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ARTICLE

Shikinefragalides A-D, new tricyclic macrolides produced by Stachybotryaceae sp. FKI-9632

Rima Koike[1] · Yoshihiro Watanabe[1,2] · Kazuho Kato[3] · Yudai Mikasa[3] · Kota Shimizu[3] · Kenichi Nonaka[1,2] · Masako Honsho[2] · Takuya Suga[1,2] · Rei Hokari[1,2] · Aki Ishiyama[1,2] · Yukihiro Asami[1,2] · Masato Iwatsuki[1,2]

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Abstract

Four new tricyclic macrolides, named shikinefragalides A (**1**), B (**2**), C (**3**) and D (**4**), were isolated by physicochemical (PC) screening from a static culture material of Stachybotryaceae sp. FKI-9632. Their structures were elucidated as new analogs of colletofragarones by MS and NMR analyses. Compounds **1** and **2** showed weak antimalarial activity and cytotoxicity.

Introduction

Numerous novel natural products have been discovered from fungal species. Some of them were developed as human medicines (e.g., penicillins, cephalosporins, statins and candins), animal drugs (e.g., penicillins and cephalosporins), and agrochemicals (e.g., afidopyropene) [1, 2]. Recent advances in whole-genome sequencing technology have revealed that the fungi have many biosynthetic gene clusters encoding unidentified secondary metabolites that surpass the number of metabolites identified so far [3, 4]. Thus, fungi are now re-recognized as one of the most important microorganisms as potential sources for new drugs and agrochemicals.

Physicochemical (PC) screening is a methodology to discover new natural compounds guided by physicochemical properties including molecular weight, molecular formula, UV profile and so on. Our research group has been searching for new fungal compounds by PC screening and discovered new fungal metabolites such as hatsusamides A and B [5] from *Penicillium steckii* FKJ-0213, pochoniolides A and B [6] from *Pochonia chlamydosporia* var. *spinulospora* FKI-7537, cipralphelin [7] from *Penicillium brevicompactum* FKJ-0123 and so on so far. After their initial discovery, many of them were found to exhibit useful biological activities.

During our recent PC screening on culture broths of 25 fungal strains by LC-DAD-ESI-MS analysis combined with dereplication by natural product database "Dictionary of Natural Products" [8], we selected a Stachybotryaceae sp. FKI-9632 strain as a producer of presumed new compounds. As a result of purification guided by LC-DAD-ESI-MS analysis, four new fungal tricyclic macrolides, named shikinefragalides A (**1**)-D (**4**), were isolated from a static culture of Stachybotryaceae sp. FKI-9632 (Fig. 1). In this paper, we report the isolation, structure elucidation, and biological activity of **1**-**4**.

Materials & methods

General experiments

The purification of **1**-**4** by an ODS column was conducted using YMC-gel ODS-A (150 µm, YMC Co., Kyoto, Japan). Preparative HPLC was performed using a Capcell pak C_{18} MG-II column (20 i.d. × 250 mm, Osaka Soda Co. Ltd., Osaka, Japan). LC-DAD-ESI-MS spectra were obtained

These authors contributed equally: Rima Koike, Yoshihiro Watanabe

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✉ Masato Iwatsuki
iwatuki@lisci.kitasato-u.ac.jp

[1] Graduate School of Infection Control Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo, 108-8641 Tokyo, Japan

[2] Ōmura Satoshi Memorial Institute, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo, 108-8641 Tokyo, Japan

[3] School of Science, Kitasato University, 1-15-1, Kitazato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan

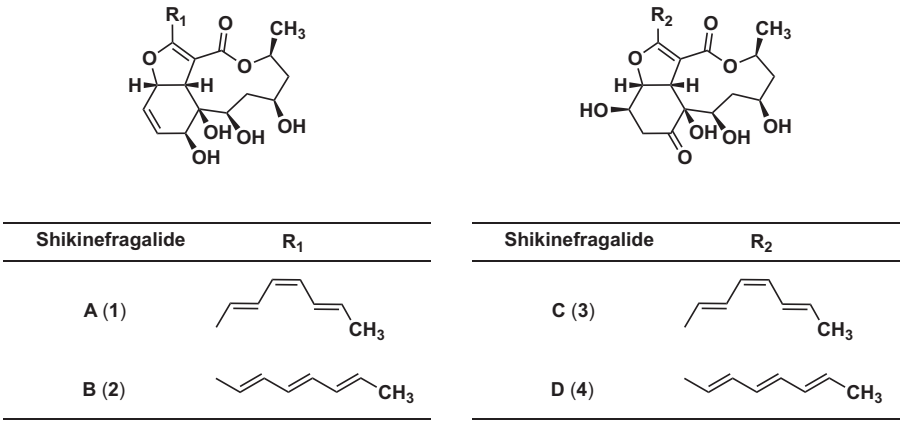


Fig. 1 Structures of shikinefragalides A (**1**)-D (**4**)

