Alkaloids From the Marine Fungus *Lecanicillium fusisporum* Using an Amino Acid-Directed Strategy

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Abstract

An amino acid-directed strategy has been developed to explore the potential of marine fungi to produce alkaloids. The marine fungus *Lecanicillium fusisporum* was cultured in glucose-peptone-yeast (GPY) medium to which were added L-tryptophan, L-phenylalanine, L-threonine, D, L-methionine, L-serine, L-lysine and L-valine. A new indole alkaloid, lecasporinoid (1), along with five known alkaloids (2−6) were discovered from the culture broth. The planar structure of lecasporinoid (1) was determined by HR-ESIMS, and 1D and 2D NMR spectroscopic data. The absolute configuration was established by optical rotation and 13C NMR calculations combining with a chemical synthetic approach.

Keywords

marine fungus, *Lecanicillium fusisporum*, alkaloids, amino acid-directed strategy, quantum chemical calculations

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Introduction

Marine-derived fungi have become an important source of structurally novel and pharmacologically active metabolites. Among them, alkaloids are a class of marine fungal natural products that show unique promise in the development of new drug leads. Biogenetically, alkaloids are derived from an amino acid pathway.1 Thus, an amino acid-directed strategy, that is adding various natural amino acids, such as L-phenylalanine, L-tryptophan, and L-valine into the regular culture medium, may induce the marine-derived fungi to produce alkaloids. For example, 23 indole alkaloids were obtained from the culture of the marine-derived fungus *Fusarium* sp. in glucose-peptone-yeast (GPY) medium supplied with L-tryptophan.2 Among them, fusaindoterpene B, JBIR-03 and 1,2-bis(1H-indol-3-yl)ethane-1,2-dione showed inhibitory activity against the Zika virus (ZIKV).3 Thirteen indole alkaloids, including two new compounds, were discovered from the marine-derived fungus *Pseudallescheria boydii* F44-1 in GPY medium with L-tryptophan, L-phenylalanine, L-methionine, and L-threonine.1,4,5 *Cyclohexylidenebis(1H-indole)* showed cytotoxic activity against various cancer cell lines.1 Seventeen new fumiquinazoline alkaloids were isolated from the marine fungus *Scedosporium apiospermum* F41-1 by feeding various amino acids.4,5 *Scedapin C* and *scequindolines D, E, J* and *quinadoline A* were found to strongly promote triglyceride accumulation in 3T3-L1 cells. Furthermore, qRT-PCR analysis suggested that *scequindoline D* might be a potent antidiabetic agent by activating the PPARγ pathway.5 These results demonstrate the great potential of alkaloid production in marine fungi.

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Recently, a marine fungus *Lecanicillium fusisporum* was isolated from the inner tissue of a sea cucumber collected from Xisha Islands in China. To induce this fungal strain to produce alkaloids, *L. fusisporum* was fermented in GPY medium supplemented with L-tryptophan, L-phenylalanine, L-threonine, D, L-methionine, L-serine, L-lysine and L-valine. By tracking the aromatic 1H NMR signals at δH 6.50–8.50 in the EtOAc extract of the culture broth, one new indole alkaloid, lecasporinoid (1), together with five known alkaloids (2–6) were discovered (Figure 1). Herein, we report the isolation, structure elucidation and bioactivity evaluation of these compounds.

**Results and Discussion**

**Structural Elucidation**

Lecasporinoid (1) was obtained as a colorless oil. The molecular formula was established as C_{16}H_{19}NO_{4} based on the potassium molecular ion detected at m/z 328.0954 (calcd 328.0946 for C_{16}H_{19}NO_{4}K) in the HR-ESIMS. The 13C NMR spectrum (Table 1), in combination with DEPT and HSQC spectra, displayed 16 carbon signals, consisting of three methyls, one sp3 methylene, five sp2 and two oxygen atom bearing methines, three nonprotonated sp2 carbons and two carbonyl carbons. The 1H-1H COSY correlations of H-4/H-5, H-5/H-6, H-6/H-7, as well as the HMBC correlations (Figure 2) from H-2 to C-3/C-4a/C-7a, from H-4 to C-7a, from H-7 to C-4a, and from H-8 to C-3/C-4a/C-9, suggested that the core structure of 1 is indole-3-acetic acid. Moreover the 1H-1H COSY correlations of Me-14/H-10, H-10/H-11, H-11/Me-15 and the HMBC correlations from H-11 to C-12, from Me-13 to C-12, combining with the deshielded signals of C-11 (δC 71.4) and H-11 (δH 5.03), displayed the presence of the substructure –CH(CH_{3})–CH(CH_{3})–O–C(=O)–CH_{3}. Then, C-8 was connected to C-10 by an ester group, which was supported by HMBC correlations from H_{2}-8 and H-10 to C-9. Accordingly, the planar structure of 1 was established, as shown in Figure 1.

