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ORIGINAL PAPER

Biological active metabolite cyclo (L-Trp-L-Phe) produced by South China Sea sponge *Holoxea* sp. associated fungus *Aspergillus versicolor* strain TS08

Dan Chu · Chongsheng Peng · Bo Ding · Fang Liu · Fengli Zhang · Houwen Lin · Zhiyong Li

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Abstract Sponge-associated fungi represent the single most prolific source of novel natural products from marine fungi. Cyclo (L-Trp-L-Phe) exhibits biological functions such as plant growth regulation, moderate cytotoxicity and thus has the application potential in pharmaceutical and agricultural biotechnologies. In this study, a fungal strain TS08 was isolated from sponge *Holoxea* sp. in the South China Sea and identified as *A. versicolor* according to its 18S rRNA gene and morphological, physiological, and biochemical characteristics. Meanwhile, cyclo (L-Trp-L-Phe) was found to be produced by *A. versicolor* strain TS08 mainly in the exponential growth phase. The highest yield of cyclo (L-Trp-L-Phe), 13.24 mg/g (per crude extract of EtOAc), 2.51% of cell dry weigh, was obtained on the tenth day of the fungal cultivation. It was the first time to find the biological active cyclo (L-Trp-L-Phe) in sponge-associated microorganism.

D. Chu · B. Ding · F. Liu · F. Zhang · Z. Li (✉)
Laboratory of Marine Biotechnology, School of Life Sciences and Biotechnology and Key Laboratory of Microbial Metabolism, Ministry of Education, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, People's Republic of China
e-mail: zyli@sjtu.edu.cn

C. Peng
School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China

H. Lin (✉)
Laboratory of Marine Drugs, Department of Pharmacy, Changzheng Hospital, Second Military Medical University, Shanghai 200003, People's Republic of China
e-mail: franklin67@126.com

Keywords *Aspergillus versicolor* · *Holoxea* sp. · Cyclo (L-Trp-L-Phe)

Introduction

In the search for new bioactive natural products increasing attention has been given to marine organisms [1]. Marine sponges harbor diverse prokaryotic and eukaryotic microbes including archaea, bacteria, cyanobacteria, microalgae, fungi and are one of the important resources for marine natural products [2]. Fungi associated with sponges display diverse biological activities and represent the single most prolific source of marine fungi-derived structurally unique and biologically active secondary metabolites to date [3, 4].

Cyclic dipeptides, also called diketopiperazines (DKPs), are quite common in nature. Many natural products with the DKPs scaffold have been isolated and exhibit a wide range of biological activities such as anti-bacteria, anti-alga, anti-virus, anti-oxidation, cytotoxicity, plant growth regulation, and immune regulation [5–7]. DKPs play an important role in regulatory mechanism of quorum sensing as signal molecules and have become a research hot point in ecological chemistry [8, 9]. As one of the DKPs, cyclo (L-Trp-L-Phe) has the biological activities of plant growth regulation and moderate cytotoxicity [10, 11], In addition, cyclo (L-Trp-L-Phe) is most likely the biosynthetic precursor to complex diketopiperazine WIN 64821, a nonpeptide neurokinin antagonist [12]. Thus, cyclo (L-Trp-L-Phe) has the application potential in pharmaceutical and agricultural biotechnologies. However, as a natural product, no research on cyclo (L-Trp-L-Phe) from sponge-associated microorganisms has been reported to date.

In this study, *Aspergillus versicolor* strain TS08 associated with South China Sea sponge *Holoxea* sp. was isolated and identified based on 18S rRNA gene and morphological, physiological characteristics. Meanwhile, the production of cyclo (L-Trp-L-Phe) by the cultivation of *A. versicolor* strain TS08 were investigated for the first time as well as the production dynamics for cyclo (L-Trp-L-Phe).

Materials and methods

Fungal isolation

A fungal strain TS08 was isolated from marine sponge *Holoxea* sp. that was collected by SCUBA diving nearby Yongxing Island (112°20′E, 16°50′N) in the South China Sea at depth of ca. 20 m in June 2007. Under sterile condition, sponge specimens were removed from the inside of the sponge body and subsequently inoculated on PDA medium (potato extract 6 g, glucose 20 g, and agar 12 g, 1 L artificial sea water (ASW): NaCl 26.518 g, $MgCl_2$ 2.447 g, $MgSO_4$ 3.305 g, $CaCl_2$ 1.141 g, KCl 0.725 g, $NaHCO_3$ 0.202 g, NaBr 0.083 g, distilled water 1,000 ml). Cultures were incubated at 28 °C and pure strain of TS08 was isolated by reinoculation on PDA plates.

