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Citation: Zhongyu Lu, Xiao Zhu, Xin Zhou, Xiaobin Li, Xiao Han, Guoqiang Li, Clavukellines AN: dolabellane diterpenes from the soft coral *Clavularia koellikeri*, Chinese Journal of Natural Medicines, 2026, 24(9), 1123−1128. doi: 10.1016/S1875−5364(26)61209−5.

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Original article

Clavukellines A–N: dolabellane diterpenes from the soft coral *Clavularia koellikeri*

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ARTICLE INFO

Article history:
Received 18 October 2025
Revised 1 February 2026
Accepted 9 February 2026
Available online 20 September 2026

Keywords:
Clavularia koellikeri
Dolabellane diterpenes
Absolute configurations
Anti-inflammatory activity
Antithrombotic activity

ABSTRACT

Chemical investigation of the soft coral *Clavularia koellikeri* led to the isolation of fourteen new diterpenoid derivatives clavukellines A–N (**1–14**) featuring the dolabellane-type skeleton. Structural characterization of these compounds was accomplished through an integrated analytical strategy combining NMR spectroscopy and HR-ESI-MS. Absolute configurations were unambiguously assigned using ECD spectral simulation and DP4 + probability analysis. Preliminary pharmacological evaluation indicated that compounds **8** and **13** exhibited anti-inflammatory activity, while compounds **1–14** showed antithrombotic activity. This study reveals promising anti-inflammatory and antithrombotic potentials that warrant further investigation.

1. Introduction

Marine soft corals have proven to be a treasure trove of novel bioactive compounds, producing an array of structurally complex secondary metabolites with significant pharmacological potential [1-3]. Among these marine invertebrates, members of the *Clavularia* [4-5] (Octocorallia, Alcyonacea, Clavulariidae) have attracted particular attention from natural product researchers due to their exceptional capacity to biosynthesize terpenoid derivatives [6-9]. Notably, this genus has been found to produce distinctive dolabellane diterpenoids, recognized by their characteristic fused 5-11 bicyclic framework [10]. Originally discovered in sea hares of the *Dolabella* genus, these architecturally intriguing molecules have shown impressive bioactivity profiles, exhibiting promising cytotoxic properties along with notable antimicrobial and antiviral effects [11-16]. Such diverse biological activities have positioned dolabellanes as valuable lead compounds for pharmaceutical development and synthetic exploration [17-19].

Building upon our ongoing research into marine natural products from the South China Sea, we focused our attention on specimens of *Clavularia koellikeri* collected from the Xisha Islands coral reef environment [20-21]. Our initial phytochemical screening led to the discovery of fourteen novel diterpenoid derivatives clavukellines A–N (**1–14**) possessing the characteristic dolabellane skeleton (Fig. 1). Notably, while compounds **8** and **13** showed anti-inflammatory activity, all isolated compounds (**1–14**) exhibited antithrombotic activity. These findings demonstrate the pharmacological potential and structural versatility of these marine metabolites [22-23].

Structurally, compounds **3**, **5–11** are characterized by oxidative modification through an epoxide group [11], whereas compounds **2, 13**, and **14** feature a tricyclic rearrangement of the classical dolabellane framework [10].

2. Results and discussion

Clavukelline A (**1**) was isolated as colorless oil. HR-ESI-MS established its molecular formula as $C_{22}H_{32}O_3$, indicating seven sites of unsaturation. The ^{13}C NMR spectrum displayed twenty-two carbon resonances, classified as six methyls, five methylenes, four methines, seven quaternary carbons, including one ketone carbonyl (δ_C 203.2), one ester carbonyl (δ_C 170.3) and methyl (δ_C 21.4). The ^1H NMR spectrum revealed characteristic signals including: two olefinic protons at δ_H 6.07 (1H, s) and δ_H 5.34 (1H, d, J = 9.0 Hz), an acetoxy-bearing methine at δ_H 5.26 (1H, d, J = 10.0 Hz), and four olefinic methyl groups at δ_H 1.66 (3H, s), δ_H 1.69 (3H, s), δ_H 1.85 (3H, s) and 1.92 (3H, s). Structural analysis indicated that five degrees of unsaturation were accounted for by three olefinic bonds and two carbonyl groups, requiring a dicyclic framework.

Key COSY correlations were H_2-3/H_2-2, H_3-16/H-5, H-7/H_3-17, H-10/H-11, H_2-13/H_2-14. The HMBC correlations of H_3-19 with C-18, H_3-17 with C-6, C-7, C-8, C-9, H_3-16 with C-3, C-4, C-5, H_3-15 with C-1, C-2, C-11, C-15, H_2-14 with C-1, C-2, C-11, C-12, C-13, H_2-13 with C-11, C-12, H-11 with C-2, C-12, C-13, C-14, H-10 with C-7, C-8, C-11, C-12, H-5 with C-6 and H-2 with C-3, C-4, C-11, connected the fragments into dicyclic skeleton. Furthermore, a propyl group moiety was confirmed by HMBC of H_3-22 (δ_H 1.69) with C-21, C-20, C-12, combined with the H_3-21 (δ_H 1.66) with C-22, C-20, C-12 (Fig. 2).