To determine the absolute configuration of 1, quantum chemical calculations were made using Gaussian 09 software. By comparing the experimental optical rotation value (+23) with the calculated value of all configurations (10S,11R-1, −53.6; 10R,11S-1, −47.5; 10S,11R-1, +52.1; 10S,11S-1, +52.3), the chirality at C-10 was deduced to be 10R. Then, the correlation coefficient (10S,11R-1: 0.99909; 10S,11S-1: 0.99877) and DP4+ probability (10S,11R-1: 98.43; 10S,11S-1: 1.57) between the calculated 13C NMR data (Figure 3) and experimental data suggested that 10S,11R-1 was a better match.

Finally, in order to validate further the calculation, we turned our attention to synthesize (10R,11R)-lecasporinoid (1a) for comparison. Synthesis of 1a was performed in two sequential steps, as illustrated in Figure 4. First, commercially available indole-3-acetic acid activated with 1-ethyl-3(3-dimethylpropyl)-2(1H)-pyrazinone (EDCI) in dichloromethane (DCM) was treated with (2R,3R)-butanol to obtain 7. In the subsequent step, the acetylation of compound 7 with acetic anhydride was carried out in the presence of N,N-diisopropylethylamine (DIPEA). The resulting 1a was purified from the reaction mixture by silica gel chromatography in good yield. Its spectroscopic data (1H and 13C NMR) differ from those of lecasporinoid (1), excluding the possibility of 10S,11R-1. Thus, the absolute configuration was assigned as 10S,11R-1.

1,1,1-Tris(3-indoly) methane (2), 3-hydroxy-β-carboline (3), 3,6-bis(1-methylethyl)-2(1H)-pyrazinone (4), deoxymutaaspergillic acid (5) and 6-[1(R)-1-methylpropyl]-3-(2-methylpropyl)-2(1H)-pyrazinone (6) were identified by comparing their 1H and 13C data with those in the literature.

**Anti-DENV Activity**

To investigate the antIdengue virus 2 activity of compounds 1 to 6 from *L. fusisporum*, in the DENV2 16681 replication assay, A549 cells were treated with compounds 1 to 6 at the indicated concentrations for 3 days. Then the effects of compounds 1 to 6 on DENV replication were analyzed by standard focus-forming assay. However, the assay revealed that compounds 1 to 6 have no apparent effect on dengue virus.

**Experimental**

**General Experimental Procedures**

1D and 2D NMR spectra were measured in CDCl_{3} using Bruker Avance II 400 and 500 spectrometers (Bruker Bio Spin AG, Industriestrasse 26, Fällanden, Switzerland), and the chemical shifts are given relative to the residual solvent signals (CDCl_{3}: δH 7.26, δC 77.2). UV spectra were recorded using a Shimadzu UV-Vis-NIR spectrophotometer (Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan), optical rotations (OR) were measured with an Anton Paar MCP500 polarimeter at 25 °C, and IR spectra were recorded on a Bruker tensor-27 spectrophotometer with KBr discs. Mass spectra were acquired

![Figure 1. Chemical formulas of compounds 1 to 6.](image-url)
Table 1. $^1$H NMR (500 MHz) and $^{13}$C NMR (125 MHz) Data for 1, 1a and 7 in CDCl$_3$ ($J$ in Hz).

