

HLE-Inhibitory Alkaloids with a Polyketide Skeleton from the Marine-Derived Fungus *Coniothyrium cereale*

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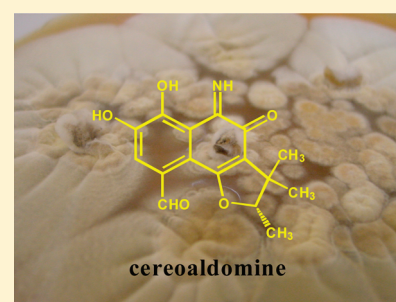
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S Supporting Information

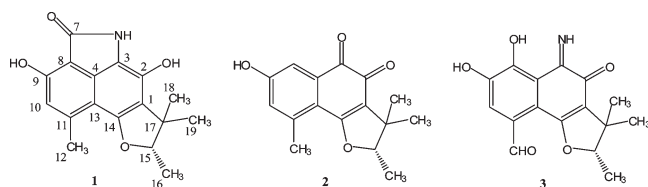
ABSTRACT: The marine endophytic fungus *Coniothyrium cereale* produces the structurally unusual polyketide-type alkaloids (–)-cereolactam (**1**) and (–)-cerealdomine (**3**), incorporating a lactam and an imine functionality, respectively, as well as the related metabolite (–)-tryptelone (**2**). Compounds **1** and **3** showed selective inhibition of human leukocyte elastase with IC₅₀ values of 9.28 and 3.01 μM, respectively. Compound **2** was found to be inhibitory toward *Mycobacterium phlei*, *Staphylococcus aureus*, and *Escherichia coli* and also cytotoxic against mouse fibroblast cells (IC₅₀ = 7.5 μM).



Marine algae harbor fungal endophytes in their inner tissue, which can be isolated from them as axenic cultures. The secondary metabolites of these ascomycetes are distinguished by a high chemical diversity and bioactivity, which render these organisms as highly useful in the drug discovery process.

In a recent study we reported the isolation of several phenalenone derivatives from the fungus *Coniothyrium cereale* obtained from the marine green alga *Enteromorpha* sp.¹ Continuing work on this algal endophyte resulted in the isolation of the novel γ -lactam-containing phenalenone derivative **1** and the imine alkaloid **3**. Both metabolites can be regarded as unprecedented structural types and may be formed by the biosynthetic degradation of phenalenone-type precursors, since the latter were also encountered in the fungal extract.¹

The protease human leukocyte elastase (HLE) is involved in the pathology of chronic obstructive pulmonary disease (COPD), pulmonary emphysema, rheumatoid arthritis, and cystic fibrosis.² Compounds **1** and **3** showed selective inhibition of HLE with IC₅₀ values of 9.28 and 3.01 μM, respectively. The biosynthetically closely related compound **2**, an enantiomer of the known metabolite tryptelone, was active against *Mycobacterium phlei*, *Staphylococcus aureus*, and *Escherichia coli* and also cytotoxic toward mouse fibroblast cells.



The molecular formula of **1** was determined to be C₁₇H₁₇NO₄, as deduced from accurate mass measurements (HRESIMS m/z 300.1236 [M + H]⁺), which requires 10 degrees of unsaturation. UV maxima at 354 and 390 nm clearly evidenced that compound **1** comprises an extended aromatic system, whereas the IR spectrum showed stretching vibrations at 3255 and at 1701 cm⁻¹ corresponding to hydroxy and carbonyl groups, respectively.

The ¹H NMR spectrum (Table 1) of **1** is characterized by four resonances due to three tertiary and one secondary methyl group (δ_{H} 2.65 for H₃-12, δ_{H} 1.36 for H₃-16, δ_{H} 1.43 for H₃-18, and δ_{H} 1.15 for H₃-19). A resonance in the ¹H NMR spectrum at δ_{H} 9.29 accounts for a nonchelated hydroxy or amino group. Two further ¹H NMR resonance signals arise from one aryl (δ_{H} 6.69 for H-10) and one aliphatic proton (δ_{H} 4.42 for H-15). The latter is downfield shifted, indicating its direct attachment to an electronegative atom. The aryl proton H-10 did not show any ¹H–¹H coupling, thus suggesting a pentasubstituted benzene ring in the molecule.