using an AB Sciex Triple TOF^{TM} 5600^{+} LC-MS/MS Systems (AB Sciex, Framingham, MA, USA). NMR spectra were obtained using a Varian XL-400 spectrometer (Agilent Technologies, CA, USA) or a JEOL JNM-ECA-500 (JEOL, Tokyo, Japan), with ^{1}H NMR at 400 or 500 MHz and ^{13}C NMR at 100 or 125 MHz in DMSO-d_6 or CD_3OD. The chemical shifts are expressed in ppm and are referred to DMSO-d_6 (2.48 ppm) or CD_3OD (3.31 ppm) in the ^{1}H NMR spectra and to DMSO-d_6 (39.5 ppm) or CD_3OD (49.0 ppm) in the ^{13}C NMR spectra. IR spectra (ATR) were taken on a FT-210 Fourier transform infrared spectrometer (Horiba Ltd., Kyoto, Japan). UV spectra were acquired with a Hitachi U-2800 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Optical rotation was measured with a JASCO P-2200 polarimeter (JASCO Co., Tokyo, Japan). CD spectra were recorded with a J-720 circular dichroism spectrometer (JASCO).

Taxonomic studies of strain FKI-9632

Soil samples around the root of plants were collected from Shikine Island, Izu Islands, Tokyo, Japan in 2018. The soil samples were diluted with Winogradsky's solution and spread on Czapek yeast extract agar (CYA) for cultivation. For isolating fungal species, that CYA was used with 50 mg l^{-1} rose bengal, 100 mg l^{-1} chloramphenicol and 100 mg l^{-1} kanamycin and kept at 25 °C for 7 days.

DNA extraction, polymerase chain reaction (PCR) amplification of the ITS region and sequencing of the strain FKI-9632 were conducted. PCR amplification products were fabricated using the QIAGEN® Fast Cycling PCR Kit (Qiagen Inc., Valencia, CA, USA). Sequencing products were purified using BigDye XTerminator Purification Kit (Applied Biosystems, Foster City, CA, USA), and samples were analyzed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Contigs were assembled using the forward and reverse sequences with the SeqMan Pro program from the Lasergene 10 package (DNASTAR Inc., Madison, WI, USA).

Fermentation

Strain FKI-9632 was grown and maintained on an agar slant consisting of 0.1% glycerol, 0.08% KH_2PO_4, 0.02% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.02% KCl, 0.2% $NaNO_3$, 0.02% yeast extract and 1.5% agar (adjusted to pH 6.0 before sterilization). A loopful of spores of the strain was inoculated into 100 ml of the GP seed medium consisting of 2.0% glucose, 0.5% Hipolypepton (Nihon Pharmaceutical Co., Tokyo, Japan), 0.2% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, and 0.1% agar (adjusted to pH 6.0 before sterilization) in a 500-ml Erlenmeyer flask. The flask was incubated on a rotary shaker (210 rpm) at 27 °C for 3 days. Fifty milliliters of the seed cultures were inoculated into each of two culture bags (Ulpack 47, Hokken Co. Ltd., Tochigi, Japan) containing a production medium (1 kg of water-sodden rice and 10 g of seaweed tea powder (Ito en Ltd., Tokyo, Japan)). Static fermentation was continued at 25 °C for 15 days.

Antimicrobial activity

The following microorganisms were used for evaluation of antimicrobial activity on a paper disc method: *Bacillus subtilis* KB 211 (ATCC 6633), *Kocuria rhizophila* KB 212 (ATCC 9341), *Escherichia coli* KB 213 (NIHJ), *Xanthomonas oryzae* KB 88, *Candida albicans* KF 1 (ATCC 64548) and *Mucor racemosus* KF 223 (IFO 4581). All compounds were prepared as 1 mg ml^{-1} MeOH solution. Each paper disk (diameter 6 mm, thin type, Advantec, Tokyo, Japan) impregnated with 10, 3, 1, 0.3, 0.1 and 0.03 µg of **1**-**4** was put into an agar plate, followed by incubation for 1–2 days at 37 °C for *B. subtilis*, *K. rhizophila* and *E. coli* KB 213 (NIHJ) or 27 °C for *X. oryzae*, *C. albicans* and *M. racemosus*.

In vitro cultivation of *Plasmodium falciparum* and antimalarial assay

In vitro cultivation and in vitro antimalarial activities against *P. falciparum* FCR3 (chloroquine-sensitive) and K1 (chloroquine-

resistant) strains were evaluated, using the method described previously [9]. This study was approved by the "Kitasato Institute Hospital Research Ethics Committee (No12102)" because of the donation of human erythrocytes from volunteers.

Cytotoxic assay against MRC-5 cells

Cytotoxic assay against human fetal lung fibroblast MRC-5 cells was carried out as described previously [10].

