Molecular Networking-Driven Discovery of Antibacterial Perinadines, New Tetracyclic Alkaloids from the Marine Sponge-Derived Fungus Aspergillus sp.

Yang Liu, Lijian Ding,* Yutong Shi, Xiaojun Yan, Bin Wu, and Shan He*

ABSTRACT: Two rare tetracyclic skeleton alkaloids named perinadines B and C (1 and 2) were isolated as mixtures of epimers from the marine-derived Aspergillus sp. LS116 driven by molecular networking. The planar structures of 1 and 2 were characterized by comprehensive spectroscopic data. Additionally, compounds 1 and 2 showed moderate in vitro antibacterial activity against Bacillus subtilis with minimum inhibitory concentration values of 32 and 64 μg/mL, respectively. Besides, both of the compounds were evaluated for anti-inflammatory activities in an in vivo zebrafish model.

INTRODUCTION

Alkaloids, a class of chemical entities with antibacterial,1 anti-inflammatory,2 anticancer,3 and antiviral properties,4 have been proved to possess enormous potential in veterinary, pharmacology, medicine, and plant protection.5 Notably, many illustrious alkaloids are marketed drugs such as sanguinarine, papaverine, morphine, and apomorphine, which were used as antimicrobial agents, muscle relaxants, and narcotic analgesics as well as the drug treatment for Parkinson’s disease.6 Moreover, bacterial infections are considered a major health problem worldwide. With increasing resistance of bacteria to antibiotics, novel antibacterial remedies are urgently needed. Alkaloids have been used as drug scaffolds and scaffold substructures in modern antibacterial chemotherapy.7 In view of those, they may become the crucial molecules for fighting microbial-associated diseases in the future.

Marine-derived fungi have gradually emerged as a prospective reservoir of structurally diverse and biologically active natural products in drug discovery progress.8 Recently, more and more alkaloids have been reported from marine fungus, which exhibited potent antibacterial activity.9 Molecular networking (MN) on the Global Natural Product Social (GNPS) platform (https://gnps.ucsd.edu), a strategy that has been demonstrated as a powerful and promising tool, can speed up greatly the dereplication and structure-based discovering for natural products. In the current study, to prioritize novel alkaloids from the crude extract of the Aspergillus genus, a workflow was employed through the combination of MN, Network Annotation Propagation (NAP) tool, and the MS2LDA web platform, which is based on latent Dirichlet allocation (LDA). Given the results of library matching in molecular networking and in silico top-ranked candidates through a consensus scoring algorithm in NAP, it enabled the prioritization of an interesting molecular family that included most of the alkaloids that have no matching MS/MS spectra (Figure S1). To further verify the results given by NAP, and to mine the substructure information in the selected cluster, we applied the text mining-based substructure algorithm MS2LDA. As a result, two rare tetracyclic alkaloids, perinadines B and C (1 and 2) were isolated and identified. The planar structures of compounds 1 and 2 were determined by high-resolution electrospray ionization mass spectrometry (HRESIMS) along with one-dimensional (1D) and two-dimensional (2D) NMR analyses. The absolute configuration of the tetracyclic moiety of 1 and 2 were assigned based on a comparison of experimental and computed electronic circular dichroism (ECD) spectra and nuclear Overhauser effect spectroscopy (NOESY) experiments. The bioactivity of compounds 1 and 2 was tested, including in vitro antibacterial and in vivo zebrafish-mode anti-inflammatory activity.
RESULTS AND DISCUSSION