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https://doi.org/10.1016/S1875-5364(26)61209-5
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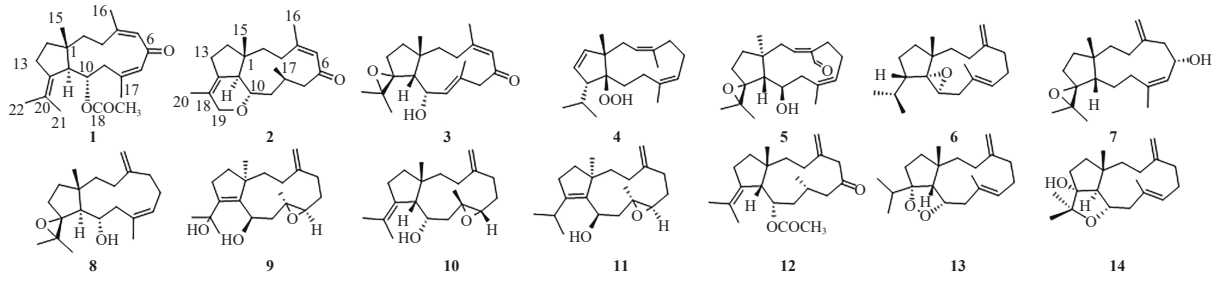


Fig. 1 Structures of clavukellines A–N (**1**–**14**).

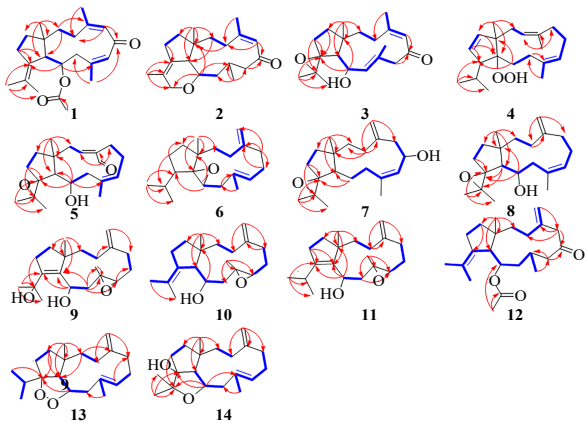


Fig. 2 Key COSY (bold lines) and HMBC (arrows) correlations of clavukellines A–N (**1**–**14**).

The NOESY correlations from H-5 to H_3-16, H-7 to H_3-17, H-10 to H_3-15, suggested the 4*Z*,7*Z*,1*S**,10*S**-configurations (Fig. 3). To determine the relative configurations of all stereogenic centers in **1**, the NMR shifts of 1*S**,10*S**,11*S**- and 1*S**,10*S**,11*R**-configurations were calculated based on the gauge independent atomic orbital (GIAO) method at the PCM/b3lyp/6-311 + G- (d,p) level, and DP4 + probability analyses were performed (100% for 1*S**,10*S**,11*R**-configurations; Fig. S1), which indicated the 1*S**,10*S**,11*R**-configurations. The absolute configuration of 1*S*,10*S*,11*R* was suggested by the ECD calculations using the time-dependent density functional theory (TDDFT) method at the RB3LYP/DGDZVP level (Fig. 4) [24-25].

Clavukelline B (**2**) was isolated as yellow oil with a molecular formula of $C_{20}H_{30}O_2$ as determined by HR-ESI-MS. Compared with compound **1**, C-19 (δ_C 67.4) was connected with C-10 (δ_C 76.6) through an oxygen atom. The Δ^7 was transferred into a single bond. NOESY correlations (H-10/H_3-15, H-17; H-11/H-8; H_3-16/H-5) established the 4*Z**,1*S**,8*R**,10*S**,11*R** relative configuration (Fig. 3), while ECD calculations (Fig. 4) confirmed the absolute configuration as 1*S*,8*R*,10*S*,11*R*.

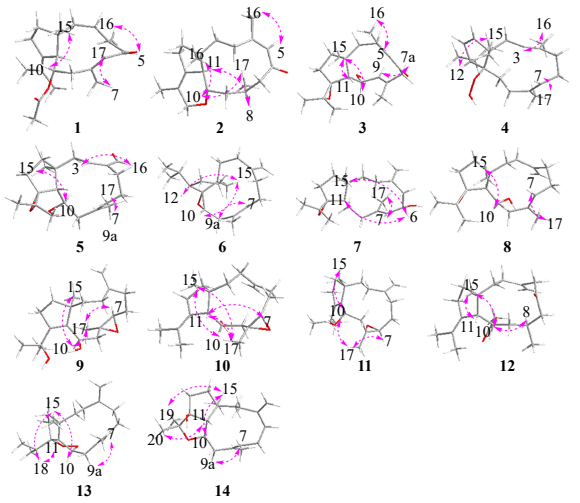


Fig. 3 Key NOESY correlations of clavukellines A–N (**1**–**14**).