Results

Identification of a fungal FKI-9632 strain as a producer of 1-4 by PC screening

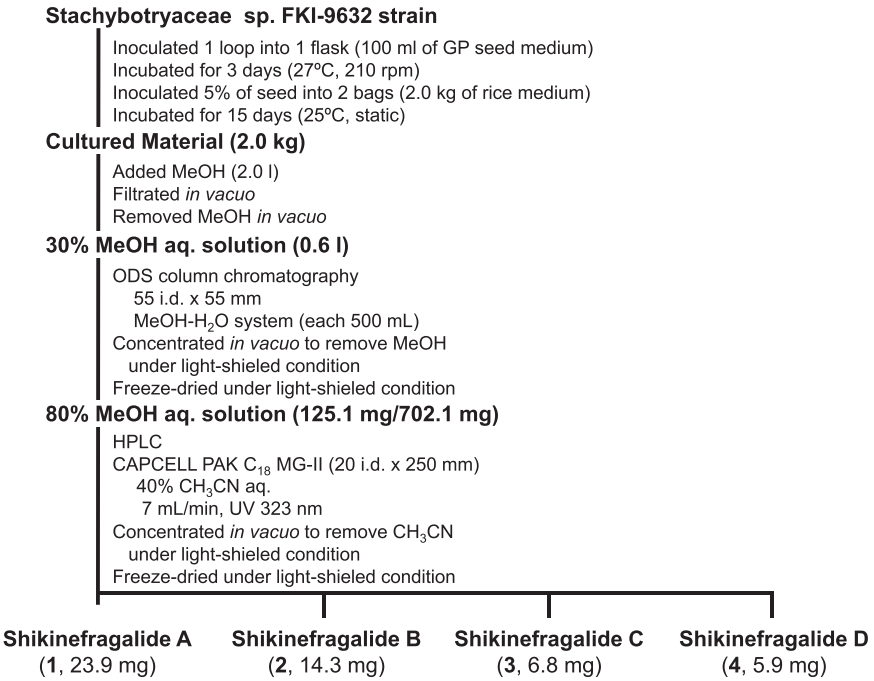
PC screening was performed as shown in Scheme S1. In this PC screening, we used 25 fungal strains that were presumed to be unknown species with homology of less than 90% compared to known species from the genetic analysis. They were selected from 600 fungal strains of FKI-9401~10000. These 25 strains were cultured on two different media to get 50 cultured broths. The obtained cultured broths were analyzed by LC-DAD-ESI-MS analysis to collect data sets of molecular weights and UV spectra. Manual dereplication by natural product database "Dictionary of Natural Products" [8], on DVD (Ver. 26.2, CRC Press) using these data sets allowed us to find 4 strains as producers of presumed new compounds (Table S1). As a result of re-culturing these strains, reproducibility was obtained only for FKI-9632 strain which produce a presumed new compound detected as a $[M + H]^+$ ion (*m/z* 405.1907) and a characteristic UV profile (λ_{max} 264 and 337 nm) by LC-DAD-ESI-MS analysis (Fig. S1).

Taxonomy of the producing strain of Stachybotryaceae sp. FKI-9632

The fungal strain FKI-9632 was isolated from a soil sample around *Smilax china* in Shikine Island, Izu Islands, Tokyo, Japan. This strain produced verticillium-like conidiophores (Fig. S2). The internal transcribed spacer (ITS) region including 5.8 S ribosomal RNA gene sequence of FKI-9632 was compared to sequences in the GenBank database by BLASTN 2.12.0 analysis [11]. The sequence of it was 87.4% similar to the sequence of CBS 143444 (holotype of *Sirastachys cyperacearum* Crous & T.I. Burgess [12], GenBank accession number MH107917). Conidiophores of FKI-9632 were different from characteristics of genus *Sirastachys*. The producing strain FKI-9632 was identified with the Stachybotryaceae based on sequence analysis.

Isolation of shikinefragalides A-D

Shikinefragalides A-D were isolated from a 15-day-old static cultured material guided UV and MS profiles using LC-DAD-ESI-MS analysis (Scheme 1). They were purified under light-shielded condition within 3 days after preparation of a cultured material due to light-sensitivity. The stationary culture (2.0 kg) was extracted with 2.0 l of MeOH. After filtration *in vacuo*, the filtrate was evaporated



Scheme 1 Fermentation and isolation of shikinefragalides A (**1**)–D (**4**)

