABA is reversibly catalyzed into SSA by GABA-AT, and then SSA is irreversibly oxidized by SSADH into succinic acid along with the conversion of NAD^+ into NADH used for the GABA-AT assay through its UV absorbance at 340 nm in above incubation system. As two key enzymes GABA-AT and SSADH were involved in above incubation system, an experiment to determine the relative efficacy of **1** inhibiting SSADH was performed using the published procedure [29]. The result (n = 5) showed that no inhibitory activity (99.3±4.6%) to SSADH when its concentration in incubation system was 200 μM, which indicated that only GABA-AT was inhibited by **1**.

Five substituted types of GABA analogs were reported (Fig. 5) according to previous works [16,19-21, 24-27], and all of them were GABA-AT inhibitors. Among approximately sixty GABA derivatives reported, no 2,3-disubstituted GABA derivatives (Fig. 5f) were designed and synthesized, and only six derivatives were 4-unsubstituted ones (Fig. 5e). Therefore, as a 2,3-disubstituted GABA derivatives, the discovery of **1** and its inhibitory activity to GABA-AT will accelerate the structure-inhibition activity researches of GABA derivatives. This together with the approximate GABA-AT inhibitory activity of **1** compared to positive control vigabatrin showed that further researches involved the toxicity, structure modification and inhibition mechanism of **1** were worthy of developing.

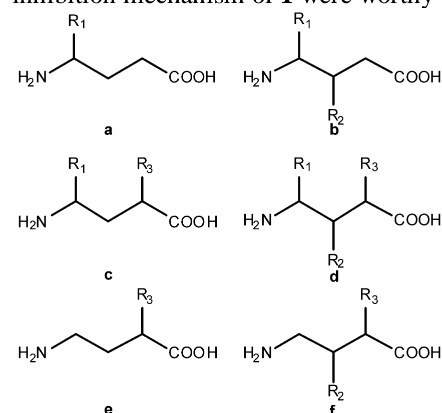

Fig. 5 Five substituted types of GABA derivatives (**a**-**f**).

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