Clavukelline C (**3**) was obtained as a colorless oil with the molecular formula $C_{20}H_{30}O_3$ (HR-ESI-MS), identical to compound **1**. NMR comparison showed three key structural modifications: the Δ^{12} double bond was oxidized to form an epoxide ring, the C-10 ester was reduced to a hydroxyl group (δ_C 70.3), and the Δ^7 position shifted to Δ^8. NOESY correlations (H_3-15/H-10, H-11; H-5/H_3-16, H_a-7/H-9) established the 4*Z**,8*E**,1*S**,10*S**,11*R** relative configuration (Fig. 3), while ECD calculations (Fig. 4) and DP4 + analysis (Fig. S2) confirmed the absolute configuration as 1*S*,10*S*,11*R*,12*R*.

Clavukelline D (**4**) was isolated as yellow oil, and its molecular formula was established as $C_{20}H_{32}O_2$ by HR-ESI-MS. Comparative NMR analysis with compound **1** revealed three principal structural differences, the first difference was a Δ^{13} double bond in **4** instead of a Δ^{12} double bond in **1**; the second difference was the carbonyl at C-6 in **1** was eliminated in **4**; the last difference was the elimination of ester group at C-10 in **1** and the formation of peroxy group at C-11(δ_C 73.9) in **4**, which was confirmed by molecular formula. The NOESY correlations of H_3-16/H-3, H_3-15/H-12, H-7/H_3-17, proved the 3*Z*,7*Z*,1*S**,12*R**-configurations (Fig. 3). Subsequently, DP4 + probability analysis showed a 100% match for the 1*S**,11*S**,12*R** diastereomer (Fig. S3). Finally, the absolute configuration was assigned as 1*S*,11*S*,12*R* based on ECD calculations.

Clavukelline E (**5**) was obtained as white powder, with HR-ESI-MS analysis confirming its molecular formula as $C_{20}H_{30}O_3$. Comparative NMR analysis revealed a close structural similarity to compound **3**, with the following key modifications. The first one was a Δ^8 bond in **3** was shifted to a Δ^7 bond in **5**, the second one was the carbonyl at C-6 in **1** was eliminated in **5**, finally, the C-16 methyl group was oxidized to an aldehyde group, evidenced by characteristic deshielded resonances at δ_C 195.6/δ_H 9.39 (d, *J* = 1.2 Hz). The NOESY showed H_3-16/H-3, H_3-17/H-7, H_3-15/H-10 exist correlation, these hydrogen in the same side, proved the 3*E*,7*Z*,1*S**,10*R**-configurations (Fig. 3), the configurations can be judged by DP4 + (1*S**, 10*R**,11*R**,12*R**-configurations 100%; Fig. S4). Finally, the 1*S*,10*R*,11*R*,12*R*-configuration was ensured by ECD calculations (Fig. 4).

Clavukelline F (**6**) was isolated as yellow oil, showing the molecular formula $C_{20}H_{32}O$ as determined by HR-ESI-MS. Its NMR spectra exhibited striking similarity to **5**, with the distinction being the positional shift of the epoxide ring from C-12/18 in **5** to C-10/11 in **6** and the aldehyde group at C-16 in **5** changed into terminal double-bond methylene group (δ_C 109.4) in **6**. The relative configuration of **6** was confirmed by analysis of the NOESY spectrum. DP4 + (Fig. S5) and ECD (Fig. 4) calculation confirmed the 7*E*,1*R*,10*S*,11*R*,12*S*-configuration.

Clavukelline G (**7**) was obtained as colorless oil, sharing the molecular formula $C_{20}H_{32}O_2$ as determined by HR-ESI-MS. Its NMR spectra exhibited striking similarity to **5** and **6**. The posi-

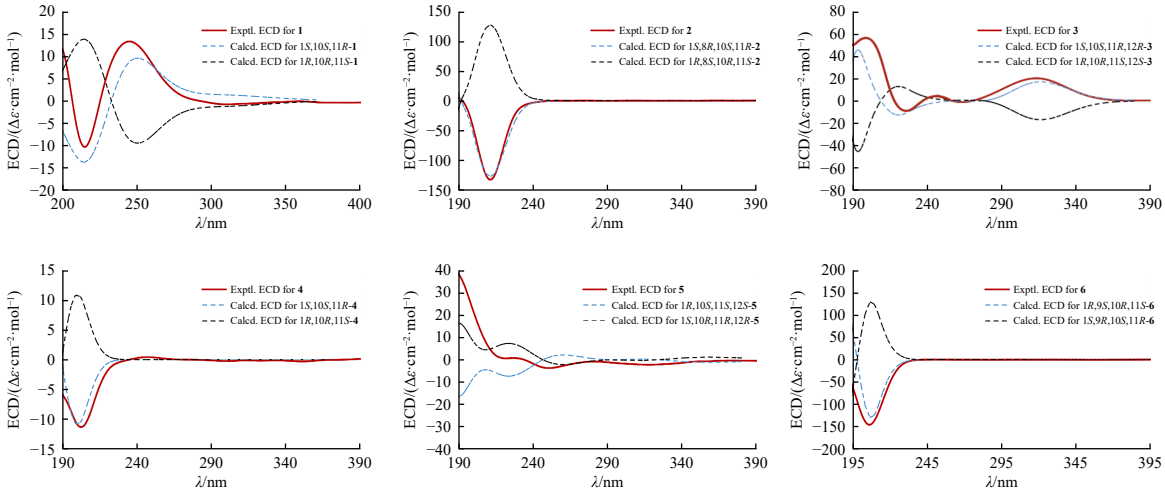


Fig. 4 Experimental and calculated ECD spectra of clavukellines A–F (**1**–**6**).