Table 1 ^{1}H and ^{13}C NMR data of **1** and **2** measured in DMSO-d_6

	Shikinefragalide A (1)		Sihikinefragalide B (2)	
position	δ_C	δ_H (int., mult., J in Hz)	δ_C	δ_H (int., mult., J in Hz)
1	77.6	5.15 (1H, dd, 10.1, 3.5)	77.5	5.13 (1H, dd, 10.0, 3.6)
2	124.4	5.66 (1H, dd, 9,9, 3.5)	124.4	5.62 (1H, dd, 10.0, 3.6)
3	130.4	5.89 (1H, dd, 9.9, 5.6)	130.3	5.87 (1H, dd, 10.0, 5.6)
4	64.5	4.01 (1H, dd, 5.6, 5.4)	64.5	4.00 (1H, dd, 5.6, 5.4)
4-OH		4.39 (1H, d, 5.4)		4.37 (1H, d, 5.4)
5	75.4	–	75.4	–
5-OH		4.12 (1H, s)		4.10 (1H, s)
6	70.6	3.37 (1H, dd, 9.5, 7.1)	70.6	3.37 (1H, dd, 9.5,7.2)
6-OH		4.09 (1H, d, 7.1)		4.08 (1H, d, 7.2)
7α	41.7	1.39 (1H, dd, 13.8, 9.5)	41.8	1.38 (1H, dd, 13.9, 9.5)
7β		2.06 (1H, dd, 13.9, 9.3)		2.05 (1H, dd, 13.9, 9.5)
8	68.5	3.74 (1H, m)	68.5	3.76 (1H, m)
8-OH		4.46 (1H, d, 4.2)		4.45 (1H, d, 4.2)
9α	45.3	1.90 (1H, br. d, 14.5)	45.3	1.89 (1H, br. dd, 13.9, 1.8)
9β		1.61 (1H, ddd, 14.5, 11.1, 10.0)		1.61 (1H, ddd, 13.9, 10.0, 9.5)
10	69.9	4.54 (1H, dqd, 11.1, 6.1, 1.9)	69.8	4.53 (1H, dqd, 10.0, 6.2, 1.8)
11	165.1	–	165.2	–
12	108.4	–	107.8	–
13	157.8	–	158.0	–
14	46.6	3.63 (1H, d, 10.1)	46.6	3.61 (1H, d, 10.0)
15	21.5	1.29 (3H, d, 6.1)	21.5	1.29 (3H, d, 6.2)
16	118.4	6.36 (1H, d, 15.3)	117.5	6.33 (1H, d, 15.5)
17	129.1	7.02 (1H, dd, 15.3, 11.6)	134.6	6.62 (1H, dd, 15.5, 11.2)
18	125.8	5.97 (1H, dd, 11.6, 11.2)	129.3	6.24 (1H, dd, 14.9, 11.2)
19	133.8	6.13 (1H, dd, 11.3, 11.2)	137.4	6.44 (1H, dd, 14.9, 10.7)
20	126.9	6.58 (1H, dd, 14.5, 11.3)	131.7	6.14 (1H, dd, 15.0, 10.7)
21	133.6	5.84 (1H, dq, 14.5, 6.9)	132.6	5.83 (1H, dq, 15.0, 6.9)
22	18.3	1.79 (3H, d, 6.9)	18.2	1.75 (3H, d, 6.9)

in vacuo to make a 30% MeOH aq. solution. The obtained 30% MeOH aq. solution (0.6 l) was applied to an ODS column (150 ml resin, 55 i.d. × 55 mm; YMC Co., Kyoto, Japan). The column was eluted stepwise with 40% MeOH aq. (500 ml), 60% MeOH aq. (500 ml), 80% MeOH aq. (500 ml) and 100% MeOH (500 ml). The 80% MeOH aq. fraction including shikinefragalides was concentrated *in vacuo* under light-shielded condition and freeze-dried. A part (125.1 mg) of the obtained material (702.1 mg) underwent HPLC using a reverse-phase column (Capcell pak C_{18} MG-II, 20 i.d. × 250 mm; Osaka Soda Co. Ltd., Osaka, Japan) with an isocratic solvent system of CH_3CN-H_2O (40:60) at a flow rate of 7.0 ml min^{-1} detected by UV 323 nm. The four fractions with retention times of 26–28, 30–32, 43–45, and 48–50 min were collected (Fig. S3), evaporated *in vacuo*, and freeze-dried under light-shielded condition to afford shikinefragalides A (**1**, 23.9 mg), B (**2**, 14.3 mg), C (**3**, 6.8 mg) and D (**4**, 5.9 mg), respectively.

Structure elucidation of shikinefragalides A-D

Physico-chemical properties of **1**-**4** are summarized in Table S4–1. Compounds **1**-**4** were expected to be analogs because they had similar physico-chemical properties (UV: λ_{max} 264-269 and 334-345, and ESI-MS: *m/z* 405 or 421).

The structure of shikinefragalide B (**2**) was elucidated at first. The molecular formula of **2** was elucidated as $C_{22}H_{28}O_7$ based on a $[M + H]^+$ ion at *m/z* 405.1880 (calcd. *m/z* 405.1907) in HR-ESI-MS with 9 degrees of unsaturation. Analyses of ^{1}H, ^{13}C, and HSQC spectra measured in DMSO-d_6 (Table 1, Figs. S4–2–4, 5 and 7) indicated the presence of one ester carbonyl carbon, ten sp^2 olefinic carbons, six sp^3 methine carbons including five oxygenated methines, two sp^3 methylene carbons, one oxygenated sp^3 tetrasubstituted carbon and two methyl carbons.

The ^{1}H-^{1}H COSY analysis of **2** revealed three partial structures **I**-**III** as shown in Fig. 2a, H-14 (δ_H 3.61)/H-1 (δ_H 5.13)/H-2 (δ_H 5.62)/H-3 (δ_H 5.87)/H-4 (δ_H 4.00)/4-OH (δ_H