Identification of the fungal strain TS08

The identification of the strain was based on standard morphological and physiological properties [13], and nucleotide sequence analysis of the 18S rRNA gene. The morphological characteristics of the substrate mycelium, aerial mycelia, sporophore, and the spore shape of strain TS08 were observed by eyes and under an optical microscope. Scanning electron microscope (SEM) was analyzed as follows: the strain was frozen in liquid nitrogen, immediately snapped, vacuum-dried, and then sputtered with gold and photographed. Images were taken using a JOEL (Tokyo, Japan) JSM-6360LV SEM. The accelerating voltage was adjusted to 15 kV. The specimen was examined at magnifications from 500 to 10,000×.

The fungal genomic DNA was extracted using a modified method based on Li et al. [14] and Van Burik et al. [15]. Its mycelia were picked out to a mortar, then 600 µl of CTAB lysis buffer (1% CTAB, 1% Triton X-100, 1% SDS, 1.4 M NaCl, 100 mM Tris, 20 mM EDTA, 2% PVP) was added. The mycelial mixture after grinding was transferred to a 1.5-ml Eppendorf tube and water-bathed at 65 °C for 30 min, and then an equal volume of phenol: chloroform:isoamyl alcohol (25:24:1) was added. After brief mixing and centrifugation (12,000*g*, 15 min, 4 °C), the supernatant was transferred to a new microtube and extracted with chloroform:isoamyl alcohol (24:1). Finally, DNA was precipitated by adding equal volume of isopropanol at −20 °C. A DNA pellet was collected by centrifugation (12,000*g*, 15 min), washed with 75% ethanol, and resuspended in sterile water. RNA was removed by adding 2 µl of RNase A (10 mg/ml) (Invitrogen) at 60 °C for 10 min.

The fragment of 18S rRNA gene was amplified from the genomic DNA using primer set nu-SSU-0817 (5′-TTAG CATGGAATAATRRAATAGGA-3′)/nu-ssu-1536 (5′-AT TGCAATGCYCTATCCCCA-3′) [16]. The PCR mixture contained 2.5 µl of 10× *Taq* Buffer with $(NH_4)_2SO_4$ and 3.5 µl 25 mM $MgCl_2$ (Fermentas EP0402, USA), 2 µl of 2 mM dNTPs (Fermentas, USA), 2 µl of each primer (10 pmol), 1 µl of fungal DNA, 0.25 µl of *Taq* DNA polymerase (5 U/µl, Fermentas), and 14.75 µl of ddH_2O. The PCR was carried out under the following conditions: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 56 °C, 1 min at 72 °C, and one final step of 10 min at 72 °C.

Sequencing analysis was performed on an ABI 3730 XL (Applied Biosystems) automated sequencer using the nu-SSU-0817/nu-SSU-1536 primer. Sequence of the fungal 18S rRNA gene was compared with those in GenBank database by BLAST algorithm to identify sequence similarity. 18S rRNA sequence was aligned using ClustalX software, and the phylogenetic tree was generated using the neighbor-joining algorithms in Mega II software. The fungal 18S rRNA gene sequence obtained in this study was deposited in GenBank under accession number FJ941881.

Cultivation of *A. versicolor* strain TS08 and production of cyclo (L-Trp-L-Phe)

A small spoon of spores growing on PDA plate was inoculated into a 250-ml Erlenmeyer flask containing 100 ml GYT culture medium (1 g glucose, 0.1 g yeast extract, 0.2 g tryptone in 100 ml ASW) and cultured at 28 °C for 1 day on a rotary shaker at 160 rpm. Then, 100 ml of the resultant seed culture was inoculated in 2-L Erlenmeyer flask containing 1L of the above culture medium for 2 weeks at 28 °C. The fungal growth was analyzed by measuring the dry weight of the mycelium (biomass) with three duplicate samples: after filtration under vacuum condition and washing with distilled water three times, the mycelia were dried to constant weight at 60 °C. The mycelia and culture filtrate were extracted three times with EtOAc. After evaporation to dryness under vacuum condition, the combined extracts were subjected to vacuum liquid chromatography (VLC); a linear gradient of petroleum ether-acetone was used as solvent system. The resulting fractions were subjected to Sephadex LH-20 column chromatography eluted with dichloromethane: methanol (1:1). HPLC was used to determine the behavior