tion of epoxide ring was same with **5**. The position of terminal double-bond methylene group was the same as **6**. The only difference between them was -OH had shifted from C-10 in **5** to C-6 (δ_C 70.7) in **7**. The relative configuration of **7** was confirmed by analysis of the NOESY spectrum. DP4 + (Fig. S6) and ECD (Fig. S7) calculation confirmed the 7*Z*,1*S*,6*S*,10*S*,11*R*-configuration.

Clavukelline H (**8**) was obtained as a colorless oil with molecular formula $C_{20}H_{32}O_2$ (HR-ESI-MS, *m/z* 305.2475 $[M + H]^+$, calcd. 305.2468). Structural comparison with **7** showed a single modification involving hydroxyl group migration from C-6 to C-10 (δ_C 70.7). The NOESY correlations of H_3-17/H-7 and H_3-15/H-10 confirmed the 7*Z*,1*S**,10*S** (Fig. 3), while DP4 + (Fig. S8) analysis and ECD calculations (Fig. S9) confirmed the 1*S*,10*S*,11*S*,12*R*-configuration.

Clavukelline I (**9**) was obtained as yellow oil. HR-ESI-MS analysis established its molecular formula as $C_{20}H_{32}O_2$ (5 degrees of unsaturation). Analysis of the 1D and 2D NMR spectra allowed the assignment of a terminal alkene at δ_C 110.7/ δ_H 4.78 (s), which was positioned based on HMBC correlations from H_2-16 to C-3, 4, 5 (Fig. 2). Compared with **8**, the position of epoxide ring from C-12/18 to C-7/8, the Δ^7 changed into Δ^{11}, and an OH was formed at C-18 (δ_C 73.0). The NOESY correlations of H-10/H_3-15, H_3-17; H_3-17/H-7 indicated the 1*R**,7*R**,8*S**,9*R**-configurations (Fig. 3). Finally, the 1*R*,7*R*,8*S*,9*R*- configuration was confirmed by the ECD calculation.

Clavukelline J (**10**) was obtained as a colorless oil. Its molecular formula was determined to be $C_{20}H_{32}O_2$ by HR-ESI-MS. Structural comparison with **9** revealed a double bond migration from Δ^{11} to Δ^{12}. With the exception of C-11, the configuration of **10** was the opposite of that in **9**. This was supported by NOESY correlations (H_3-15/H-10, H_3-17; H-11/H_3-17, H-7; Fig. 3), which was similar to **9**, through ECD (Fig. S11) calculation can confirmed the configuration of **10** was 1*S*,7*S*,8*R*,10*S*,11*S*.

Clavukelline K (**11**) was isolated as colorless oil, with HR-ESI-MS establishing its molecular formula as $C_{20}H_{32}O_2$. This compound shares close structural relationships with both **9** and **10**. Specifically, compared to **9**, it lacks the hydroxyl group at C-18. Compared to **10**, a double bond was transferred. The absolute configuration was unequivocally assigned as 1*R*,7*R*,8*S*,10*R* based on NOESY and ECD calculations (Fig. S12).

Clavukelline L (**12**) was identified as a colorless oil. HR-ESI-MS established the molecular formula was $C_{22}H_{34}O_3$. The structure was similar to **1** except Δ^7 was removed, NOESY correlations (H_3-15/H-10, H-11; H-10/H-8; Fig. 3) confirmed the relative configurations of 1*S**,8*S**,10*S**,11*S**. The 1*S*,8*S*,10*S*,11*S*-configuration was assigned by the ECD calculation (Fig. S13).

Clavukelline M (**13**) was showed as yellow oil. HR-ESI-MS confirmed the molecular formula was $C_{20}H_{32}O_2$. The structure of **13** was similar to **6**, but features a peroxy bridge between C-10 and C-12. The 7*E*,1*S*,10*S*,11*R*,12*S* configuration was established through ECD calculations (Fig. S14).

Clavukelline N (**14**) was identified as colorless oil, gave the molecular formula $C_{20}H_{32}O_2$ as deduced from the HR-ESI-MS. Comprehensive analysis of the ^1H and ^{13}C NMR data, in conjunction with 2D NMR spectra, confirmed that the structure of **14** is similar to that of **13**. The differences between them were the shift and kind at C-18. Based on the integration of NOESY, DP4 + (Fig. S15), ECD calculation (Fig. S16), the configuration of **14** was 7*E*,1*S*,10*S*,11*S*,12*R*.

Anti-inflammatory activity of compounds **8**, **13** was evaluated in transgenic fluorescent zebrafish (*Tg:zlyz-EGFP*) induced by $CuSO_4$ (Fig. 5).

Antithrombotic activity of compounds **1**–**14** was evaluated in wild-type AB zebrafish induced by arachidonic acid. Compounds **1**–**4**, **7**–**10**, **12**–**13** exhibited activities superior to that of the positive control (Fig. 6).

