Novel 1,3-Benzodioxole From Marine-Derived Actinomycete in East Vietnam Sea

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Abstract
Analysis of an antimicrobial extract from the culture broth of the marine-derived actinomycete Streptomyces sp. G261 led to the isolation of a new 1,3-benzodioxole derivative (1), together with 10 known compounds 2-11. The actinomycete strain G261 was isolated from sediment, collected at Cu Lao Cham, Quang Nam in Vietnam. The taxonomic identification of the strain G261 was achieved by analysis of 16SrRNA gene sequences. On the basis of morphological and phylogenetic evidence, the actinomycete strain G261 was assigned to the genus Streptomyces. The structures of the isolated compounds were established by their spectral data analysis, including mass spectrometry, 1-dimensional nuclear magnetic resonance (1D-NMR), and 2D-NMR. The structure of 1 was confirmed by comparison of the calculated with experimental 13C NMR data. Compound 1 exhibited antimicrobial activity against Enterococcus faecalis and Staphylococcus aureus with minimum inhibitory concentration values of 128 and 256 µg/mL, respectively. Whereas, compound 1 had a weak inhibition when tested against 4 cancer cell lines, KB, LU-1, HepG-2, and MCF-7.

Keywords
actinomycete, Streptomyces, benzodioxole, antibacterial, antiyeast

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Marine-derived actinomycetes are rich sources of novel secondary metabolites and have diverse biological activities such as antibacterial, antifungal, antiviral, antitumor, antiprotozoal, and immunosuppressive activities.¹² Among actinomycetes, Streptomyces species are the richest source of antibiotics and other industrially important compounds.³⁴ The East Vietnam Sea covers an area of approximately 3 million km² and traces 3000 km of coastline. The marine biodiversity of the East Vietnam Sea is considered to be some of the most extensive in the world, and it remains poorly understood and explored. Previously, we reported the isolation and structural characterization of new flavonoids⁵⁶ and phenolic compounds⁷ from several strains of marine Streptomyces. In continuation of our ongoing search for bioactive metabolites from marine-derived actinomycetes, herein we report the isolation and structural characterization of a new 1,3-benzodioxole derivative (1) and 10 known metabolites (2-11) (Figure 1) from the fermentation broth of an actinomycete strain G261, which was isolated from sediment collected at a depth of 14 m, from the coast of Cu Lao Chao, Quang Nam in Vietnam. The ethyl acetate (EtOAc) extract of a G261 fermentation exhibited antimicrobial activity against both gram-positive (Enterococcus faecalis—ATCC13124 and Bacillus cereus—ATCC13245) and gram-negative (Pseudomonas aeruginosa—ATCC27853) bacteria strains, and the yeast strain (Candida albicans - ATCC1023). G261 strain was identified using 16SrRNA gene sequence analysis (GenBank registration code: MG917691).

Compound 1 was obtained as a white amorphous solid. Its infrared (IR) spectrum exhibited absorption bands of carbonyl (1747 and 1732 cm⁻¹) functionalities. Its high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) data (supplemental Figure 4S) showed a deprotonated molecular ion [M - H]⁻ at m/z 178.0152, which together with 13C nuclear magnetic resonance (NMR) data are consistent with a molecular formula of C₈H₅NO₄. Seven degrees of unsaturation were thus assigned for 1. The ¹H NMR spectrum of 1 in dimethyl sulfoxide