| Position | $^1$H NMR (500 MHz) | $^{13}$C NMR (125 MHz) |
|----------|---------------------|------------------------|
|          | $\delta$, type      | $\delta$, type         |
| 1-NH     | 8.09, brs           | 8.29, brs              |
| 2        | 123.1, CH, 7.18, m  | 123.3, CH, 7.15, m     |
| 3        | 108.7, C            | 108.3, C               |
| 4a       | 127.4, C            | 127.2, C               |
| 4        | 119.1, CH, 7.62, brd (8.0) | 118.9, CH, 7.64, brd (8.0) |
| 5        | 119.8, CH, 7.13, brt (8.0) | 119.7, CH, 7.12, brt (8.0) |
| 6        | 122.4, CH, 7.19, brt (8.0) | 122.2, CH, 7.20, brt (8.0) |
| 7        | 111.3, CH, 7.37, brd (8.0) | 111.3, CH, 7.32, brd (8.0) |
| 7a       | 136.2, C            | 136.2, C               |
| 8        | 31.6, CH$_2$, 3.78, s | 31.7, CH$_2$, 3.78, s |
| 9        | 171.5, CO           | 171.6, CO              |
| 10       | 71.7, CH, 5.03, dq (6.5, 3.5) | 71.9, CH, 5.01, m     |
| 11       | 71.4, CH, 4.97, dq (6.5, 3.5) | 71.6, CH, 4.96, m     |
| 12       | 170.6, CO           | 170.6, CO              |
| 13       | 21.2, CH$_3$, 1.95, s | 20.9, CH$_3$, 1.86, s |
| 14       | 15.0, CH$_3$, 1.21, d (6.5) | 16.1, CH$_3$, 1.22, d (6.5) |
| 15       | 15.2, CH$_3$, 1.16, d (6.5) | 16.2, CH$_3$, 1.16, d (6.5) |

Figure 2. Key $^1$H–$^1$H COSY and HMBC correlations of compound 1.

Fungal Strain and Culture Method

The marine fungus Lecanicillium fusisporum was isolated from the inner tissue of a sea cucumber collected from Xisha Islands in China. The fungal strain was maintained on potato dextrose agar (PDA) slants. Analysis of the ITS rDNA by BLAST database screening provided a 99.9% match with Lecanicillium fusisporum. The fermentation medium contained glucose 10 g/L, peptone 5 g/L, yeast extract 2 g/L, sea salt 20 g/L, L-tryptophan 2 g/L, L-phenylalanine 2 g/L, L-threonine 2 g/L, L-methionine 2 g/L, L-serine 2 g/L, L-lysine 2 g/L, L-valine 2 g/L, and H$_2$O 1 L (pH 7.5). The mycelia were aseptically transferred to 500 mL Erlenmeyer flasks containing 200 mL of the liquid medium, then sterilized at 120 °C for 30 min. The flasks were statically incubated at 28 °C for 40 days.

Extraction and Isolation

Fifty liters of culture broth were filtered through cheesecloth. The culture broth was extracted three times with 60 L EtOAc. The combined EtOAc extract was concentrated under reduced pressure to afford 17 g crude extract. This was chromatographed on a silica gel column with a stepwise gradient of light petroleum–EtOAc (100:0–0:100), followed by EtOAc–MeOH (100:0–0:100). The eluents were collected every 400 mL, and similar fractions were pooled to afford six fractions (code Fr.1–Fr.6) by TLC monitoring. Fr.3 was further purified by preparative HPLC (73% MeOH–H$_2$O) to yield compounds 4 (2.2 mg) and 5 (3.2 mg). Fr.4 was subjected to silica gel column chromatography eluting with a gradient of light petroleum–EtOAc to afford five subfractions (Fr.4.1–Fr.4.5). Fr.4.3 was further purified by preparative HPLC (71% MeOH–H$_2$O) to yield compounds 1 (0.5 mg), 2 (1.8 mg) and 3 (1.0 mg).
Fr.4-4 was further purified by preparative HPLC (68% MeOH–H₂O) to obtain compound 6 (2.0 mg).