The ¹³C NMR spectrum (Table 1) disclosed 17 resonances resulting from four methyl groups (CH₃-12, CH₃-16, CH₃-18, and CH₃-19), one sp² methine group (C-10), and a further sp³ methine group (C-15), all resulting from structural elements already deduced from the ¹H NMR data. All other carbon atoms in **1** were quaternary, including the six sp² aromatic carbon atoms C-1, C-3, C-4, C-8, C-11, and C-13, as well as C-2, C-9, and C-14, which were, according to their ¹³C NMR chemical shifts, attached to oxygen. C-7 resonated at δ 165.3, indicating a carbonyl moiety, whereas

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Table 1. NMR Spectroscopic Data for Compounds 1 and 3

position	1 ^b		3 ^c	
	δ_C , mult. ^a	δ_H (mult, J in Hz)	δ_C , mult. ^a	δ_H (mult, J in Hz)
1	119.5, C		124.3, C	
2	137.4, C		183.1, C	
3	112.2, C		176.5, C	
4	128.5, C		110.0, C	
7	165.3, C			
8	105.1, C		149.0, C	
9	151.1, C		149.7, C	
10	117.4, CH	6.69, s	112.4, CH	7.37, brs
11	142.1, C		126.7, C	
12	20.8, CH ₃	2.65, s	191.5, CH	10.71, s
13	106.8, C		122.2, C	
14	156.3, C		168.2, C	
15	88.9, CH	4.42, q (6.6)	92.7, CH	4.73, q (6.6)
16	14.2, CH ₃	1.36, d (6.6)	14.6, CH ₃	1.47, d (6.6)
17	44.0, C		44.0, C	
18	25.6, CH ₃	1.43, s	25.6, CH ₃	1.41, s
19	21.0, CH ₃	1.15, s	20.4, CH ₃	1.22, s
NH		9.29, s		7.46, brs ^d
OH-8				11.50, brs ^d
OH-9				9.25, brs ^d

^aImplied multiplicities determined by DEPT. ^bIn DMSO-*d*₆. ^cIn acetone-*d*₆. ^dSignals exchangeable.

C-17 proved to be an aliphatic sp³ quaternary carbon atom. The nitrogen atom, which was evident from the molecular formula, had to be present in the form of an amide group, since ¹H–¹⁵N HMBC measurements (Figure S4 in the Supporting Information (SI)) revealed the nitrogen resonance signal at δ_N 128.0.

In the ¹H–¹³C HMBC spectrum H-10 showed strong correlations to C-8, C-12, and C-13, whereas CH₃-12 displayed heteronuclear couplings to C-10, C-11, and C-13 (see Table S1 and Figure S5 in the SI). This pattern of HMBC correlations, together with the UV and ¹H NMR data, indicated a naphthalene-type compound, substituted at C-11 with a methyl group. The NH proton was found to have correlations with C-3, C-4, C-7, and C-8, supporting the presence of a γ -lactam ring. The direction of the amide bond is as shown; the NH is attached to C-3 and not to C-8, due to the correlation from H-10 to C-7 in the ¹H–¹³C HMBC spectrum.

The ¹H NMR spectrum contained two singlet resonances at δ_H 1.43 (H₃-18) and 1.15 (H₃-19) due to geminal dimethyl groups attached to the quaternary carbon C-17, which was corroborated by the ¹H–¹³C HMBC cross-peak correlations from both H₃-18 and H₃-19 to C-17. C-17 is attached to C-1 due to the correlations from both H₃-18 and H₃-19 to C-1. Finally, correlations between the CH₃-16 resonance signal and both carbons C-15 and C-17 established a prenyl moiety. CH-15 can be attached to either C-2 or C-14 via oxygen due to its downfield chemical shift (δ_C 88.9). Although ¹H–¹³C HMBC cross-peaks of H-15 to C-2 or C-14 are missing, CH-15 has to be connected to C-14 due to comparable ¹³C NMR chemical shifts of compound 1 to those of compounds 2 and 3. Compound 1 is thus composed of a dihydroxy-methyl-naphthalene nucleus fused to a trimethyldihydrofuran ring and a γ -lactam ring. We give the name (–)-cereolactam to compound 1.