3. Conclusion

Fourteen new dolabellane-type diterpenoid derivatives were isolated from the soft coral *Clavularia koellikeri*. All compounds showed antithrombotic activity and compounds **8** and **13** exhibited anti-inflammatory activity.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured using a Jasco P-1020 polarimeter with CD spectra recorded on a Jasco J-815 spectropolarimeter. NMR spectra were acquired on a JEOL JNM-ECP600 spectrometer (600 MHz for ^1H, 150 MHz for ^{13}C) using $CDCl_3$ as solvent (δ_H 7.26, δ_C 77.2). HRESIMS were obtained in positive ion mode using a Thermo LTQ-Orbitrap mass spectrometer. HPLC separations were performed on an Agilent 1100 system equipped with a DAD detector and YMC-packed C18 column (5 μm, 250 × 10 mm) at 2 mL·min^{-1} flow rate. Column chromatography employed 200–400 mesh silica gel with TLC monitoring on GF_{254} plates using UV visualization (254/365 nm) and 10% ethanolic H_2SO_4 staining.

4.2. Soft coral material

The investigated specimen of *Clavularia koellikeri* was ob-

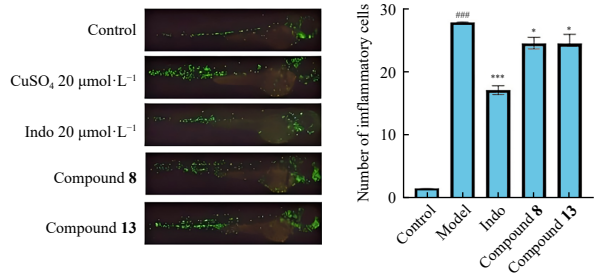


Fig. 5 Anti-inflammatory assays of compounds **8** and **13**. Data are expressed as mean ± SEM (n = 30). $^{###}P < 0.001$*vs* the blank control group ; * indicates a highly significant difference between the positive drug group/sample group and the $CuSO_4$ model group ($P < 0.01$); *** indicates an extremely significant difference between the positive drug group/sample group and the $CuSO_4$ model group ($P < 0.001$); Statistical significance was determined by one-way ANOVA. Compared group was indomethacin control group.

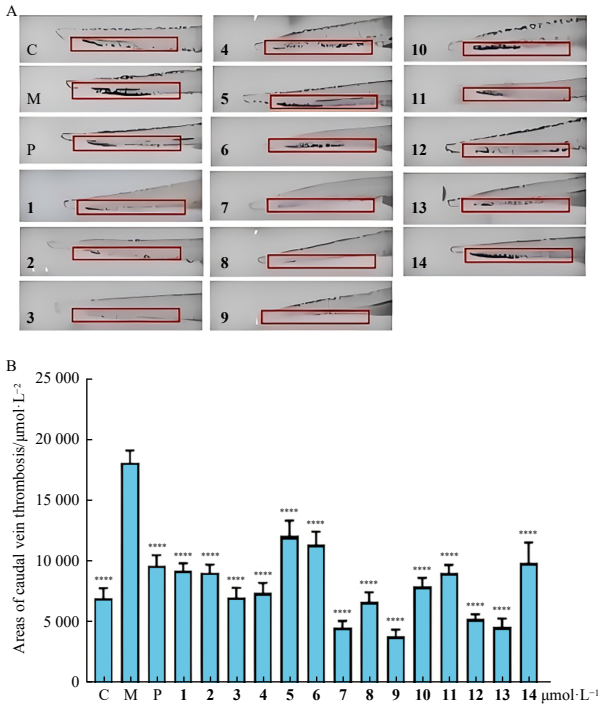


Fig. 6 Antithrombotic assays of compounds **1**–**14**. The experimental data are expressed as mean ± SEM (n = 30). Compared with the model group, $^{****}P < 0.0001$, indicating that the difference in tail vein staining of zebrafish was statistically significant. Statistical significance was determined by one-way ANOVA. Compared group was aspirin positive control group.

tained from Xisha Islands waters in the South China Sea during marine organism sampling. Species authentication was conducted through morphological examination by the author Dr. Xiubao Li (Hainan University), with comparison to authenticated reference samples. The preserved specimen (voucher code: xs-sh-28) is currently maintained at −20 °C in the marine natural products repository at the School of Medicine and Pharmacy, Ocean University of China, Qingdao, China. Detailed photographs of the coral are provided in the Supporting Information.

4.3. Extraction and isolation

The biomass of *Clavularia koellikeri* (9.7 kg, wet weight) was homogenized and extracted with methanol (5 × 25 L) under ultrasonication. The concentrated crude extract (120.0 g) was desalted and initially fractionated on a silica gel column. Elution with stepwise gradients of PE/acetone and dichloromethane/methanol yielded six fractions (A–E). Based on TLC and LC–MS analysis, fraction E (eluted with PE/acetone 5∶1) was prioritized for its diverse metabolite profile.