The crude extract of *Aspergillus* sp. LS116 was analyzed by high-performance liquid chromatography quadrupole time-of-flight tandem mass spectrometry (HPLC-Q/TOF-MS/MS), and the acquired data was processed following the classical molecular networking workflow on the GNPS platform. With the process of GNPS MS/MS spectral library matching, it gave only 19 nodes with annotations, including six alkaloids (highlighted with a blue circle in Figure 1). To further annotate the other cluster without any spectral library matching and find some novel alkaloids with interesting structures, NAP was utilized in this molecular network. Then, with the observation of the first ranked NAP consensus candidates, the molecular family with 10 putative alkaloids (molecular family A) was selected as a priority. To unearth further substructure information from the selected cluster, we applied the Web-based MS2LDA application to explore the results. This process revealed that most of the members in molecular family A shared a Mass2Motif (motif_289, which was annotated as *Streptomyces* and one block of species motif, with no clear substructure annotation, Figure S2). Taking into account the results given by the NAP, this motif was most likely an alkaloid-related substructure. It encouraged us to perform the isolation for the nodes with motif_289 in molecular family A. Hence, with the liquid chromatography diode array detector electrospray ionization mass spectrometry (LC-DAD-ESIMS tracking for the node in molecular family A, we achieved the purification of a pair of stereoisomers, Perinadine B (1) and Perinadine C (2), both corresponding to the node at $m/z$ 456.273 ($[M + H]^{+}$ ion) and actually made from a series of parent ions with the same MS/MS spectra (Figure S4).

Perinadine B (1) was isolated as a colorless solid. Its molecular formula, C$_{27}$H$_{37}$NO$_{5}$, corresponding to 10 indices of hydrogen deficiency, was deduced by the (+)-HR-ESI-MS ion peak at $m/z$ 456.2752 [$M + H]^{+}$ and the $^{13}$C NMR data (Figures S5 and S12). Although it was repeatedly isolated as a

![Figure 1. Prioritization of perinadine derivatives using MS/MS molecular networking combined with the NAP and MS2LDA. (1) Molecular network of *Aspergillus* sp. LS116 crude extract with library hits (including the alkaloids in library matching). (2) NAP analyzes all nodes in the molecular family A, and the nodes identified as alkaloids in the first ranked candidate were highlighted according to the network consensus algorithm. (3) MS2LDA analysis for the selected cluster and targeted isolation of perinadine derivatives.](https://pubs.acs.org/doi/10.1021/acsomega.2c00402)
Table 1. $^1$H (600 MHz) and $^{13}$C (150 MHz) NMR Data of 1 and 2 in CDCl$_3$

| No. | δ$_H$ (ppm) | δ$_C$ (ppm) | $^1$H mut. (J in Hz) | 1 | 2 | 3 | 4 | 5 | 6 |
|-----|-------------|-------------|---------------------|---|---|---|---|---|---|
| 1   | 70.7, CH    | 65.5, CH    | 4.25, d (3.8)       | 4.36, d (8.5) |
| 3   | 78.5, CH    | 75.1, CH    | 3.72–3.77, m        | 4.15, q (6.8) |
| 4   | 37.4, CH    | 34.6, CH    | 2.82, p (6.8)       | 2.61–2.64, m |
| 4a  | 139.1, C    | 136.9, C    |                     |               |
| 5   | 116.9, C    | 116.9, C    |                     |               |
| 6   | 154.6, C    | 154.1, C    |                     |               |
| 7   | 102.3, CH   | 102.3, CH   | 6.33, s             | 6.31, s       |
| 8   | 149.0, C    | 149.3, C    |                     |               |
| 8a  | 116.1, C    | 115.4, C    |                     |               |
| 9   | 21.7, CH    | 18.2, CH$_3$| 1.34, d (6.3)       | 1.26, d (6.8) |
| 10  | 19.6, CH$_3$| 21.7, CH$_3$| 1.22, d (7.0)       | 1.26, d (6.8) |
| 11  | 11.4, CH$_3$| 10.0, CH$_3$| 2.14, s             | 2.11, s       |
| 2'  | 85.4, CH    | 88.2, CH    | 5.82, d (5.3)       | 5.43, d (6.8) |
| 3'  | 47.5, CH$_2$| 48.5, CH$_2$| 2.57–2.62, m        | 2.67–2.71, m |
| 4'  | 26.9, CH$_2$| 27.2, CH$_2$| 2.04–2.09, m        | 2.19–2.25, m |
| 4'β | 2.29–2.35, m| 2.19–2.25, m|                     |               |
| 5'α | 45.1, CH$_2$| 45.0, CH$_2$| 3.72–3.77, m        | 3.67–3.71, m |
| 5'β | 3.53–3.59, m| 3.85–3.89, m|                     |               |