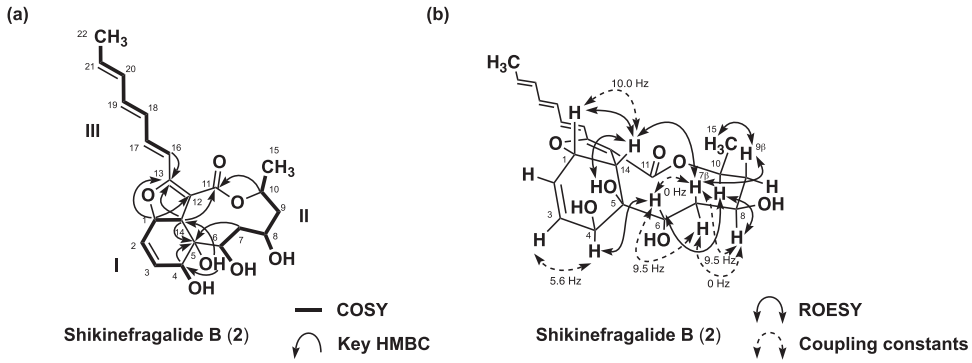


Fig. 2 Structure elucidation of shikinefragalide B (**2**). (**a**) ^{1}H-^{1}H COSY and key HMBC cross peaks of **2**. (**b**) Key ROESY correlations and ^{1}H-^{1}H coupling constants of **2**

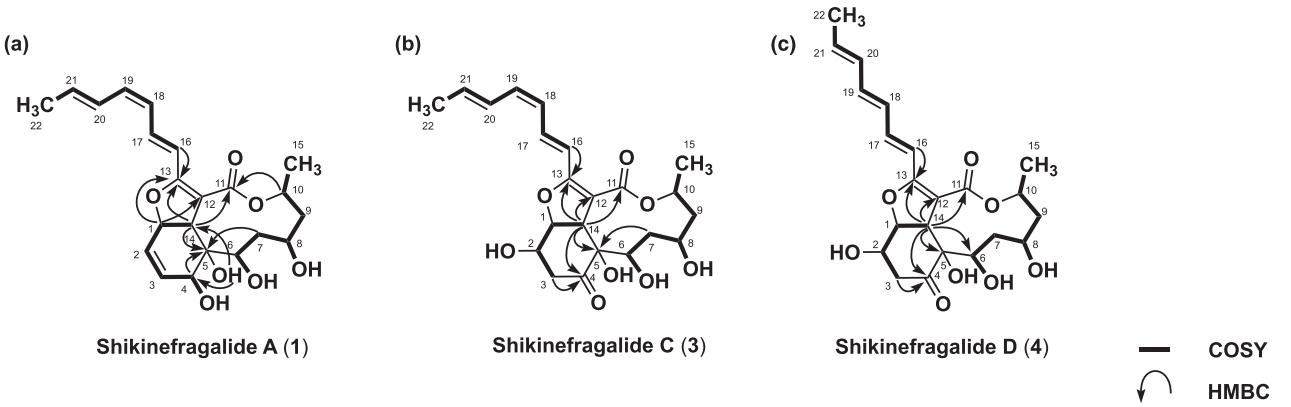


Fig. 3 ^{1}H-^{1}H COSY and key HMBC cross peaks of shikinefragalides A (**1**), C (**3**) and D (**4**). (**a**) shikinefragalide A (**1**), (**b**) shikinefragalide C (**3**) and shikinefragalide D (**4**)

4.37) as **I**, H-6 (δ_H 3.37)/H_2-7 (δ_H 1.38, 2.05)/6-OH (δ_H 4.08)/H-8 (δ_H 3.76) /8-OH (δ_H 4.45)/H_2-9 (δ_H 1.61, 1.89)/H-10 (δ_H 4.53)/H_3-15 (δ_H 1.29) as **II**, and H-16 (δ_H 6.33)/H-17 (δ_H 6.62)/H-18 (δ_H 6.24)/H-19 (δ_H 6.44)/H-20 (δ_H 6.14)/H-21 (δ_H 5.83)/H_3-22 (δ_H 1.75) as **III**. The geometry of three double bonds in **III** was determined to be all *E* by large ^{1}H-^{1}H coupling constants (15.5, 14.9 and 15.0 Hz). Based on HMBC cross peaks from H-1 to C-13 (δ_C 158.0) and C-12 (δ_C 107.8), from H-14 to C-13 and C-5 (δ_C 75.4), from 5-OH (δ_H 4.10) to C-14 (δ_C 46.6) and C-4 (δ_C 64.5), from H-4 to C-5, and from H-16 to C-13, it was suggested **2** has a dihydrofuran-fused cyclohex-2-enol ring moiety including **I**, which is connected to a 1,3,5-heptatrienyl moiety **III** at C-13 position. Finally, the correlations in HMBC from H_2-7 to C-5, from H-10 to C-11 (δ_C 165.2), and from H-14 to C-11, suggested the presence of a 10-membered macrolactone ring including **II**, which was connected at C-12 and C-5 positions of a dihydrofuran-fused cyclohexene ring moiety. From all results described above, **2** was elucidated as a new analog of colletofragarones [13], and designated shikinefragalide B (Fig. 1).