Fraction C (8.6 g) underwent primary separation via silica gel column chromatography (300–400 mesh) with PE-acetone gradient elution (100∶1 to 1∶1, *V*/*V*; total eluent volume 5 L), yielding four subfractions (C1–C4). The diterpenoid-rich C4 (3.6 g) was further purified by MPLC (C_{18} column, MeOH-H_2O 90∶10, *V*/*V*; flow rate 7.0 mL·min^{-1}) to obtain five fractions (C41–C45). Final isolation of C43 (0.5 g) using semipreparative HPLC (YMC-Packed C18 column, 5 μm, 250 × 10 mm, MeOH–H_2O 80∶20, *V*/*V*; flow rate 2.0 mL·min^{-1}; UV detection at 210 nm) afforded compounds **12** (4.7 mg) and **13** (11.5 mg).

Fraction D (18.5 g) was first chromatographed on silica gel (200–300 mesh) with PE-acetone (50∶1 to 1∶1, *V*/*V*; total eluent volume: 10 L) and through TLC (silica gel GF_{254}, visualized with vanillin-H_2SO_4) to give five subfractions (D1-D5). The LC-MS active D2 (9.2 g) was processed by MPLC (80∶20 *V*/*V*; flow rate: 7 mL·min^{-1}; UV detection at 210 nm) to obtain seven fractions (D21-D27). Further purification of D22-D25 *via* HPLC (YMC-Packed C-18 column, 5 μm, 250 × 10 mm; isocratic ACN–H_2O 80∶20, *V*/*V*; 2.0 mL·min^{-1}; UV 210 nm; injection volume: 200 μL) isolated compounds **6** (898 mg), **9** (10.9 mg), **11** (3.6 mg) and **14** (13.4 mg).

Fraction E (15.6 g) underwent silica gel chromatography (PE-acetone 20∶1 to 1∶1, *V*/*V*) to yield five subfractions (E1–E5). MPLC purification of E2 (8.6 g) and E3 (5.0 g) with MeOH–H_2O (70∶30, *V*/*V*, flow rate: 7 mL·min^{-1}; UV detection at 210 nm) produced fractions E21-E28 and E31-E37, respectively. HPLC separation of E23/E28 (ODS column, 5 μm, 250 × 10 mm; isocratic ACN/H_2O, 75∶25, *V*/*V*; 2.0 mL·min^{-1}, UV 210 nm) gave compound **8** (11.4 mg), while E33–E36 processing (same column; ACN–H_2O 70∶30, *V*/*V*) afforded compounds **1** (3.4 mg), **2** (9.8 mg), **3** (4.5 mg), **4** (13.5 mg), **5** (1.9 mg), **7** (7.2 mg) and **10** (6.0 mg).

4.4. Identification of compounds

Clavukelline A (**1**): colorless oil; $[\alpha]_D^{20}$ 13.6 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 212 (−8.52), 241 (12.74) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table 1; HR-ESI-MS *m/z* 367.2236 $[M + Na]^+$ (Calcd. for $C_{22}H_{32}O_3Na$, 367.2244).

Clavukelline B (**2**): yellow oil; $[\alpha]_D^{20}$ −4.2 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 210 (−148.02) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table 1; HR-ESI-MS *m/z* 303.2313 $[M + H]^+$ (Calcd. for $C_{20}H_{31}O_2$, 303.2319).

Clavukelline C (**3**): colorless oil; $[\alpha]_D^{20}$ 118.0 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 200 (58.95), 322 (16.93) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table 1; HR-ESI-MS *m/z* 341.2086 $[M + Na]^+$ (Calcd. for $C_{20}H_{30}O_3Na$, 341.2087).

Clavukelline D (**4**): yellow oil; $[\alpha]_D^{20}$ 10.2 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 200 (−10.52) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table 1; HR-ESI-MS *m/z* 305.2473 $[M + H]^+$ (Calcd. for $C_{20}H_{33}O_2$, 305.2475).

Clavukelline E (**5**): white powder; $[\alpha]_D^{20}$ −64.4 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 190 (38.62), 228 (0.83), 258 (−4.05) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table 1; HR-ESI-MS *m/z* 319.2261 $[M + H]^+$ (Calcd. for $C_{20}H_{31}O_3$, 319.2268).

Clavukelline F (**6**): yellow oil; $[\alpha]_D^{20}$ −182.3 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 205 (−138.62) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table S1; HR-ESI-MS *m/z* 289.2522 $[M + H]^+$ (Calcd. for $C_{20}H_{33}O$, 289.2526).

Clavukelline G (**7**): colorless oil; $[\alpha]_D^{20}$ 2.8 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 210 (−37.91) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table S1; HR-ESI-MS *m/z* 305.2471 $[M + H]^+$ (Calcd. for $C_{20}H_{33}O_2$, 305.2475).

Clavukelline H (**8**): colorless oil; $[\alpha]_D^{20}$ −38.6 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 206 (−82.21) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table S1; HR-ESI-MS *m/z* 305.2468 $[M + H]^+$ (Calcd. for $C_{20}H_{33}O_2$, 305.2475).

Table 1 ^{13}C NMR and ^{1}H NMR Data of clavukellines A–E (**1**–**5**) in $CDCl_3$.