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(DMSO)-d$_6$ (supplementary Figure 9S) indicated the resonances at $\delta_H$ 7.25 (dd, $J = 2.0$, 7.5 Hz, H-5), 7.19 (dd, $J = 7.5$, 8.0 Hz, H-6), and 7.24 (dd, $J = 1.5$, 8.0 Hz, H-7), and 2 exchangeable protons at $\delta_H$ 11.97 (br. s) and 10.42 (br. s). The presence of the 2 exchangeable protons was confirmed by the $^1$H NMR spectrum of 1 in deuterated methanol (CD$_3$OD) (supplemental Figure 5S) in which the resonances of these 2 exchangeable protons were not observed. Analysis of the $^{13}$C NMR (supplemental Figures 6S and 10S) with the aid of heteronuclear single quantum coherence (HSQC) spectra (supplemental Figure 7S) revealed the presence of 8 sp$^2$ carbon resonances, including 3 methines and 5 quaternary carbons. This observation suggested the presence of a 1,2,3-trisubstituted benzene ring. The assignment of carbon signals corresponding to each proton signal was achieved by an HSQC experiment (Table 1). In the heteronuclear multiple bond correlation (HMBC) spectrum of 1 (supplemental Figures 8S and 11S), the presence of the 1,2,3-trisubstituted benzene ring was confirmed by cross-peaks of H-7 ($\delta_H$ 7.24) with C-3a ($\delta_C$ 142.5) and C-7a ($\delta_C$ 145.0) and H-5 ($\delta_H$ 7.25) with C-4 ($\delta_C$ 115.6) and C-3a. The carbonyl C-8 at ($\delta_C$ 161.6) was linked to C-4 of the benzene ring by its HMBC correlation with H-5 (Figure 2).

Taking into account the molecular formula of C$_8$H$_5$NO$_4$ was established above, 4 possible structures 1, 1a, 1b, or 1c could be suggested for 1 (Figure 3). Since the 2 exchangeable broad singlet protons had no correlation in the HMBC spectrum, the structural assignment of 1 could not be resolved by 2D NMR data. Comparison of the NMR data of 1 with that previously reported for 1a indicated significant differences, especially for the carbon chemical shifts of C-3a ($\delta_C$ 142.5 for 1 and 133.0 for 1b), C-2 ($\delta_C$ 147.2 for 1 and 156.7 for 1b) and C-8 ($\delta_C$ 161.6 for 1 and 167.8 for 1b). The carbon chemical shifts of four structures 1, 1a, 1b, and 1c were calculated by using the Gaussian 09 program. To obtain minimum energy conformers, geometry optimization of each possible isomer of these compounds was conducted (supplemental Figures 1S-4S, supplemental Tables 1S-4S). The NMR data of compounds 1, 1a, 1b, and 1c were generated by using the Gaussian 09 program. In dimethyl sulfoxide-d$_6$.

Table 1. Nuclear Magnetic Resonance Data of Compound 1 ($^1$H: 500 MHz, $^{13}$C: 125 MHz).

| Position | $\delta_C$ | $\delta_H$ mult. ($J$ in Hz) | $\delta_C$ | $\delta_H$ mult. ($J$ in Hz) |
|----------|------------|-------------------------------|------------|-------------------------------|
| 2        | 147.2      | 149.2                         |            |                               |
| 3a       | 142.5      | 144.2                         |            |                               |
| 4        | 115.6      |                               | 116.7      |                               |
| 5        | 116.2      | 7.25 dd (2.0, 7.5)            | 118.1      | 7.49 dd (2.0, 7.0)            |
| 6        | 125.0      | 7.19 dd (7.5, 8.0)            | 126.4      | 7.22 dd (7.5, 8.0)            |
| 7        | 122.0      | 7.24 dd (1.5, 8.0)            | 123.6      | 7.25 dd (2.0, 8.0)            |
| 7a       | 145.0      |                               | 146.5      |                               |
| 8        | 161.6      |                               | 163.4      |                               |
| NH$_2$   | 11.97      | br. s                         | 10.42      | br. s                         |

In dimethyl sulfoxide-d$_6$.  
In deuterated methanol.
(Δδ_{exp}–Δδ_{calcd}: −8.4), and C-7 (Δδ_{exp}–Δδ_{calcd}: +8.7) of 1c (supplementary Table 8S). Whereas the calculated chemical shifts of 1 were close to the experimental values (Table 2 and supplementary Table S5). Additionally, chemical shifts difference between C-3a and C-7a for 1 was around 2.3 ppm (Table 1), while this value was 11.0 (for 1a) and 11.7 ppm (for 1b) by theory calculation (supplementary Tables 6S and 7S). This analysis allowed determining the structure of 1 as 1,3-benzodioxole-2-one-4-carboxamide. The NH2 protons had different chemical shifts at δH 11.97 and 10.42, probably due to hydrogen bonding of 1 proton of the position 3 making these 2 NH2 protons inequivalent. This was supported by a conformational calculation of 1 which indicated 1 proton of NH2 group was oriented toward the oxygen O-3 in the structure of the most stable conformer (94.1%) (supplementary Figure 1S and Table 1S). The calculated carbon chemical shifts of compound 1a (supplementary Table S5) were in good agreement with those reported in the literature.