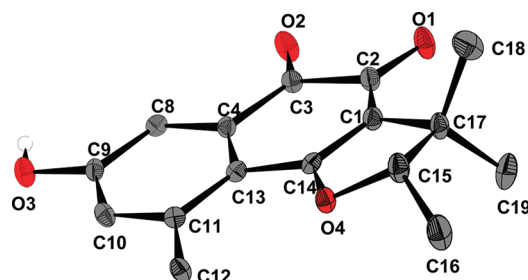


Figure 1. X-ray crystallographic structure of compound 2.

The molecular formula of compound 2 was established as C₁₆H₁₆O₄ from accurate mass measurements (HRESIMS *m/z* 295.0941 [M + Na]⁺). Interpretation of the NMR data (Table S6 and Figures S7 and S8 in the SI) indicated that compound 2 has the same planar structure as the known fungal metabolite tryptelone. The general structure of compound 2 was confirmed by single-crystal X-ray crystallography (Figure 1). However, the specific rotation of –355 indicated that compound 2 is the enantiomer.³ For compound 2 the trivial name (–)-tryptelone is suggested.

Compound 3, with a molecular formula of C₁₆H₁₅NO₅ (HRESIMS *m/z* 324.0842 [M + Na]⁺), has a similar skeleton to 1 and 2; however, the methyl group CH₃-12 is replaced by an aldehyde group. In contrast to compound 2, there is only one carbonyl group and one aromatic proton in the molecule. The presence of the aldehyde was deduced from the chemical shifts $\delta_{H/C}$ 10.71/191.5 in the ¹H and ¹³C NMR spectra being representative for CHO-12. The aldehyde group was determined to be attached to C-11 from cross-peak correlations in the ¹H–¹³C HMBC spectrum between the aldehyde proton H-12 and carbons C-9, C-10, C-11, and C-13. The molecular formula deduced for 3, the presence of only a single resonance signal for an aromatic proton (δ_H 7.37, H-10), and the ¹³C NMR chemical shift of C-9 (δ_C 149.7) all indicated that C-9 is hydroxylated. Comparing our spectroscopic data (Table 1 and Figures S11 and S12 in the SI) with those of the reference compounds 8-methoxytryptelone methyl ether and 4'-hydroxy-8-methoxytryptelone methyl ether revealed closely similar chemical shifts and further confirmed the substitution pattern of the aromatic ring comprising carbons C-4 and C-8 to C-13.² Compound 3 is further distinguished by an imine functionality (IR 1624 cm⁻¹) replacing one of the carbonyls found in compound 2. The position of the imine group is deduced from calculations of the ¹³C NMR chemical shifts of both possible regioisomers using the ACD NMR predictor software (ACD laboratories). Comparison of the calculated data with the measured ¹³C NMR data of compound 3 (Figure S18 in the SI) clearly indicates that the imine group is positioned at C-3, which is the same position of transamination as in compound 1. We give the name (–)-cerealdomine to compound 3.

Compounds 1–3 contain a single stereogenic center at C-15, have the same substitution pattern around this center, and display comparable NOESY correlations (see Tables S1, S6, and S10 in the SI). Additionally, all compounds have a negative specific rotation ($[\alpha]_D$ –70 for compound 1, –355 for compound 2, –320 for compound 3), suggesting that 1–3 have the same absolute configuration at C-15. For compound 2, the absolute configuration at C-15 was determined as 15*S* by comparing its CD spectrum (see Figure S14 in the SI) with that of the reference compounds (+)-tryptelone³ and (+)-dunnione.^{4,5}