No.	1		2		3		4		5	
	δ_C, type	δ_H (*J*)	δ_C, type	δ_H (*J*)	δ_C, type	δ_H (*J*)	δ_C, type	δ_H (*J*)	δ_C, type	δ_H (*J*)
1	44.2, C		43.6, C		43.4, C		23.5, C		44.4, C	
2	38.7, CH_2	1.39, m	37.4, CH_2	1.88, m; 1.42, m	41.6, CH_2	1.67, m; 1.55, m	44.0, CH_2	1.86, m; 1.58, m	41.6, CH_2	2.55, m; 2.03, m
3	28.4, CH_2	2.69, m; 2.32, m	28.7, CH_2	2.34, m; 2.16, m	36.5, CH_2	2.46, m; 2.11, m	127.8, CH	5.21, t (8.0)	152.9, CH	6.56, dd (18.0, 16.4)
4	151.6, C		148.8, C		152.4, C		132.9, C		144.2, C	
5	129.2, CH	6.07, s	130.2, CH	5.92, t (1.4)	129.3, CH	6.28, s	39.0, CH_2	2.07, m	24.3, CH_2	2.58, m; 2.05, m
6	203.2, C		207.1, C		196.8, C		23.7, CH_2	2.22, m; 2.08, m	45.1, CH_2	2.31, m
7	128.8, CH	5.34, d (9.0)	49.3, CH_2	2.45, m	46.5, CH	3.71, dd (10.4, 9.8)	124.9, CH	5.03, m	126.8, CH	5.22, m
8	131.6, C		32.3, CH	2.13, m	128.0, C		132.6, C		134.1, C	
9	47.0, CH_2	3.55, d (17.0); 2.91, d (17.0)	41.9, CH_2	1.81, m; 1.34, m	131.8, CH_2	5.71, d (10.0)	36.9, CH_2	2.01, m	25.8, CH_2	2.30, m; 2.05, m
10	73.7, CH	5.26, d (10.0)	76.6, CH	3.35, d (10.0)	70.3, CH	4.76, d (10.4)	28.0, CH_2	1.67, m; 1.21, m	71.5, CH	3.76, dd (12.0, 10.4)
11	48.8, CH	2.65, s	54.6, CH	1.82, m	48.1, CH	1.66, m	73.9, C		56.7, CH	2.37, m
12	136.0, C		132.8, C		74.4, C		46.1, CH	1.62, m	90.0, C	
13	28.8, CH_2	2.22, m; 2.17, m	24.3, CH_2	2.26, m; 2.16, m	28.5, CH_2	2.05, m; 1.64, m	127.0, CH	5.28, dd (16.0, 14.0)	37.2, CH_2	2.07, m; 1.67, m
14	42.0, CH_2	1.49, m; 1.28, m	39.0, CH_2	1.48, m; 1.36, m	40.9, CH_2	1.64, m; 1.25, m	138.8, CH	5.55, d (12.0)	34.9, CH_2	2.07, m; 1.67, m
15	24.0, CH_3	1.02, s	17.7, CH_3	0.69, s	22.0, CH_3	1.44, s	29.3, CH_3	1.28, s	26.2, CH_3	1.16, s
16	23.6, CH_3	1.85, s	22.9, CH_3	1.81, s	17.2, CH_3	1.81, s	15.1, CH_3	1.58, s	195.6, CH	9.39, d (1.2)
17	27.0, CH_3	1.92, s	22.9, CH_3	1.10, d (6.8)	25.8, CH_3	1.82, s	14.8, CH_3	1.52, s	18.7, CH_3	1.57, s
18	170.3, C		124.0, C		61.9, C		33.0, CH	1.48, m	74.9, C	
19	21.4, CH_3	1.97, s	67.4, CH_2	3.82, dd (16.0, 14.0)	20.9, CH_3	1.25, s	19.4, CH_3	0.80, d (8.0)	27.0, CH_3	1.44, s
20	124.4, C		14.4, CH_3	1.54, s	21.4, CH_3	1.34, s	20.7, CH_3	0.80, d (8.0)	26.8, CH_3	1.38, s
21	21.5, CH_3	1.66, s								
22	22.9, CH_3	1.69, s								

Clavukelline I (**9**): yellow oil; $[\alpha]_D^{20}$ 34.8 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 208 (−2.82) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table S1; HR-ESI-MS *m/z* 321.2425 $[M + H]^+$ (Calcd. for $C_{20}H_{33}O_3$, 321.2424), 343.2244$[M + Na]^+$ (Calcd. for $C_{20}H_{32}O_3Na$, 343.2244).

Clavukelline J (**10**): colorless oil; $[\alpha]_D^{20}$ 14.2 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 200 (−14.16) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table S1; HR-ESI-MS *m/z* 305.2469 $[M + H]^+$ (Calcd. for $C_{20}H_{33}O_2$, 305.2475).

Clavukelline K (**11**): colorless oil; $[\alpha]_D^{20}$ 20.8 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 206 (−2.98) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table S2; HR-ESI-MS *m/z* 305.2473 $[M + H]^+$ (Calcd. for $C_{20}H_{33}O_2$, 305.2475).