Other known compounds, 2-[(5-methyl-1,4-dioxan-2-yl) methoxy]ethanol (2),11 1H-pyrrole-2-carboxylic acid (3),12 norharman (4),13 3-hydroxy-adenosine (5),14 2-acetamidobenzamide (6),15 cyclo-[Pro-Gly] (7),16 cyclo-[Pro-Ala] (8),17 cyclo-[Pro-Leu] (9),18 cyclo-trans-4-OH-[Pro-Phe] (10),18 and cyclo-[Leu-Tyr] (11)19 were also isolated and characterized from the culture broth of G261 strain. Their structures were determined by spectral data analysis and comparison with those reported in the literature.

All the isolates were evaluated for their antibacterial activity against Enterococcus faecalis (ATCC13124), Staphylococcus aureus (ATCC25923), Bacillus cereus (ATCC13245), Escherichia coli (ATCC25922), Pseudomonas aeruginosa (ATCC27853), and Salmonella enterica (ATCC12228), and antifungal property against Candida albicans (ATCC1023). Compound 1 exhibited inhibitory activity against E. faecalis and S. aureus with minimum inhibitory concentration (MIC) values of 128 ± 5.7 and 256 ± 7.8 µg/mL, respectively. Compound 2 showed activity against E. faecalis, E. coli, and C. albicans with MIC values of 32 ± 3.1, 128 ± 4.6 and 64 ± 3.4 µg/mL, respectively. Whereas, compound 4 was selectively inhibited B. cereus with MIC value of 32 ± 2.9 µg/mL, and compounds 10 and 11 had selective inhibition against S. aureus and E. coli with MIC values of 256 ± 7.2 and 32 ± 2.3 µg/mL, respectively (Table 3). Additionally, compound 1 displayed weak cytotoxicity against 4 cancer cell lines, KB (mouth epidermal carcinoma cells), HepG-2 (human liver hepatocellular carcinoma cells), LU-1 (human lung adenocarcinoma cells) and MCF-7 (human breast cancer cells) with half-maximal inhibitory concentration (IC_{50}) of 71.8 ± 4.1, 73.8 ± 5.3, 52.0 and 82.0 ± 5.9 µg/mL, respectively.

**Experimental**

**General**

HR-ESI-MS were recorded on an FT-ICR 910 MS TQFTMS-7T mass spectrometer. IR spectra were recorded on a Nicolet Impact

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### Table 2. Δ_{exp}–Δ_{calcd} Values of Carbon Chemical Shift Differences Between Experimental Values of 1 with Those of Calculated by Theory in Methanol of 1, 1a, 1b and 1c.

| Position | 1   | 1a  | 1b  | 1c  |
|----------|-----|-----|-----|-----|
| 2        | −1.1| −5.6| −1.3| −7.0|
| 3a       | +2.4| +11.2| +4.9| +1.72|
| 4        | −0.3| +5.8| +6.1| +5.2|
| 5        | +2.4| −3.1| −4.9| −8.4|
| 6        | +0.6| +4.7| +3.8| +2.5|
| 7        | −3.8| +7.8| +9.7| +8.7|
| 7a       | +2.6| +2.5| +18.9| +2.6|
| 8        | +1.5| −1.7| +2.1| +0.9|

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**Figure 2.** Key heteronuclear multiple bond correlations of 1.

**Figure 3.** Four possible structures of 1.
FT-IR spectrometer and NMR spectra on a Bruker AM500 MHz spectrometer operating at 125.76 MHz for 13C NMR and at 500.13 MHz for 1H NMR. 1H chemical shifts were referenced to DMSO- d6 and CD3OD at δ 2.50 and 3.31 ppm, respectively, while the 13C chemical shifts were referenced to the central peak at δ 39.5 (DMSO- d6) and 49.0 ppm (CD3OD).