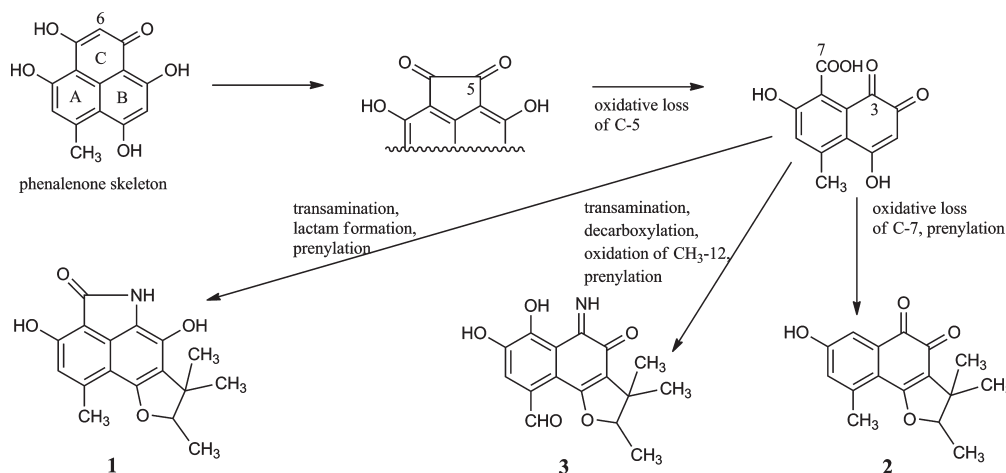


Figure 2. Proposed biosynthesis of compounds 1–3.

Compound 2 had a negative Cotton effect at λ_{\max} 480 (–1.8), which is opposite that described for the reference compounds. Furthermore, the levorotatory specific rotation ($[\alpha]_D$ –355 for compound 2 and +364 for (+)-tryptelone) showed that compound 2 is the enantiomer of the known metabolite (+)-tryptelone.³ As for 2, compounds 1 and 3 also displayed negative Cotton effects, even though at different λ_{\max} (390 and 360 nm for 1 and 3, respectively) due to their different chromophores. On the basis of their chiroptical properties, the stereogenic center at C-15 for compounds 1 and 3 is also proposed to have the *S* configuration.

Compounds 1 and 3 showed inhibition of human leukocyte elastase, an enzyme of therapeutic interest,² and exhibited IC_{50} values of 9.28 ± 2.77 and $3.01 \pm 0.23 \mu\text{M}$, respectively (Figures S16 and S17 in the SI). Compound 2 did not inhibit HLE ($IC_{50} > 20 \mu\text{M}$), but it showed cytotoxic activity with an IC_{50} value of $7.5 \mu\text{M}$ using an MTT assay with mouse fibroblast cells. In addition, in agar diffusion assays with *Mycobacterium phlei*, *Staphylococcus aureus*, and *E. coli*, compound 2 ($20 \mu\text{g}/\text{disk}$) showed inhibition zones of 18, 14, and 12 mm, respectively. Noteworthy, (+)-tryptelone, the reported enantiomer of compound 2, was described to have significant antibacterial activity against *Bacillus subtilis* (ATCC 6633) and showed modest antibacterial activity against *Staphylococcus aureus* (MRSA) (CGMCC 1.2465).⁶

Compounds 1 and 3 had no cytotoxic activity in the MTT assay and were not active in the agar diffusion assays. Additionally, compounds 1–3 did not show inhibition of bovine chymotrypsin, bovine trypsin, human thrombin, papain from *Carica papaya*, porcine cholesterol esterase, and acetylcholinesterase from *Electrophorus electricus* ($IC_{50} > 50 \mu\text{M}$).