Clavukelline L (**12**): colorless oil; $[\alpha]_D^{20}$ 18.6 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 202 (−81.43), 289 (51.21) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table S2; HR-ESI-MS *m/z* 347.2574 $[M + H]^+$ (Calcd. for $C_{22}H_{35}O_3$, 347.2581), *m/z* 369.2390 $[M + Na]^+$ (Calcd. for $C_{22}H_{34}O_3Na$, 369.2400).

Clavukelline M (**13**): yellow oil; $[\alpha]_D^{20}$ 4.0 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 195 (3.55) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table S2; HR-ESI-MS *m/z* 305.2466 $[M + H]^+$ (Calcd. for $C_{20}H_{33}O_2$, 305.2475).

Clavukelline N (**14**): colorless oil; $[\alpha]_D^{20}$ −28.4 (*c* 0.5, MeOH); ECD (1.0 mmol·L^{-1}, MeOH) λmax (Δε): 203 (−63.42) nm; ^{13}C and ^{1}H NMR data ($CDCl_3$) see Table S2; HR-ESI-MS *m/z* 305.2470 $[M + H]^+$ (Calcd. for $C_{20}H_{33}O_2$, 305.2475).

4.5. In vivo assay

4.5.1. Zebrafish maintenance

Zebrafish were reared separately by sex under a 14-h light/10-h dark cycle and fed regularly with artificial pellet feed and newly hatched Artemia nauplii. For egg collection, sexually mature healthy zebrafish were placed in breeding tanks at a female-to-male ratio of 1∶2 or 2∶2. Fertilized eggs were collected between 10:00 and 11:00 the following day. After disinfection and rinsing, the fertilized eggs were transferred to zebrafish embryo culture water (containing 5.0 mmol·L^{-1} NaCl, 0.17 mmol·L^{-1} KCl, 0.4 mmol·L^{-1} $CaCl_2$, and 0.16 mmol·L^{-1} $MgSO_4$) and cultured under controlled light conditions at 28 °C.

4.5.2. $CuSO_4$-induced model of zebrafish

At 3 days post fertilization, normal zebrafish embryos were selected under a stereomicroscope and transferred into a 24-well culture plate, with 10 embryos per well. Two replicate wells were set up for each group per experiment. The positive drug group was treated with 20 μmol·L^{-1} indomethacin, while the sample group was treated with 20 μmol·L^{-1} of the test sample. No drug was added to the blank control group or the model group. Each group was supplemented with culture water to a final volume of 1.0 mL. The 24-well plate was then placed in a light-incubator at 28 °C. After 2 h of incubation, except for the blank control group,

the model group, positive drug group, and sample group were treated with 20 μmol·L^{-1} $CuSO_4$ and continued to be cultured in the dark at 28 °C.

4.5.3. The source of wild-type AB zebrafish

The zebrafish were provided by the Zebrafish Drug Screening Platform, Institute of Biology, Shandong Academy of Sciences.

4.5.4. Arachidonic acid -induced model of zebrafish

Wild-type AB zebrafish larvae at 72 h post-fertilization (hpf) were selected as the experimental animals. To reduce the interference of melanin on quantification, an appropriate amount of PTU was added to inhibit melanin synthesis. The zebrafish larvae were placed in 24-well cell culture plates and randomly divided into four groups: a blank control group, a model group (80 μmol·L^{-1} arachidonic acid, AA), a positive control group (80 μmol·L^{-1} AA + 124.9 μmol·L^{-1} aspirin), and various sample treatment groups (80 μmol·L^{-1} AA + 25 μmol·L^{-1} of each sample). Each well contained 10 fish, and each group was set up with three replicates.

After incubation at 28 ± 0.5 °C for 6 h with aspirin/test compounds, all groups except the blank control were exposed to AA for 1.5 h. Larvae were then rinsed and stained with o-dianisidine in the dark for 15 min. Following washes, bright-field images were captured for analysis of tail vein staining using Image-Pro Plus to determine statistical significance.

All experiments were repeated three times, and the experimental data are expressed as mean ± SEM (n = 30). Statistical analysis was performed using GraphPad Prism 9.1.0 software, and intergroup differences were compared using one-way ANOVA. A value of $P < 0.05$ was considered statistically significant for intergroup differences.

4.5.5. ECD calculations

Conformational analysis was performed using the following workflow: MMFF conformer generation (Spartan'10) was followed by DFT geometry optimization at the B3LYP/6-31 + G(d,p)/D3BJ level. Conformers exceeding a 1% Boltzmann population were subjected to TD-DFT ECD calculations (RB3LYP/DGDZVP, 30 states) and GIAO NMR shift prediction (B3LYP/6-311 + G(d,p)). The ECD and NMR results were evaluated using the RMSD of the fit and DP4 + analysis, respectively.

Funding

This work was supported by the National Key R&D Program of China (No. 2024YFC2815900), China Postdoctoral Science Foundation (No. 2024M763097), Shandong Postdoctoral Science Foundation (No. SDCX-ZG-202400077), and Qingdao Postdoctoral Science Foundation (No. QDBSH20240102034).

Declaration of competing interests

These authors have no conflict of interest to declare.

Data availability

Data will be available on request by sending E-mail to the corresponding authors.

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