Isolation and Taxonomic Identification of the Actinomycete Strain G261

Strain G261 was isolated from a sediment sample collected by PONAR at a depth of 15 m, from the coast of Cu lao Cham, Quang Nam in Vietnam in October 2016, strain G261 (GenBank accession number MG917691). The 16SrRNA gene sequence analysis together with morphological and phylogenetic evidence (supplemental Figures 12S and 13S), the actinomycete strain G261 was identified to the genus *Streptomyces*.

Fermentation of G261

Strain G261 was activated and inoculated into 1 L of A1 medium pH 7.0 comprising starch (10.0 g), yeast extract (4.0 g), peptone (2.0 g), and instant ocean (30.0 g) in 1.0 L of distilled water. After 7 days of incubation at 28°C with agitation, the culture broth was used to inoculate the fermentation in 50 L of high-nutrient medium A1 + (soluble starch: 10 g/L, yeast extract: 4.0 g/L, peptone: 2.0 g/L, instant ocean: 30.0 g/L, 5 mL KBr, 20 mg/mL, 5 mL FeSO4 (8 mg/mL), 1.0 g/L CaCO3: 1.0 g/L). The fermentation was incubated at 28°C with agitation of 200 rpm and harvested on the 10th day.

Isolation and Purification Procedures

The fermentation broth (50 L) was extracted with n-butanol (BuOH) (5 × 30 L). The combined n-BuOH extract were concentrated under reduced pressure. The crude extract (39.9 g) was fractionated by column chromatography (CC) on silica gel CC eluted with n-hexane/acetone gradient to yield 17 fractions. Fraction F3 (4.94 g) was purified by CC on silica gel, eluted with dichloromethane (CH2Cl2)/methanol (MeOH) gradient to give 7 subfractions F3.1–F3.7. Subfraction F3.3 (589 mg) was separated by CC on silica gel (0%-30% MeOH in CH2Cl2), followed by preparative thin-layer chromatography (TLC) (CH2Cl2/Methanol: 8/2) to furnish 7 (10.9 mg). Fraction F4 (269 mg) was subjected to a CC on silica gel (0%-100% EtOAc in n-hexane) furnishing 3 subfractions (F4.1–F4.3). Subfraction F4.1 (37.2 mg) was purified by preparative TLC (n-hexane/acetone: 95/5) to provide 4 (5.5 mg). Fraction F5 (364 mg) was subjected to a CC on silica gel (0%-100% of EtOAc in n-hexane) to afford 6 subfractions (F5.1–F5.6). Subfraction F5.3 (54 mg) was chromatographed on a silica gel CC (0%-50% of acetone in n-hexane), yielding 2 (5.1 mg). Subfraction F5.4 (66 mg) was separated on a Sephadex LH-20 column (MeOH/CH2Cl2: 9/1), followed by recrystallization in a solvent mixture n-hexane/acetone (8/2, v/v), to afford 3 (3.9 mg). Subfraction F5.6 (75 mg) was purified by Sephadex LH-20 column (MeOH), furnishing 1 (4.1 mg). Fraction F6 (187 mg) was subjected to a silica gel CC (5%-100% acetone in n-hexane) to give 5 (5.2 mg). Fraction F11 (279 mg) was separated by CC on Sephadex LH-20 column (MeOH/CH2Cl2: 9/1), followed by recrystallization in a solvent mixture n-hexane/acetone (8/2, v/v), to afford 6 (8.1 mg). Fraction F13 (700 mg) was separated by CC on Sephadex LH-20 (MeOH) giving 11 (2.3 mg). Finally, subfraction F17.5 (150 mg) was purified on silica gel CC (CH2Cl2/MeOH: 9/1) to give 9 (9.9 mg).