$a^*$Two sets of signals.

single peak by high-performance liquid chromatography (HPLC), an NMR spectral analysis of 1 suggested a pair of epimers (Table 1). It was the same with perinadine A, but the chemical shifts for each set of carbon signals of perinadine B (1) were close to each other. The slightly obvious chemical shift difference existed in the atoms near C-7', may have been caused by a mixture of epimers at a chiral center (C-7'). To simplify this, one set of signals was chosen to clarify the gross structure. An analysis of the NMR data of 1 (Table 1) showed signals of one aromatic proton at δ$_H$ 6.33 (1H, s, H-7), a pair of olefin proton at δ$_H$ 5.37 (2H, overlap, H-13', 14'), one ketone at δ$_C$ 207.5 (C, C-8'), and one carboxyl at δ$_C$ 171.0 (C, C-6'). These groups accounted for 7 out of the 10 degrees of unsaturation, requiring three additional rings for 1. Furthermore, with the observation of the $^{13}$C and HSQC spectra (Figures S6 and S9), the remaining 25 carbons were attributable to five sp$^2$ quaternary carbons ($\delta_C$ 154.6, 149.0, 139.1, 116.9, and 116.1), three sp$^2$ methines (δ$_C$ 131.0, 125.3, and 102.3), six sp$^3$ methines (δ$_C$ 85.4, 78.5, 70.7, 52.9, 47.5, and 37.4), and six sp$^3$ methylenes (δ$_C$ 45.1, 39.9, 32.4, 29.1, 26.9, and 23.1) as well as five methyls (δ$_C$ 21.7, 19.6, 18.0, 14.2, and 11.4). These aforesaid data showed great similarity with perinadine A, and revealed the similarity skeleton between them. A detailed comparison of the NMR data displayed that the absence of the signal of the carboxylic acid unit, and the sp$^2$ quaternary carbon located at C-7 was displaced by an sp$^2$ methine of 1. The gross structure of 1 was eventually accomplished by analysis of $^1$H–$^1$H correlated spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) data (Figures S6 and S8).

The presence of the 2-methyl-3-keto C10 acyl group was evident by the cross-peaks of H-2'- (δ$_H$ 2.48, overlap)/H-10' (δ$_H$ 1.51, overlap)/H-2'-11' (δ$_H$ 1.28, m)/H-12' (δ$_H$ 1.93, m) /H-13' (δ$_H$ 5.37, overlap)/H-14' (δ$_H$ 5.37, overlap)/H-15' (δ$_H$ 1.61, d, 7.0) and H-7' (δ$_H$ 3.87, q, 7.0)/H-16' (1.48, d, 7.0) in the $^1$H–$^1$H COSY spectrum and the HMBC correlations of H-7', H-10', and H-16' with C-8' (δ$_C$ 207.5).

In addition, the double bond between C-13 and C-14 was determined as an E-configuration on account of the $^{13}$C NMR shift of the allylic carbon of 1-chlorobut-2-ene, which demonstrated a close with the $^{13}$C NMR shift of the methyl of C-15) corresponded to the (E)-configuration, while δ$_C$ 17.6 corresponded to the (Z)-configuration.