of the crude extract and the cyclo (L-Trp-L-Phe): Agilent 1200 (USA), chromatography C18 RP-column, ODS-Φ4.6. Gradient elution mobile phase: 0–100% acetonitrile: 100–0% deionized water; eluting conditions 0–20 min; flow rate 1.0 ml/min; sample 10 mg/ml; inpouring 20 μl; detected at 280 nm. The final purification was achieved by semi-preparative reversed-phase HPLC: Waters 1,525/2,996 liquid chromatography, VWD G1314A detector (UV) 210 nm, ODS-Φ10; mobile phase 80% acetonitrile:20% deionized water; eluting conditions 0–10 min; flow rate of 1.0 ml/min; inpouring 20 μl; detected at 280 nm.

Identification of cyclo (L-Trp-L-Phe) by spectroscopic analyses

Mass spectroscopy was done on cyclo (L-Trp-L-Phe) using the electrospray technique with a JMS-300 spectrometer. The sample dissolved in methanol was injected with a spray flow of 2 ml/min and a spray voltage of 2.2 kV using the loop injection method. Nuclear magnetic resonance (NMR) spectroscopy was done on cyclo (L-Trp-L-Phe) dissolved in 100% DMSO-d_6 with a Bruker 400 MHz instrument. The sample was subjected to 2,048 scans with a sweep width of 6,024 and 8K real points.

Results

Identification of the fungal strain TS08 associated with sponge *Holoxea* sp

The detailed morphological and physiological properties of the fungal strain TS08 are shown in Table 1. The colonies were green and the morphological characteristics of the substrate mycelium, aerial mycelia, sporophore, and the spore shape of the strain TS08 could be clearly observed. In an observation using the SEM, the typical *Aspergillus* conidial heads, 20–50 mm in diameter, were observed; conidiophores were 600–900 mm long, thick-walled and smooth (Fig. 1). The morphological characteristics indicated the strain as *Aspergillus* sp. In addition, ergosterol and ergosterol peroxide were found in the fungal crude extract, which were the main components in the cell wall of the fungi. About 760 bp of the 18S rRNA gene of strain TS08 was successfully PCR-amplified and sequenced. Based on BLAST analysis, the 18S rRNA gene sequence of strain TS08 showed 99% similarity with that of *A. versicolor* relatives. In Fig. 2, strain TS08 was grouped in one cluster with *A. versicolor* references among which the closest reference is from Arabian Sea and some are uncultured marine eukaryote clones from Baltic Sea. Thus, according to the results above, the strain IS08 was identified as *A. versicolor.*

Table 1 Physiological and biochemical properties of strain TS08

Properties	Results
Growth temperature	20–37°
Degradation of urea	+
Nitrite reduction	+
Ammonia sulfate reduction	+
D-Fructose	+
D-galactose	−
Malto-dextrin	+
Glycerol monostearate	+
Maltose	−
Sucrose	+
Inositol	−
Sorbitol D-Mannitol	−

+ Positive; − Negative

Production of cyclo (L-Trp-L-Phe) by *A. versicolor* strain TS08

As shown in Fig. 3, the crude extract with EtOAc of the *A. versicolor* strain TS08 mainly included three peaks at retention time 1.290 ($P_{1.290\ min}$), 1.422 ($P_{1.422\ min}$), and 1.631 min ($P_{1.631\ min}$); the latter peak ($P_{1.631\ min}$) was much higher than the former peaks (Fig. 3a). The crude extracts were subjected to VLC and resulted in seven fractions. The sixth fraction was subjected to Sephadex LH-20 column chromatography. The final purification was achieved by semi-preparative reversed-phase HPLC at the UV absorption spectrum of 280 nm, and gave a single peak with retention time of 1.613 min (Fig. 3b).