The relative configuration of **2** was established by ROESY and ^{1}H-^{1}H coupling constant analyses (Fig. 2b). Key ROESY correlations between H-1/H-14, H-14/5-OH, H-4/H-6, H-6/H-10, H-8/H-10, H_β-9/H_β-7, H_β-9/H_3-15 and H_β-7/H-14 and coupling constants between 1/14 (10.0 Hz), 3/4 (5.6 Hz), 6/7β (0 Hz), 6/7α (9.5 Hz), 7β/8 (9.5 Hz) and 7α/8 (0 Hz) suggested the relative configuration of **2** to be 1*S**, 4*S**, 5*S**, 6*R**, 8*S**, 10*S**, 14*S**. The absolute configuration of **2** was elucidated by circular dichroism (CD) spectra. Compared with reported CD spectrum of colletofragarone A2 [14], **2** had same positive cotton effect around 240 nm (Fig. S4–2–16), the absolute configuration of **2** should be 1*S*, 4*S*, 5*S*, 6*R*, 8*S*, 10*S*, 14*S*.

The molecular formula of shikinefragalide A (**1**) was determined to be $C_{22}H_{28}O_7$, which was same to that of **2**, based on a $[M+H]^+$ ion at *m/z* 405.1866 (calcd. *m/z* 405.1907) in HR-ESI-MS. ^{1}H, ^{13}C, and HMQC spectra of **1** measured in DMSO-d_6 (Table 1, Figs. S4–1–4, 5 and 7), resembled those of **2**, except for the proton and carbon signals of a 1,3,5-heptatrienyl moiety. The interpretation of ^{1}H-^{1}H COSY and HMBC of **1** suggested **1** has a same planar structure to **2**. (Fig. 3a). The difference was found in geometry of three double bonds, which was determined to be 16*E*, 18*Z* and 20*E* by ^{1}H-^{1}H coupling constants between 16/17 (15.3 Hz), 18/19 (11.2 Hz), and 20/21 (14.5 Hz), respectively. The relative configuration of **1** was established by ROESY and ^{1}H-^{1}H coupling constant analyses (Fig. 4a) same as **2**. The absolute configuration of **1** was also elucidated to be 1*S*, 4*S*, 5*S*, 6*R*, 8*S*, 10*S*, 14*S* by CD spectrum

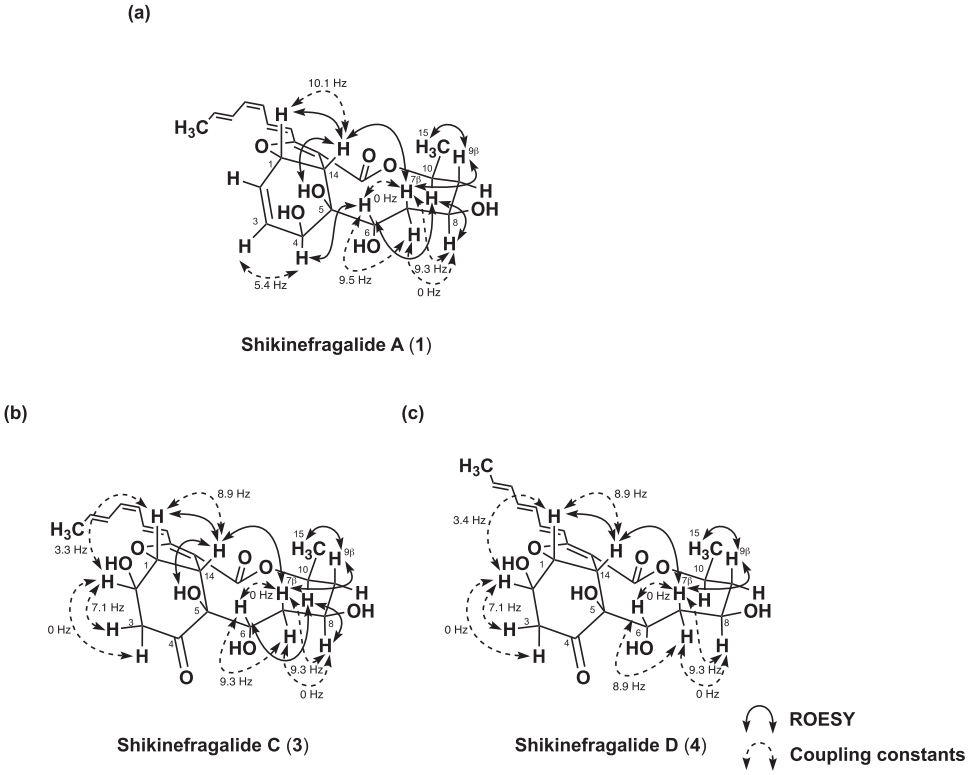


Fig. 4 Key ROESY correlations and ^{1}H-^{1}H coupling constants of shikinefragalides A (**1**), C (**3**) and D (**4**). (**a**) shikinefragalide A (1), (**b**) shikinefragalide C (3) and shikinefragalide D (4)

(Fig. S4–1–16). From these observations described above, **1** was elucidated as a new analog, which was a 18*Z* isomer of **2** and designated shikinefragalide A (Fig. 1).