Compounds 1–3 are related to metabolites with a phenalenone skeleton (Figure 2 and Scheme S19, see SI), which are also present in this fungus.¹ The phenalenone nucleus was found through feeding experiments with labeled acetate to originate from a heptaketide, which cyclizes to a tricyclic aromatic ring system.^{7,8} Phenalenone derivatives with a heterocyclic or contracted ring C are formed by oxidative loss of carbons; for the fungal metabolites sclerodione and sclerodin it was shown by labeling studies that C-6 is missing.⁹

For the formation of the basic skeleton of compound 1 the oxidative loss of C-5, transamination at the carbonyl-substituted position C-3, and closure of the lactam ring can be proposed. Compound 3 may arise from an amine-containing precursor of

metabolite 1 through oxidation. The skeleton of compound 2 would result from loss of C-5 and C-7. Although unlikely, a hexaketide instead of a heptaketide origin may also be proposed for 1–3. In the latter case, however, the C-7 carboxyl moiety of compound 1 would have to be introduced by methylation using *S*-adenosylmethionine and subsequent oxidation.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were measured on a Büchi 535 apparatus. Optical rotations were measured with a Jasco DIP 140 polarimeter. UV and IR spectra were obtained employing Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instruments, respectively. CD spectra were taken on a Jasco J-810 spectropolarimeter. All NMR spectra were recorded on a Bruker Avance 300 DPX spectrometer and a Bruker Avance 500 DRX spectrometer. Spectra were referenced to the residual solvent signals with resonances at $\delta_{\text{H/C}}$ 2.04/29.8 (acetone-*d*₆), $\delta_{\text{H/C}}$ 3.35/49.0 (methanol-*d*₄), and $\delta_{\text{H/C}}$ 2.50/39.5 (DMSO-*d*₆). The ¹H–¹⁵N HMBC spectrum was referenced externally to urea (¹⁵N chemical shift is reported relative to liquid ammonia). VLC grade (Macherey-Nagel, Polygoprep 60-50 C18) was used for vacuum liquid chromatography. All organic solvents were distilled prior to use. HPLC was carried out using a Waters system, controlled by Waters Millennium software, consisting of a 600E pump, a 996 PDA, and a 717 Plus autosampler. ESI mass spectra were obtained on an Applied Biosystems/MDS Sciex API 2000 MS spectrometer. HRESIMS were recorded on a Bruker Daltonik micrOTOF-Q time-of-flight mass spectrometer with ESI source. Dark violet-red single crystals of compound 2 were grown in methanol/water (1:1). The data collection was performed on a Bruker X8-KappaApexII diffractometer (area detector) using graphite-monochromated Mo K α radiation ($\lambda = 0.71073 \text{ \AA}$). The diffractometer was equipped with a low-temperature device (Kryoflex I, Bruker AXS GmbH, 100 K). Intensities were measured by fine-slicing ω - and φ -scans and corrected for background, polarization, and Lorentz effects.

Fungal Material, Culture, Extraction, and Isolation. General procedures were as described before (see also the SI).¹ Compounds 1–3 were isolated from subfraction 8 by RP-HPLC (column: Waters Atlantis C₁₈, 250 × 4.6 mm, 5 μm ; CH₃CN/H₂O (32:68), 2 mL/min).

(–)-Cereolactam (**1**): green, amorphous solid (11.5 mg); $[\alpha]_D^{23}$ –70 (*c* 0.10, MeOH); UV (MeOH) λ_{\max} (log ϵ) 213 nm (5.1), 274 nm (5.0), 339 nm (4.3), 354 nm (4.3), 390 nm (4.1); CD (*c* 0.2 mg/mL, MeOH), λ ($\Delta\epsilon$) = 390 (–0.3); IR (ATR) ν_{\max} 3255, 2922, 2851, 1701, 1608, 1379, 1062, 873, 824 cm^{-1} ; ¹H NMR and ¹³C NMR (Table 1 and SI);

HRESIMS m/z 300.1236 $[M + H]^+$ (calcd for $C_{17}H_{18}NO_4$, m/z 300.1230).

(–)-*Trypethelone* (**2**): dark violet-red, crystalline solid (21.5 mg); mp 250–253 °C; $[\alpha]_D^{23}$ –355 (c 0.10, MeOH); CD (c 0.2 mg/mL, MeOH), λ ($\Delta\epsilon$) = 480 (–1.8); UV, IR, NMR, and MS data (see SI).