Further convincing spectroscopic evidence for the identification of the isolated compound was obtained by electrospray mass spectroscopy, and ^{1}H, ^{13}C NMR. ESI–MS (+) data of m/z 356.15 in Fig. 4 suggested the pseudomolecular ion $C_{20}H_{19}N_3O_2Na$ [M + Na^+] which was supported by NMR data (Table 2). The color of the isolated compound turned red from white when 5% vanillin sulfuric acid solution was added, indicating that the structure may include indole group. ^{1}H NMR (Table 2) not only indicated an indole ring related to proton signals (δ 6.94, 7.47, 6.98, 7.05, 7.30), but also implied a mono-substituted benzene ring related to proton signals δ 7.16 (1H, m), δ 7.15 (2H, m), and δ 6.71 (2H, m). The proton resonances at δ 7.71 (1H, s) and δ 7.91 (1H, s) combined with carbon resonances at δ 166.22 (s), δ 166.88, δ 55.29, δ 55.64 (Table 2) conformed the structure with a diketopiperazine skeleton. Finally, the structure of the isolated compound was determined to be cyclo (L-Trp-L-Phe) by comparison with the reported data (Fig. 5) [10].

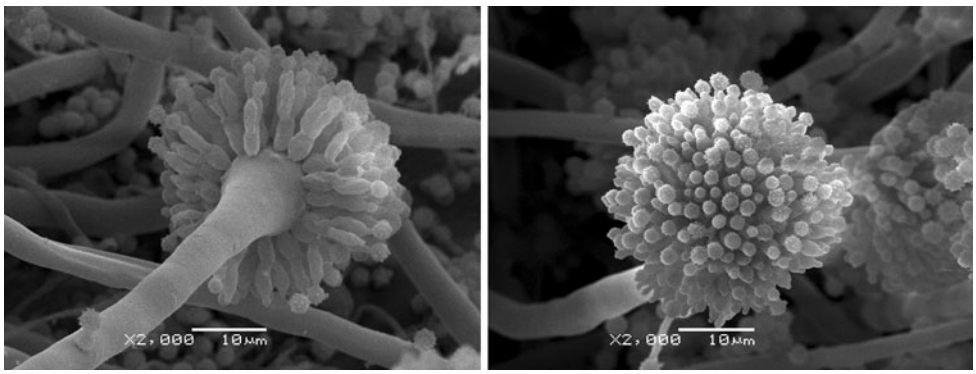

Fig. 1 The morphological characteristics of strain TS08 (×2000 SEM)

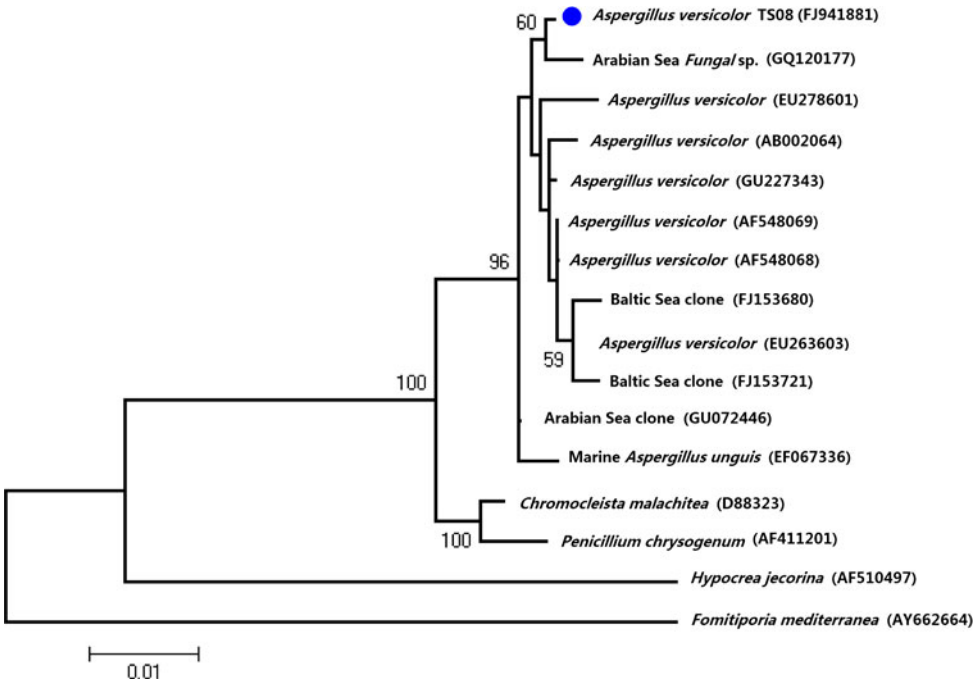

Fig. 2 Neighbor-joining phylogenetic tree of strain TS08 from sponge *Holoxea* sp. based on 18S rRNA gene sequences. The values at each node represent the bootstrap values from 100 replicates and the *scale bar* represents 0.0005 substitutions per nucleotide. The bootstrap values under 50% were cut off. *Penicillium* sp. FJ716242 was used as outgroup