The molecular formula of shikinefragalide C (**3**) was determined to be $C_{22}H_{28}O_8$, based on a $[M + H]^+$ ion at *m/z* 421.1839 (calcd. *m/z* 421.1856) in HR-ESI-MS with 9 degrees of unsaturation, indicating the presence of an additional oxygen atom compared with that of **1**. ^{1}H, ^{13}C, and HSQC spectra of **3** measured in DMSO-d_6 (Table 2, Fig. S4–3–4, 5 and 7), resembled those of **1**, except for the proton and carbon signals of a partial structure **I**. The interpretation of ^{1}H-^{1}H COSY and HMBC of **3** (Fig. 3b), suggested the **3** has a 3-hydroxylcyclohexan-1-one ring moiety, instead of a cyclohex-2-enol ring moiety of **1**.

The relative configuration of **3** was established by ROESY and ^{1}H-^{1}H coupling constant analyses (Fig. 4b). Key ROESY correlations between H-1/H-14, H-14/5-OH, H-6/H-10, H-8/H-10, H_β-9/H_β-7, H_β-9/H_3-15 and H_β-7/H-14 and coupling constants between 1/14 (8.9 Hz), 1/2 (3.3 Hz), 2/3α (7.1 Hz), 2/3β (0 Hz), 6/7β (0 Hz), 6/7α (9.3 Hz), 7β/8 (9.3 Hz) and 7α/8 (0 Hz) suggested the relative configuration of **3** to be 1*R**, 2*R**, 5*S**, 6*R**, 8*S**, 10*S**, 14*S**. The absolute configuration of **3** was also elucidated by the comparison with reported CD spectrum of colletoin B [14] to be 1*R*, 2*R*, 5*S*, 6*R*, 8*S*, 10*S*, 14*S* (Fig. S4–3–10). From all results described above, **3** was elucidated as a new analog, and designated shikinefragalide C (Fig. 1).

The molecular formula of shikinefragalide D (**4**) was determined to be $C_{22}H_{28}O_8$, which was same to that of **3**, based on a $[M + H]^+$ ion at *m/z* 421.1845 (calcd. *m/z* 421.1856) in HR-ESI-MS. ^{1}H, ^{13}C, and HMQC spectra of **4** measured in DMSO-d_6 (Table 2, Figs. S4–4–4, 5 and 7), resembled those of **3**, except for the proton and carbon signals of a 1,3,5-heptatrienyl moiety. The interpretation of ^{1}H-^{1}H COSY and HMBC of **4** suggested **4** has a same planar structure to **3**. (Fig. 3c). The difference was observed in geometry of three double bonds, which was determined to be all *E* by large ^{1}H-^{1}H coupling constants between 16/17 (15.3 Hz), 18/19 (15.0 Hz), and 20/21 (15.1 Hz), respectively. The relative configuration of **4** was established by ROESY and ^{1}H-^{1}H coupling constant analyses (Fig. 4c) same as **3**. The absolute configuration of **4** was also elucidated to be 1*R*, 2*R*, 5*S*, 6*R*, 8*S*, 10*S*, 14*S* by CD spectrum (Fig. S4–4–10). From these results described above, **4** was elucidated as a new analog, which was a 18*E* isomer of **3** and designated shikinefragalide D (Fig. 1).

Biological activity

Compounds **1** and **2**, isolated with over 10 mg, were tested for in vitro antimalarial activity against both a chloroquine-sensitive FCR3 strain and a chloroquine-resistant K1 strain of *P. falciparum*, as well as for cytotoxicity in human MRC-5 cells (Table 3, Fig S6). Compound **1** displayed in vitro

Table 2 ^{1}H and ^{13}C NMR data of **3** and **4** measured in DMSO-d_6

	Shikinefragalide C (3)		Sihikinefragalide D (4)	
position	δ_C	δ_H (int., mult., *J* in Hz)	δ_C	δ_H (int., mult., *J* in Hz)
1	83.4	4.84 (1H, dd, 8.9, 3.3)	83.8	4.81 (1H, dd, 8.9, 3.4)
2	67.9	4.39 (1H, br. s)	67.9	4.34 (1H, br. d, 7.1)
2-OH		4.08 (1H, br. d)		Not observed
3α	44.8	2.70 (1H, dd, 17.3, 7.1)	44.8	2.64 (dd, 17.3, 7.1)
3β		2.90 (1H, d, 17.3)		2.88 (d, 17.3)
4	210.9	–	210.8	–
5	82.1	–	82.1	–
5-OH		4.14 (1H, br. s)		Not observed
6	68.7	3.28 (1H, dd, 9.3, 4.0)	68.7	3.26 (1H, d, 8.9)
6-OH		4.25 (1H, br. d 4.0)		Not observed
7α	41.9	1.49 (1H, dd, 13.9, 9.3)	41.9	1.46 (1H, dd, 14.2, 8.9)
7β		2.09 (1H, dd, 13.9, 9.3)		2.09 (1H, dd, 14.2, 9.5)
8	68.3	3.72 (1H, m)	68.2	3.70 (1H, m)
8-OH		4.55 (1H, br. s)		Not observed
9α	42.3	1.89 (1H, br. d, 14.6)	41.9	1.87 (1H, br. d, 14.4)
9β		1.63 (1H, ddd, 14.6, 8.0, 8.0)		1.61 (1H, ddd, 14.4, 8.0, 8.0)
10	70.2	4.80 (1H, m)	70.1	4.78 (1H, m)
11	163.8	–	163.8	–
12	103.5	–	102.8	–
13	163.8	–	163.9	–
14	52.8	3.80 (1H, d, 8.9)	52.7	3.77 (1H, d, 8.9)
15	20.9	1.29 (3H, d, 6.4)	20.9	1.28 (3H, d, 6.4)
16	117.7	6.77 (1H, d, 15.3)	116.7	6.74 (1H, 15.3)
17	133.1	7.18 (1H, dd, 15.3, 11.6)	138.6	6.75 (1H, dd, 15.3, 9.0)
18	125.6	6.02 (1H, dd, 11.6, 11.1)	129.1	6.29 (1H, dd, 15.0, 9.9)
19	136.0	6.24 (1H, dd, 11.4, 11.1)	139.8	6.54 (1H, dd, 15.0, 10.8)
20	127.0	6.63 (1H, dd, 15.1, 11.4)	131.7	6.20 (1H, dd, 15.1, 10.8)
21	135.0	5.93 (1H, dq, 15.1, 6.8)	134.3	5.90 (1H, dq, 15.1, 6.9)
22	18.4	1.79 (3H, d, 6.8)	18.4	1.76 (3H, d, 6.9)