Table 3. Antibacterial and Antifungal Activities of Compounds 1-11 (Minimum Inhibitory Concentration: μg/mL).

| Compounds | Gram-positive | Gram-negative | Yeast |
|-----------|---------------|---------------|------|
|           | Enterococcus faecalis | Staphylococcus aureus | Bacillus cereus | Escherichia coli | Pseudomonas aerogenous | Salmonella enterica | Candida albicans |
| 1         | 128 ± 5.7     | 256 ± 7.8     | >256           | >256          | >256                | >256               | >256         |
| 2         | 32 ± 3.1      | >256          | >256           | >256          | >256               | >256               | 64 ± 3.4     |
| 3         | >256          | >256          | >256           | >256          | >256               | >256               | >256         |
| 4         | >256          | >256          | 32 ± 2.9       | >256          | >256               | >256               | >256         |
| 5         | >256          | >256          | >256           | >256          | >256               | >256               | >256         |
| 6         | >256          | >256          | >256           | >256          | >256               | >256               | >256         |
| 7         | >256          | >256          | >256           | >256          | >256               | >256               | >256         |
| 8         | >256          | >256          | >256           | >256          | >256               | >256               | >256         |
| 9         | >256          | >256          | >256           | >256          | >256               | >256               | >256         |
| 10        | >256          | 256 ± 7.2     | >256           | >256          | >256               | >256               | >256         |
| 11        | >256          | >256          | >256           | 32 ± 2.3      | >256               | >256               | >256         |
| Streptomycin | 256 ± 7.2     | 256 ± 6.8     | 128 ± 4.1      | 32 ± 2.8      | 256 ± 6.5          | 128 ± 5.1          | 8 ± 0.7      |
| Nystatin  |               |               |                |               |                    |                    |              |
Antimicrobial Assay

Antimicrobial assays for compounds 1-11 were carried out using E. coli (ATCC 25922), P. aeruginosa (ATCC 27853), S. enterica (ATCC 12228), E. faecalis (ATCC 13124), S. aureus (ATCC 25923), B. cereus (ATCC 13245), and C. albicans (ATCC 1023). Stock solutions of samples were prepared in DMSO and the antimicrobial assays were carried out in 96-well microtiter plates against the microbial strains (5 × 10^5 CFU/mL) using a modification of the published method. After incubation for 24 hours at 37°C, the absorbance at 650 nm was measured using a microplate reader. Streptomycin and nystatin were used as reference compounds.

Cytotoxicity Assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cytotoxic activity of compound 1 with human cancer cell lines (KB, LU-1, HepG2, and MCF-7) acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA) using a modification of the published method. Cells were cultured in medium RPMI 1640 supplemented with 10% fetal bovine serum under a humidified atmosphere of 5% CO2 at 37°C. Compound 1 was dissolved in DMSO at a concentration of 20 mg/mL. A series of dilutions was prepared to final concentrations of 128, 32, 8, 2, and 0.5 mg/mL. Sample (100 µL) of the complexes with different concentrations was added to the wells on 96-well plates. Cells were separated with trypsin and EDTA and seeded in each well with 3 × 10^4 cells per well. An MTT solution (20 µL, 4 mg/mL) of phosphate buffer saline (8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, and 0.24 g KH2PO4 per liter) was added to each well after being incubated for 48 hours. The cells were further incubated for 4 hours and a purple formazan precipitate was formed, which was separated by centrifugation. The precipitate was dissolved by adding DMSO (100 µL) to each well. The optical density of the solution was determined by a plate reader (TECAN) at 540 nm. The inhibition ratio was achieved on the basis of the optical densities from the calculation of three replicate tests. Ellipticine (IC50: 1.2-2.4 µM) was used as a reference compound.

1,3-Benzodioxole-2-One-4-Carbocyclamide (1)

White amorphous solids.

Rf: 0.43 (CH2Cl2/EtOAc: 2/1).
IR (KBr): 3419, 2954, 2900, 1921, 1869, 1747, 1732, 1616, 1502, 1440, 1340 cm⁻¹.
NMR data see Table 1.
HR-ESI-MS: m/z [M − H]⁻ calcd for C8H4NO4: 178.0140; found: 178.0152.

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Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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