Interestingly, the hypothesis regarding the existence of pyrrolidine ring D and its linkage with the 2-methyl-3-keto C10 acyl group moiety at C-7' through a secondary amide carbonyl group was evidenced by combining the $^1$H–$^1$H COSY spectrum [cross-peaks of H-2'- (δ$_H$ 5.28, d, 5.3)/H-3' (δ$_H$ 2.60, m)/H-4'α (δ$_H$ 2.07, m)/H-5'α (δ$_H$ 3.75, m)] and the HMBC spectrum [correlations from H-2' to C-5' (δ$_C$ 45.1)], along with the correlations for H-2', H-5'α, and H-7' to the amide carbonyl carbon (δ$_C$ 171.0, C-6').

The key HMBC correlations of H-7 with C-5 (δ$_C$ 116.9), C-6 (δ$_C$ 154.6), C-8 (δ$_C$ 149.0), and C-8α (δ$_C$ 116.1) revealed the
existence of benzene ring A. The methyl proton signal at H-11 (δH 2.14, s) showed HMBC correlations to C-4a (δC 139.1), C-5 (δC 116.9), and C-6 (δC 154.6), together with the observation of the chemical shift C-6, suggesting that the methyl at C-11 (δC 10.0) was tethered to C-5 and the hydroxyl group was attached to C-6. The 3,4-dimethyl-2,3H-pyran ring B was established based on the HMBC correlations of H-1 (δH 4.25, d, 3.8) with C-4a, C-8, and C-8a and H-3 (δH 3.75, m) with C-1 (δC 70.7) and C-4a. In addition, the methyls at C-9 (δC 21.7) and C-10 (δC 19.6) substituted at C-3 (δC 78.5) and C-4 (δC 37.4), respectively, were deduced according to the correlations of H-9 (δH 1.34, d, 6.3)/H-3 and H-10/H-4 (δH 2.82, p, 6.8) in the 1H-1H COSY spectrum, along with the HMBC correlations of H-9 with C-4 and H-10 with C-3.

It is worth mentioning that the relatively deshielded sp2 quaternary carbon at δC 149.0 (C-8) was shown as a small induced shift, thus revealing that this carbon was linked with an ether linkage. And the relatively deshielded carbon chemical shifts for C-2′ (δC 85.4) showed that an oxygen atom was also attached to C-2′ in addition to N-1′. Considering the fragments mentioned above required the presence of six oxygen atoms, while only five were present based on the molecular formula C27H37NO5, there was a need for the existence of benzene ring A. The methyl proton signal at H3-11 (δH 2.14, s) showed HMBC correlations to C-4a (δC 116.9), and C-6 (δC 149.0) substituted at C-3 (δC 70.7) and C-4 (δC 37.4), respectively, were deduced according to the correlations of H-9 (δH 1.34, d, 6.3)/H-3 and H-10/H-4 (δH 2.82, p, 6.8) in the 1H-1H COSY spectrum, along with the HMBC correlations of H-9 with C-4 and H-10 with C-3.

The correlation from H-1 to H1-10 in the NOESY spectrum and the absence of correlation between H-1 and H2-9 implied that the relationship of H2-9 and H1-10 was trans. Meanwhile, since NOESY correlations for H-3′/H-4′β and H-1′/H-4′α were observed, the trans relationship between H-1 and H-3′ also was considered. On the basis of the observation of the NOESY correlations of H-2′/H-3′ and H-2′/H-5′β (Figure 4), the cis relationship between H-2′ and H-3′ was deduced. In conclusion, the five sp3 methine protons in the tetracyclic core of 1 were summarized as 1β, 3β, 4α, 2′α, and 3′α orientations. The absolute stereochemistry of the tetracyclic core was deduced by a comparison of the experimental circular dichroism (CD) spectrum of 1 with the simulated ECD spectra generated by the time-dependent density functional theory (TDDFT). Since the chiral center at C-7 had the property of being easy to rotate, simplified structures (1S,3R,4S,2′R,3′S)-1A and (1R,3S,4R,2′S,3′R)-1B were used for the ECD calculation (Figures 4 and 5). The experimental CD spectrum of 1 was nearly identical to the calculated ECD curve of (1R,3S,4R,2′S,3′R)-1B. Therefore, the absolute configurations at C-1, C-3, C-4, C-2′, and C-3′ were assigned as R, S, R, S, and R.