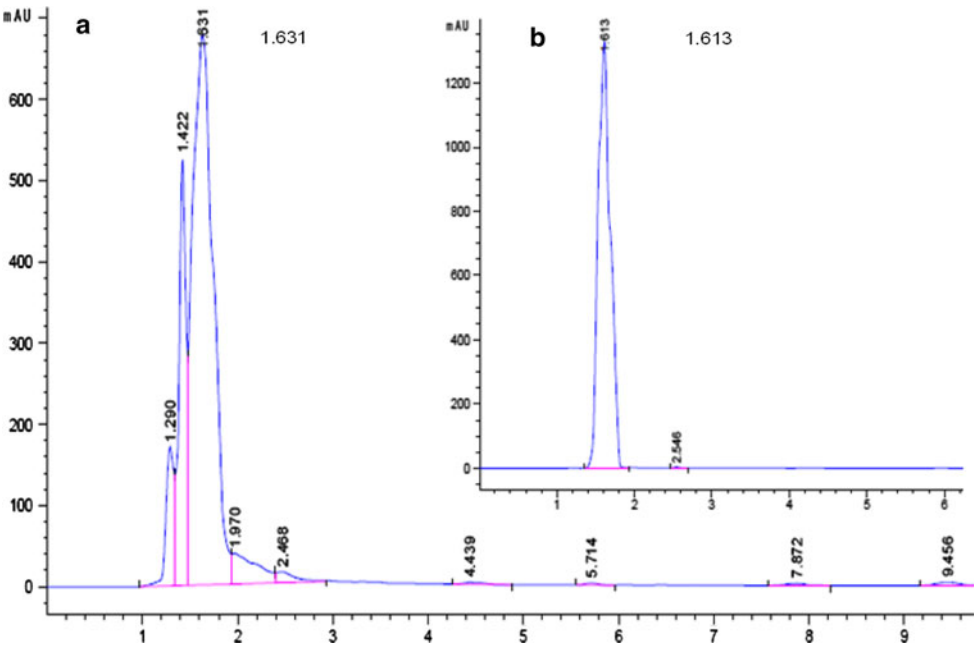

Fig. 3 HPLC chromatogram of the crude extract (**a**) and the cyclo (L-Trp-L-Phe) (**b**)

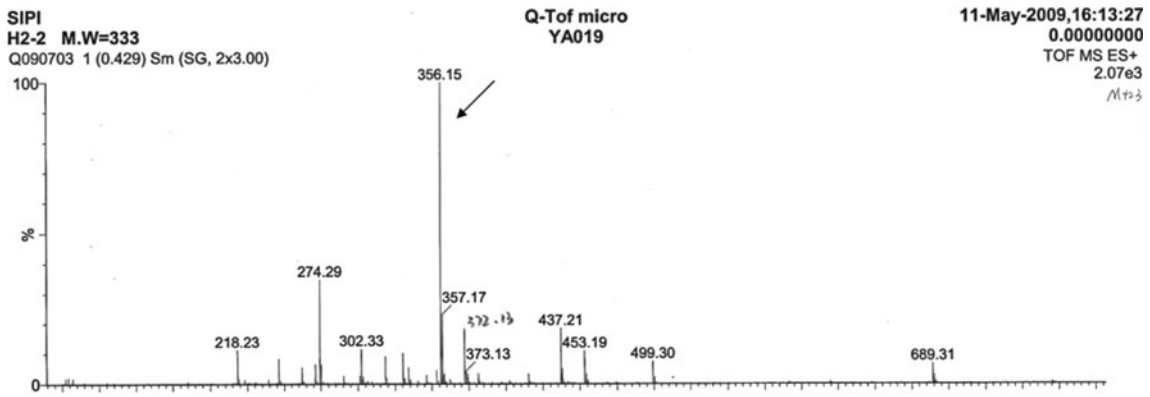

Fig. 4 ESI-MS spectrometry of cyclo (L-Trp-L-Phe) isolated from *A. versicolor* strain TS08. An *arrow* shows the electrospray mass peak with $[M + Na]^+ = 356.15$ of the cyclo (L-Trp-L-Phe)