(–)-*Cereoaldomine* (**3**): bright blood red, crystalline solid (6.0 mg); $[\alpha]_D^{23}$ –320 (c 0.30, MeOH); UV (MeOH) λ_{max} ($\log \epsilon$) 268 nm (5.2), 501 nm (4.5); CD (c 0.2 mg/mL, MeOH), λ ($\Delta\epsilon$) = 360 (–0.4); IR (ATR) ν_{max} 3314, 2922, 2891, 1735, 1712, 1624, 1567, 1461, 1380, 1273, 1097, 1032, 877 cm^{-1} ; 1H NMR and ^{13}C NMR (Table 1 and SI); HRESIMS m/z 324.0842 $[M + Na]^+$ (calcd for $C_{16}H_{15}NNaO_5$, m/z 324.0848).

X-ray Diffraction Structure Determination for Compound 2. Crystal data: $C_{16}H_{16}O_4$; crystal size (mm) 0.60 × 0.30 × 0.08, dark red-violet needle; crystal system monoclinic; space group $P2_1$; unit cell dimensions $a = 7.2659(5)$ Å, $b = 14.0034(9)$ Å, $c = 13.9617(9)$ Å, $\alpha = \gamma = 90^\circ$, $\beta = 97.446(2)^\circ$, $V = 1408.59(16)$ Å³; $Z = 4$, $\rho = 1.284$ g cm^{-3} ; $\mu = 0.092$ mm^{–1}; $F(000) = 576$; θ range for data collection 2.83 to 26.00°; reflections collected/unique 7990/4342 [$R(int) = 0.0446$]; completeness to θ 26.00° = 99.9%; refinement method, full-matrix least-squares on F^2 ; final R indices [$I > 2\sigma(I)$] $R_1 = 0.0399$, $wR_2 = 0.1045$; largest difference Fourier peak and hole 0.250 and –0.208 e Å^{–3}.

The crystals turned out to be non-merohedral twins. The diffraction pattern could be indexed using the least-squares procedure implemented in the CELL_NOW program system (Bruker AXS, 2009). Subsequent multidomain integration of the data set led to a refined twin law of (0.99997 0.00052 –0.00012 0.00133 –1.00000 –0.00025 –0.49853 0.00012 –0.9997); thus, a rotation of 180° around the reciprocal axis (1.000 0.001 –0.249) converts the HKLs from one to the other twin domain (SAINT, Bruker AXS, 2009). The data reduction and empirical absorption correction with TWINABS (Bruker AXS, 2009) have a HKLF 5 file suitable for the twin refinement in ShelX.¹⁰ The absolute structure of **2** could not be determined using Mo $K\alpha$ radiation due to the lack of heavier atoms. The unit cell of crystals of **2** shows the presence of a pseudosymmetry element (c glide plane; see Figure S20b, SI). Applying this would change the overall space group from $P2_1$ to $P2_1/c$, which would be incompatible for chiral molecules in enantiomeric pure samples. This glide plane fits for all atoms except the chiral atoms C-12 and C-28 (see Figure S20b; numbering according to crystallographic standard procedures), which are either both S,S -configured or R,R -configured, but not R,S - or S,R -configured. Since compound **2** was crystallized from an enantiomeric pure sample, a refinement of the structure of **2** in $P2_1/c$ gives R -values much too high to be correct.

CCDC 819315 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via <http://www.ccdc.cam.ac.uk/cgi-bin/catreq.cgi>.

Bioassays. Agar diffusion assays, MTT assays, and enzyme assays were done as described before (see also the SI).¹

■ ASSOCIATED CONTENT

📄 **Supporting Information.** Experimental procedures, NMR and CD spectra, and data for compounds **1–3**, as well as X-ray crystallographic data (CIF file) for compound **2**. Dose-dependent inhibition of HLE by compounds **1** and **3** and other relevant information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ DEDICATION

This work is dedicated to Prof. Gerhard Bringmann on the occasion of his 60th birthday.

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