Perinadine C (2), a colorless solid, had the same molecular formula of C27H37NO5 as 1, based on the (+)-HR-ESI-MS ions at m/z 478.2574 [M + Na]+ (Figure S23). Analysis of the key 1H-1H COSY, HSQC, and HMBC spectra suggested that the planar structure of 2 was identical to those of 1. Similar to 1, compound 2 was also repeatedly isolated as a single peak by HPLC; an analysis of its NMR spectral data suggested a pair of epimers (Table 1). A comparison of the 1H and 13C NMR data (Table 1) with those of 1 showed that the chemical shift was similar except for small discrepancies at C-1, C-3, C-4, C-9, and C-10 in the 13C NMR spectrum, which showed that 2 was an isomer of 1 with different stereochemistry on the corresponding chiral center. Different from 1, the relationship between H-1 and H-3 was considered as a cis-configuration based on the NOESY correlations of H-1/H-2′ and H-3′/H-2′. However, the cross-peaks of δH 4.36/1.26 in the NOESY spectrum cannot be enough to determine the relative orientation of H-1/H-9/H-10 because, while H-1 and H-9 may be the cis-configuration, H-1 and H-10 may also be. Taking into account the biosynthesis, the trans-configuration between H-3 and H-4 was deduced to be the same as those in 1. At this time, there are four possible configurations of compound 2 that need to be considered, as follows: (1R, 3S, 4R, 2′R, 3′S)-2A, (1R, 3R, 4S, 2′R, 3′S)-2B, (1S, 3R, 4S, 2′S, 3′R)-2C, and (1S, 3S, 4R, 2′S, 3′R)-2D. Then, the computed ECD of the four configurations was performed separately. The absolute configurations of 2 at C-1, C-3, C-4, C-2′, and C-3′ were eventually determined as R, R, S, R, and S on the basis of the good agreement between calculated and the experimental ECD curves (Figure S5).

In addition, the isolated new compounds 1 and 2 were evaluated for their antimicrobial activities against four human pathogenic microbes and their anti-inflammatory activity. The results suggested that compounds 1 and 2 exhibited moderate inhibitory activity (32 and 64 μg/mL) against Bacillus subtilis, while no significant bioactivity was observed in the rest of the...
three pathogens (Table 2), and none of them exhibited the bioactivity in the \textit{in vivo} zebrafish anti-inflammatory assay.

Biogenetically, perinadine B (1) and perinadine C (2) were presumably derived from citrinin (a well-known mycotoxin) and scalusamide B (a pyrrolidine alkaloid isolated from \textit{Penicillium brevicaespactum}) according to the proposal given by Sasaki et al.\textsuperscript{10} Moreover, scalusamide B may be derived from glutamic acid or proline and a pentaketide.\textsuperscript{12} Finally, perinadines B and C (1 and 2) were generated via an intermolecular cyclization, separately.

\section*{CONCLUSION}

In this study, two new rare tetracyclic alkaloids, namely, perinadines B and C (1 and 2), were isolated from the sponge-associated fungus \textit{Aspergillus} sp. On a skeleton, they are the first reported for derivatives of a rare type of tetracyclic alkaloids.
Table 2. Antibacterial Activity of Compounds 1 and 2

| compounds | MIC (μg/mL) |
|-----------|------------|
|           | B. subtilis | S. aureus | E. coli | P. aeruginosa |
| perinadine B (1) | 32         | >256      | >256    | >256         |
| perinadine C (2) | 64         | >256      | >256    | >256         |
| Gentamicin*     | 0.31        | 5         | 2.5     | 10           |

*Gentamicin was used as positive control in an antibacterial activity assay.

alkaloids—perinadine A. The isolation process was driven by a semiautomatic workflow composed of molecular networking, NAP, and a substructure-informed MS2LDA tool. Given the bioactivity of perinadine A and scalusamide A, the antibacterial activity of perinadines B and C were close with them, and consequently, the activity against bacteria of these perinadine classes may actually be responsible by the substructure scalusamide A.