Table 2 Comparison of experimental NMR data with literature for cyclo (L-Trp-L-Phe) [10]

Position	Data from literature[a]		Data from experiment [a]	
	δ_C	δ_H^b	δ_C	δ_H^b
1	–	10.89 (1H, s)	–	10.89 (1H, s)
2	120.89	6.96 (1H, d, 2.20)	120.92	6.94 (1H, s)
3	108.85	–	108.80	–
4	118.42	7.48 (1H, dd, 7.17, 1.00)	118.45	7.47 (1H, d, 7.7)
5	118.76	6.98 (1H, ddd, 7.17, 7.45, 1.45)	118.78	6.98 (1H, d, 7.33)
6	124.41	7.08 (1H, ddd, 7.73, 7.45, 1.00)	124.44	7.05 (1H, m)
7	111.33	7.32 (1H, dd, 7.73, 7.45)	111.36	7.30 (1H, d, 8.07)
8	127.54	–	127.53	–
9	136.07	–	136.05	–
10	29.69	2.52 (1H, dd, 14.47, 5.68)	29.70	2.52 (1H, d, 5.50)
		2.81 (1H, dd, 14.47, 4.46)		2.78 (1H, dd, 3.67, 3.30)
11	55.29	3.98 (1H, m)	55.27	3.95 (1H, s)
12	–	7.91 (1H, d, 2.00)	–	7.91 (1H, s)
13	166.22	–	166.21	–
14	55.64	3.89 (1H, m)	55.62	3.83 (1H, s)
15	–	7.71 (1H, d, 2.00)	–	7.71 (1H, s)
16	166.88	–	166.83	–
17	39.89	1.85 (1H, dd, 13.49, 7.02)	39.71	1.82 (1H, m)
		2.45 (1H, dd, 13.49, 4.70)		2.43 (1H, m)
18	136.56	–	136.53	–
19	128.03	7.16 (1H, m)	128.06	7.15 (1H, m)
20	129.70	6.71 (1H, m)	129.72	6.68 (1H, d, 6.59)
21	126.36	7.17 (1H, m)	126.40	7.16 (1H, m)
22	129.70	6.71 (1H, m)	129.72	6.68 (1H, d, 6.59)
23	128.03	7.16 (1H, m)	128.06	7.15 (1H, m)

[a] Taken in DMSO-d_6, [b]Intensities, multiplicities and J values (Hz) shown in parentheses

The growth curve of *A. versicolor* strain TS08 and its production dynamics for cyclo (L-Trp-L-Phe) are summarized in Fig. 6. The fungus reached its highest cell density on the tenth day, and cyclo (L-Trp-L-Phe) was mainly biosynethsized during the exponential growth phase. The peak area on the seventh day (8,318 mAUs) increased more than threefold than that of the fourth day (2,027 mAUs). The peak area on the tenth day was 9,681

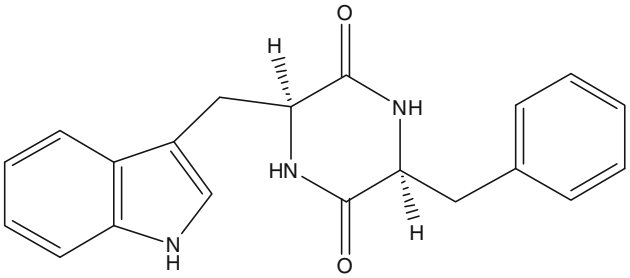

Fig. 5 The constitutional formula of cyclo (L-Trp-L-Phe)

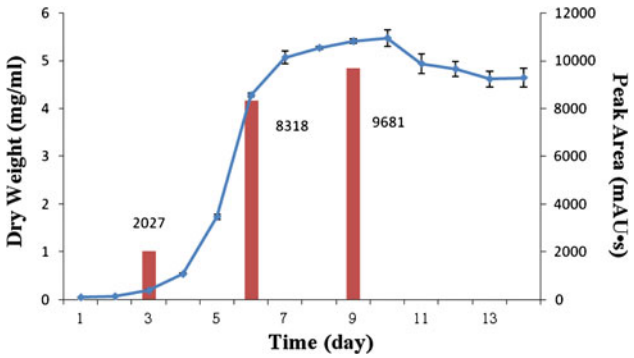

Fig. 6 The growth curve of *A. versicolor* strain TS08 and production of cyclo (L-Trp-L-Phe)

mAUs, which was about 1.16 times greater than that of the seventh day. The highest yield of cyclo (L-Trp-L-Phe), 13.24 mg/g (per crude extract of EtOAc) and 2.51% of cell dry weigh, produced by *A. versicolor* strain TS08 was achieved on the tenth day of the fungal cultivation.