Procedure for the Preparation of Compounds 7 and 1a

(2R,3R)-3-Hydroxybutan-2-yl-2-(1H-indol-3-yl)acetate (7). To a solution of 3-indoleacetic acid (350 mg, 2 mmol) and (2R,3R)-butanediol in dry dichloromethane (10 mL) at room temperature was added EDCI (383 mg, 2 mmol) and DMAP (cat.). After 12 h, the reaction mixture was quenched with water (10 mL) and extracted with dichloromethane (3 × 8 mL). The combined organic phases were dried with anhydrous sodium sulfate and evaporated to dryness. Purification by column chromatography afforded 7 (236 mg, 48%) as a white solid. ¹H NMR and ¹³C NMR data see Table 1.

(2R,3R)-Lecasporinoid (1a). To a solution of 7 (94 mg, 0.38 mmol) and acetic anhydride (77 mg, 0.76 mmol) in dry dichloromethane (6 mL) at room temperature was added DIPEA (54 mg, 0.42 mmol) and DMAP (4.6 mg, 0.038 mmol). After 1.5 h, the reaction mixture was quenched

Figure 3. ¹³C NMR calculation results for two possible isomers of 1. (a) Linear correlation plots of calculated versus experimental ¹³C NMR chemical shift values for each potential configuration. (b) Relative errors between the predicted ¹³C NMR chemical shifts of two potential structures and recorded ¹³C NMR data. (c) The DP4+ probability of chemical shifts about isomer 1 (10S,11S-I) and isomer 2 (10S,11R-I).

Figure 4. The synthetic route of 1a. Reagents and conditions: (a) (2R,3R)-butanediol, EDCI, DMAP, DCM, rt, 12 h; (b) acetic anhydride, DMAP, DIPEA, DCM, rt, 1.5 h.
with water (8 mL) and extracted with dichloromethane (3 × 6 mL). The combined organic phases were dried with anhydrous sodium sulfate and evaporated to dryness. Purification by column chromatography afforded 1a (99 mg, 90%) as a colorless oil. 1H NMR and 13C NMR data see Table 1.

Spectroscopic Data

Lecasporinoid (I): colorless oil; [a]20 D +23.0 (c 1.0, MeOH); UV (MeOH) λmax (log ε) 280 (2.21) nm; IR (KBr) νmax 3401, 2976, 1087, 1049, 1024, 1002 cm−1; 1H NMR and 13C NMR data see Table 1; HRESIMS m/z 328.0954 [M + K]⁺ (calcld for C16H19NO4K, 328.0946).

Antiviral Activity Against DENV2

A549 cells were pretreated with compounds 1 to 6 (5 μM or 50 μM) for 1 h, followed by infection with DENV2 16681 strains at MOI 3. The cells were incubated with the chemicals throughout the entire experiment. Supernatants were harvested at 24 h p.i. for virus titration by standard focus-forming assay (FFA) on Vero cells. Samples were diluted in DMEM supplemented with 2% FBS. Vero cells were inoculated with serial 10-fold dilutions of each virus sample. Overlay containing 1% methylcellulose (Sigma) and 2% DMEM (Invitrogen) was then added and incubated at 37 °C. Cells were washed with phosphate-buffered saline (PBS) and fixed with 1% paraformaldehyde (PFA) in PBS at 3 days p.i. Cells were incubated with anti-WNV E18 monoclonal antibody (MAb), followed by incubation with goat anti-mouse HRP-conjugated secondary antibody (CST). Cells were incubated with TrueBlue peroxidase substrate (KPL). The number of spots was determined by a CTL-BioSpot analyzer.

Computational Section

The absolute configuration of compound 1 was determined by quantum chemical calculations using Gaussian 09 software. Conformational analysis of diastereoisomers was performed by using the Merck Molecular Force Field (MMFF). They were further optimized by the density functional theory (DFT) method at the B3LYP/6-31G(d) level. The optical rotations were calculated using the TDDFT method at the B3LYP/6-311 +G(d) level in methanol (α = 589.3 nm). The 13C NMR were calculated using the TDDFT at the B3LYP/6-31G* (d, p) level in chloroform. The predicted chemical shifts and optical rotations were obtained according to the Boltzmann distribution theory and their relative Gibbs free energy. All the theoretical NMR values were analyzed by using linear regression and DP4+. Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

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Trial Registration

Not applicable, because this article does not contain any clinical trials.

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Supplemental Material

Supplemental material for this article is available online.

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