## MATERIALS AND METHODS

### General Experimental Procedures.

Optical rotations were measured with a Jasco P-2000 digital polarimeter. UV spectra were obtained on a NADE Evolution 201 spectrophotometer. IR spectra were obtained with a Nicolet iS5 spectrometer using KBr pellets. ECD data were measured on a Circular Dichroism Spectrometer (JASCO J-810, Jasco Inc.). NMR spectra were obtained at room temperature on a Varian 600 MHz instrument, using tetramethylsilane (TMS) as internal standard. HRESIMS spectra were recorded on an Agilent 6545 HPLC Q-TOF mass spectrometer. Column chromatography (CC) was performed with the following chromatographic substrates: silica gel (200 mesh, Amersham Biosciences). Medium-pressure liquid chromatography (MPLC) was performed on FLEXA Purification System using an ODS column. Semipreparative HPLC was run on an Agilent HPLC 1260 Infinity instrument equipped with a 1260 DAD detector using a C18 column (NanoChrom, 10 × 250 mm, 5 μm, China).

### Fungal Material.

The fungal strain Aspergillus sp. LS116 in this work was isolated from a marine sponge Haliclona sp. collected in Lingshui, Hainan Province, China, and was identified by an Internal Transcribed Spacer (ITS) rDNA sequence. An analysis of the ITS rDNA (GenBank ID: FJ864703) by a BLAST database screening provided a 99% similarity to Aspergillus versicolor. The fungal strain LS116 was identified eventually as Aspergillus sp. With the universal primers ITS1 (5′-TCC GTA GGT GAA CCT GGC G-3′) and ITS4 (5′-TCC TCC GCT TAT GAT TGA TAT GC-3′) in the reaction system, a polymerase chain reaction (PCR) was conducted, and the DNA fragment of the fungal ITS regions was amplified. Then, the verified PCR product based on gel electrophoresis was sequenced on equipment from Sangon Biotech Co., Ltd. This fungal strain was preserved at −80 °C in a 15% (v/v) glycerol aqueous solution. The voucher specimen was deposited at the College of Food and Pharmaceutical Sciences, Ningbo University.

### Fermentation and Extraction.

The seed medium PDB (consisting of 8.0 g of potato extract, 20 g of dextrose, 35 g of artificial sea salt, and 1000 mL of distilled H₂O) in 500 mL Erlenmeyer flasks (200 mL/flask) was inoculated with the strain LS 116 and incubated at 28 °C for 3 d on a rotating shaker with 180 rpm. Subsequently, the 100 × 1000 mL flask with rice medium (80 g of rice, 35 g of sea salt, 120 mL of distilled water for each flask) were inoculated with a 10% seed solution. A large-scale fermentation was performed at 28 °C for 40 d. Finally, the fermentation-finished broth was extracted three times with ethyl acetate (EtOAc) to afford 36 g of crude extract.

### Isolation and Purification.

The crude extract was analyzed by LC-MS/MS. Elution was conducted with a mobile phase consisting of water + 0.1% formic acid (A) and CH₃CN + 0.1% formic acid (B), following the gradient from 10% to 100% B in 30 min, maintaining 100% B for 10 min, then gradient from 10% to 100% B in 0.1 min and maintaining 100% B for 5 min at a flow rate of 0.7 mL/min. Samples were dissolved in MeOH with 1 mg/mL. A 5 μL aliquot of the crude sample was injected. The samples (5.0 μL of 1 mg/mL crude extract were injected) were analyzed in the data-dependent acquisition (DDA) mode. The full mass spectrometry (MS) survey scan was performed with positive electrospray ionization (ESI) mode in the range of 100–1700 Da; then the five most intense ions were further scanned for MS/MS fragmentation spectra with the fixed collision energy (CE) at 40 ev. The MS/MS data were converted to .mzXML files using MSConvert software. The converted MS/MS file was submitted to the GNPS platform for molecular networking for dereplication (https://gnps.ucsd.edu). With the help of the Cytoscape software, the nodes and tables file generated by the classic molecular networking workflow in GNPS (METABOLONICS-SNENTS-V2) were visualized to form molecular networking. Parameters for the molecular network generation were set as follows: both precursor mass and MS/MS fragment ion tolerance were set as 0.02 Da, minimum pairs cosine score 0.7, minimum matched fragment ions 6, minimum cluster size 2, network TopK 10. The spectral library searching was performed in the default parameter with analogues search.