Discussions

It is known that cyclic dipeptides have many potential biological functions. The investigation of the preferred conformations of cyclic dipeptides is very important to explore the functionary mechanism and discover the biological characteristics of cyclic dipeptides. Cyclic peptides are more bioavailable and more stable against degradative peptidases than linear peptides, the relevant research is fundamental to many aspects of peptide chemistry. In 1988, Stierle et al. [17] first discovered DKPs in a *Micrococcus* sp. isolated from sponge *Tedania ignis*, which was the first demonstration that a bacterium associated with sponge produces secondary metabolites ascribed to the sponge host. In the case of cyclo (L-Trp-L-Phe), it was first isolated from an unidentified *Penicillium* sp. and proved to be with the biological function of regulating the growth of plant by Kimura et al. [10]. In 2008, cyclo (L-Trp-L-Phe) was isolated from fungal EF8 which was isolated from the conchocelis of *Porphyra yezoensisit* by Ding et al. [11] and proved to exhibit a moderate cytotoxicity against 37 human tumor cell lines with the average IC_{50} 3.3 μg ml^{-1}. But till now, few reports on the production of DKPs by microbial cultivation have been found [18]. This study was the first to find the cyclo (L-Trp-L-Phe) from the sponge-associated microorganisms and provide a preference for the production of cyclo (L-Trp-L-Phe) by microbial cultivation.

Cyclic dipeptides are commonly biosynthesized from amino acids by different organisms and considered to be secondary functional metabolites or side products of terminal peptide cleavage. From the perspective of biogenic speculation, the cyclo (L-Trp-L-Phe) is formed through a simple condensation of the amino acids L-Trp and L-Phe, which are essential nutrients for fungal growth. As Fig. 6 shows, cyclo (L-Trp-L-Phe) is synthesized in the company of the growth of *A. versicolor* strain TS08. It is supposed that the cyclo (L-Trp-L-Phe) is the essential substance during the growth of this fungus. According to the experimental data, the yield of the cyclo (L-Trp-L-Phe), 13.24 mg/g (crude extract of EtOAc), is still not very high. Some possible reasons are as follows: first, the EtOAc is semi-polarity extractant, which can not extract the high-polarity cyclo (L-Trp-L-Phe) absolutely. Second, the extraction process has suffered from serious losses. Further study should be done to optimize the cultivation conditions for improvement of the yield of cyclo (L-Trp-L-Phe) from *A. versicolor* strain TS08.

Studies on sponge-associated fungi have typically concentrated on natural product chemistry [1, 4], while investigations on biology of sponge-associated fungi have been started recently and are rare. More than 20 genera fungi have been revealed in sponges from Kaneohe Bay and Gurraig Sound Kilkieran Bay [19–22]; however, to date, no research on the diversity of fungi associated with China Sea Sponges has been reported. At least 17 genera of culturable fungi within ten taxonomic orders of two phyla in two South China Sea Sponges *Clathrina luteoculcitella* and *Holoxea* sp. have been found by us (data not shown), suggesting the diverse fungal community associated with South China Sea sponges. Today, marine fungi are still one of the most understudied marine ecological groups and our knowledge concerning the diversity and function of fungi associated with sponge is still very limited. According to Baker et al. [19], some sponge-derived fungal isolates showed antimicrobial activities, which were also found for the fungi associated with South China Sea sponges by us (data not shown). Fungus of the genus *Aspergillus* have been isolated from sponges *Gelliodes fibrosa*, *Haliclona caerulea*, *Mycale armata* [21] and proved to produce biological natural products, for example, cytotoxic alkaloid and meroterpenoids [23, 24]. *A. versicolor*, which produced new angular tricyclic chromone derivatives, was also isolated from sponge *Xestospongia exigna* [25]. The study

results, from previous studies and the current study, suggest the potential ecological role that the sponge-associated fungi play in the host's chemical defense.

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