weak antimalarial activity against both a chloroquine-sensitive *P. falciparum* FCR3 strain and a chloroquine-resistant *P. falciparum* K1 strain with IC_{50} values of 62.7 and 186.6 μM, respectively. Compound **2** showed weaker antimalarial activity with IC_{50} values of 104.5 and 209.4 μM than **1**. In addition, **1** and **2** showed weak cytotoxicity against human MRC-5 cells, with IC_{50} values of 48.2 and 76.3 μM, respectively, indicating selectivity indices (IC_{50} values against MRC-5 cells/IC_{50} values against *P. falciparum* strains) ranging from 0.3 to 0.8.

Compounds **1**–**4** demonstrated no antimicrobial activity against *B. subtilis*, *K. rhizophila*, *E. coli*, *X. oryzae*, *C. albicans* and *M. racemosus* at 10 μg on a paper disc method.

Discussion

In this study, four new fungal macrolides, named shikinefragalides A (**1**)–D (**4**), were discovered from a static cultured material of a Stachybotryceae sp. FKI-9632 by PC screening. To data, there have been several analogs reported such as colletofragarones [13], colletoins [14], and dictyosphaeric acids [15] (Fig. S5). To our best knowledge, the biosynthetic pathway of colletofragarone-related compounds has not been clarified yet. The main differences among colletofragarone-related compounds and **1**-**4** are degrees of oxidation at C-2, C-3, C-4, C-5, C-6, and C-8 positions, the decipher and comparison of their biosynthetic pathway would reveal responding enzymes for oxidation.

Colletofragarone A2 and colletoin A were reported to show cytotoxicity against Saos-2 (p53R175H) cells, with IC_{50} values of 0.35 and 0.36 μM, respectively, whereas colletoins B and C were less active, with IC_{50} values of 21 and 12 μM, respectively [14]. Dictyosphaeric acid A was reported to have antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci*, and *C. albicans*, while dictyosphaeric acid B was reported to show no significant antibacterial activity [15, 16]. These reports suggest that an α, β-unsaturated carbonyl group in their six-membered ring is essential for their activity (Fig. S5) [14, 16]. Compounds **1**-**4** without an α, β-unsaturated carbonyl group, showed no antibacterial activity, which also support its importance.

This is the first report of antimalarial activity of colletofragarone-related compounds against *P. falciparum*. Compounds **1** and **2** showed weak in vitro antimalarial activity. Recently, dictyosphaeric acid A was passed *in silico* screening against COVID-19 and was reported to be a potential inhibitor of the host enzyme, transmembrane protease serine 2 (TMPRSS2) [17]. Since **1**-**4** are sensitive to light and gradually degraded even under light-shielded condition, they are not suitable for long-term storage, and it would be difficult to evaluate their activity against various targets. Therefore, it is necessary to chemically modify colletofragarone-related compounds including **1**-**4** to improve their stability for pharmaceutical applications by inducing them.

In this study, we used 25 unidentified fungi having less than 90% homology with known species for sources of PC screening. As a result, we could select 4 producers of

Table 3 In vitro antimalarial activity, cytotoxicity, and selectivity index of **1**, **2** and chloroquine (an antimalarial drug)

Compound	IC_{50} [μM]				
	Antimalarial activity		Cytotoxicity	Selectivity index (SI)	
	K1 strain*	FCR3 strain**	(MRC-5)	MRC-5/K1	MRC-5/FCR3
Shikinefragalide A (**1**)	186.6	62.7	48.2	0.3	0.8
Shikinefragalide B (**2**)	209.4	104.5	76.3	0.4	0.7
Chloroquine***	0.577	0.047	58.2	101	1,238

*chloroquine-resistant strain

**chloroquine-sensitive strain

***drug commonly used to treat malaria

presumed new compounds with higher rate of 16% than those of identified species (less than 5%) in our research team, suggesting that fungal strains with high novelty are very good sources for PC screening to improve efficacy of discovery new fungal secondary metabolites.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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