In the NAP workflow, a MetFrag in silico fragment analysis was performed by matching the candidate structures with the exact mass filter of 5 ppm from the structural databases of DNP, GNPS, and SUPNAT. The molecular networking job on the GNPS and NAP annotation job are accessible with the following links: http://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=dd77856239848cfd0b32ae867d6c4b and https://proteomics2.ucsd.edu/ProteoSAFe/status.jsp?task= cda883b8e4b64c21b3243a6fe227a723.

In the MS2LDA unsupervised substructure annotation workflow, the fragmentation spectra were mined for co-occurring fragments on the MS2LDA Web application. The working parameters were set as follows: isolation_window (0.5), min_ms2_int (5000), n_its (1000), K (300), mz tol (5.0). The result of MS2LDA annotation is available at the following link: http://ms2lda.org/basicviz/summary/1691/.

Detailed separation steps are as follows: First, the organic extract (36 g) was separated to seven fractions (Fr.1–Fr.7) with the aid of vacuum liquid chromatography (VLC) on silica gel by gradient elution eluted with a mixture of petroleum ether/EtOAc (100:1 to 0:1, v/v). Then the Fr.3 was further separated by chromatography over a Sephadex LH-20 column eluting with CH₂OH and CH₃Cl₂ (1:1, v/v) to afford six subfractions (Fr.3.A–Fr.3.F). Subsequently, the subfraction Fr.3.B was further fractionated into 10 major fractions (Fr.3.B.1–Fr.3.B.10) through MPLC (MeOH/H₂O, 30–100%, v/v) with the reversed-phase ODS (Octadecylsil) substrates. Finally, Fr.3.B.6 was purified by semipreparative
HPLC with CH$_3$CN–H$_2$O (68:33, v/v) and flow 2 mL/min to yield 1 (2.0 mg, t$_R$ = 24 min) and 2 (5.0 mg, t$_R$ = 22 min).

**Perinidine B** (1). Colorless solid; [α]$_D^{25}$ +25 (c 1.0, MeOH); ECD (MeOH) $\lambda_{ext}$ (Δε) 219 (+8.51), 227 (+5.26), 235 (+6.07), 281 nm (−3.18); IR (KBr) $\nu_{max}$ 3290 (br), 2930, 2357, 2312, 1639, 1628, 1011 cm$^{-1}$; $^1$H NMR (CDCl$_3$, 600 MHz) and $^{13}$C NMR (CDCl$_3$, 150 MHz) data, see Table 1; HRESIMS m/z [M + Na]$^+$ peak at 478.2580 (calcd for C$_{27}$H$_{37}$NO$_5$Na, m/z 478.2569).

**Perinidine C** (2). Colorless solid; [α]$_D^{25}$ +31 (c 1.0, MeOH); ECD (MeOH) $\lambda_{ext}$ (Δε) 219 (−14.59), 251 (−0.02), 283 nm (−2.65); IR (KBr) $\nu_{max}$ 3290 (br), 2930, 2357, 2312, 1639, 1628, 1011 cm$^{-1}$; $^1$H NMR and $^{13}$C NMR data, see Table 1. HRESIMS m/z [M + Na]$^+$ peak at 478.2574 (calcd for C$_{25}$H$_{37}$NO$_5$Na, m/z 478.2569).

**ECD Calculations.** The relative configuration of compounds 1 and 2 was established through NOESY spectra and Chem3D simulations. Then a conformation search was performed of its simplified structures using Spartan software on the Merck molecular force field (MMFF) with an energy cutoff of 2 kcal/mol. The predominant conformers were optimized at the B3LYP/6-31G level using Gaussian 09. TDDFT calculations for the optimized conformers were performed at the B3LYP/6-31G level with the integral equation formalism polarizable continuum model (IEF-PCM) solvent model in MeOH. A Boltzmann distribution was used for weighting the ECD curves of simplified structures of compounds 1 and 2.

**Antibacterial Assay.** The antibacterial assays for determination of the minimum inhibitory concentration (MIC) were performed following the broth microdilution method according to a standard protocol with a slight modification. Staphylococcus aureus ATCC 6538, Bacillus subtilis JCM 1465, Escherichia coli JCM 1649, and Pseudomonas aeruginosa ATCC 10145 were used as the test strains for the antibacterial assay. In 96-well plates, a final volume of 100 µL/well of Mueller-Hinton broth as the basic medium with the compound at concentrations from 256 to 1 μg/mL along with the bacterial inoculum was added at turbidity of 5 × 10$^8$ CFU/mL. Gentamicin was used as a positive control. The plates were incubated overnight at 37 °C, and then we observed the plates, the MIC was determined as the smallest concentration in which bacteria cannot be observed.

**In Vivo Anti-Inflammatory Activity Assay.** This in vivo anti-inflammatory experiment used the same zebrafish model as that of the previously described protocol. The method was performed by monitoring the number of neutrophils in zebrafish, which are highly similar to mammals in morphology and physiology, and its embryo's optical transparency makes it possible for one to noninvasively obtain dynamic imaging of the inflammation in vivo. Indomethacin was used as a positive control.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00402.

Nodes identified as alkaloids in the first ranked candidate, analysis result of parent ions, MS/MS spectra, NMR spectra, IR spectra, UV spectra, Boltzmann populations of low-energy conformers (PDF)

**AUTHOR INFORMATION**

**Corresponding Authors**

Lijian Ding – Li Dak Sum Yip Yio Chin Kenneth Li Marine Biopharmaceutical Research Center, College of Food and Pharmaceutical Sciences, Ningbo University, Ningbo 315832 Zhejiang, People’s Republic of China; orcid.org/0000-0002-2259-9830; Email: dinglijian@nbu.edu.cn

Shan He – Li Dak Sum Yip Yio Chin Kenneth Li Marine Biopharmaceutical Research Center, College of Food and Pharmaceutical Sciences, Ningbo University, Ningbo 315832 Zhejiang, People’s Republic of China; Ningbo Institute of Marine Medicine, Peking University, Ningbo 315800, People’s Republic of China; Email: shanhe@nbu.edu.cn

**Authors**

Yang Liu – Li Dak Sum Yip Yio Chin Kenneth Li Marine Biopharmaceutical Research Center, College of Food and Pharmaceutical Sciences, Ningbo University, Ningbo 315832 Zhejiang, People’s Republic of China

Yutong Shi – Li Dak Sum Yip Yio Chin Kenneth Li Marine Biopharmaceutical Research Center, College of Food and Pharmaceutical Sciences, Ningbo University, Ningbo 315832 Zhejiang, People’s Republic of China

Xiaojun Yan – Li Dak Sum Yip Yio Chin Kenneth Li Marine Biopharmaceutical Research Center, College of Food and Pharmaceutical Sciences, Ningbo University, Ningbo 315832 Zhejiang, People’s Republic of China

Bin Wu – Ocean College, Zhejiang University, Hangzhou 310058, People’s Republic of China; orcid.org/0000-0002-7638-2696

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c00402

**Notes**

The authors declare no competing financial interest.

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