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Varioxepine A, a 3*H*-Oxepine-Containing Alkaloid with a New Oxa-Cage from the Marine Algal-Derived Endophytic Fungus *Paecilomyces variotii*

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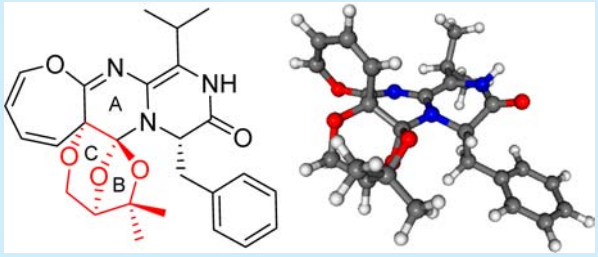
Supporting Information

ABSTRACT: A new 3*H*-oxepine-containing alkaloid, varioxepine A (**1**), characterized by a structurally unprecedented condensed 3,6,8-trioxabicyclo[3.2.1]octane motif, was isolated from the marine algal-derived endophytic fungus *Paecilomyces variotii*. Due to the low proton/carbon ratio, the unambiguous assignment of the planar structure and relative configuration was precluded by NMR experiments and solved by single crystal X-ray analysis. The absolute configuration was established by DFT conformational analysis and TDDFT-ECD calculations. Compound **1** inhibited plant pathogenic fungus *Fusarium graminearum*.



Oxepine- or 3*H*-oxepine-containing diketopiperazine alkaloids are new members of a rarely reported class of natural products.[1] This class of alkaloids generally possesses an oxepine−pyrimidinone−ketopiperazine tricyclic core derived from different amino acids. Although a very limited number of natural products containing these structural features are described,[2] there is increasing fascination with their structural determination, bioactivity, and biogenesis. Due to various biological activities such as antiplasmodial, anti-inflammatory, growth inhibitory, and transcriptional activation on LXRα (Liver X Receptor),[2] the oxepine-containing natural products have attracted much attention from synthetic chemists.[3]

As a continuation of our investigation on structurally novel and biologically active metabolites from marine-derived fungi,[4] a new 3*H*-oxepine-containing diketopiperazine-type alkaloid, varioxepine A (**1**, Figure 1), was isolated from an extract of *Paecilomyces variotii* EN-291, an endophytic fungus obtained from marine red alga, showing antimicrobial activity. Compound **1** is a 3*H*-oxepine-containing alkaloid having a condensed 3,6,8-trioxabicyclo[3.2.1]octane unit, which has not been reported yet in natural products. Structure elucidation for **1** proved to be difficult, particularly for the central parts (rings A and B), due to a low proton/carbon ratio and lack of descriptive 2D NMR signals. Herein, the isolation, structure elucidation including the determination of absolute configuration, bioactivity evaluation, and possible biogenetic pathway of compound **1** are described.

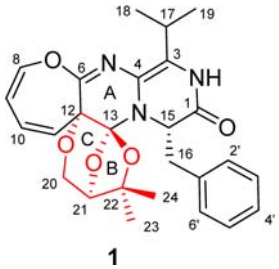


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Figure 1. Chemical structure of compound **1**.

Varioxepine A (**1**)[5] was initially obtained as a yellow amorphous solid. Its molecular formula, $C_{26}H_{29}N_3O_5$, was established from a prominent pseudomolecular ion peak at m/z 464.2184 $[M + H]^+$ (calcd 464.2180) in its HRESIMS, implying the presence of 14 degrees of unsaturation.

The ^{1}H NMR and HSQC spectra (measured in $CDCl_3$) indicated the presence of 29 protons, including three aromatic resonances comprising five protons (δ_H 7.14−7.22, H-2′−H-6′), eight signals between δ_H 3.74 and 6.53 due to protons deshielded as a result of unsaturation or attached heteroatoms (H-8−H-11, H-15, H_2-20, and H-21), four methyl groups [δ_H 1.54 (s, H_3-23), 1.52 (s, H_3-24), and 0.86 (d, J = 7.1 Hz, H_3-18 and H_3-19)], one methine signal at δ_H 3,22 (m, H-17), and one methylene at δ_H 3.08/3.24 (H_2-16) as well as an exchangeable proton signal at δ_H 7.80 (NH-2). The ^{13}C NMR spectroscopic data, in conjunction with DEPT experiments, exhibited the

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presence of 26 resonances which were assigned to four methyl, two methylene (with one oxygenated), 12 methine (with nine aromatic/olefinic), and eight quaternary (with one carbonyl and four aromatic/olefinic) carbons.

The placement of the NH unit was established by its correlations to C-1, C-4, and C-15 in the HMBC spectrum (Figure 2), while the characteristic signals for an α-carbon/

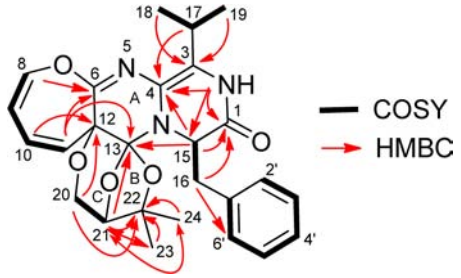

Figure 2. Selected 2D NMR correlations of compound **1**.

proton (CH-15, $\delta_{C/H}$ 56.1/4.56) and an amide carbon (C-1, δ_C 165.5) indicated the incorporation of an amino acid unit in the molecule. Detailed analysis of the COSY and HSQC data led to the identification of five spin systems, including an oxepin residue containing four methine protons (H-8−H-11), an isopropyl moiety (H-17−H-19), two −CH−CH_2− units (H-15 and H_2-16, and H_2-20 and H-21), and a monosubstituted phenyl group (H-2′−H-6′).

Further analysis of the 1D and 2D NMR data of **1** resulted in the connection of most of the above substructures (Figure 2). The ^{13}C NMR chemical shift of C-15 (δ_C 56.1) indicated that this carbon was attached to a nitrogen atom, and HMBC correlations of NH-2, H-15, and H_2-16 with the downfield sp^2 carbon (C-1, δ_C 165.5) positioned this carbon at C-1. Further HMBC correlations of H-15 and H-17 with C-4, in turn, led to the completion of the ketopiperazine ring in the molecule. The presence of the 3*H*-oxepine ring was deduced by the HMBC correlations of H-8 with C-6, along with the consideration of the downfield chemical shifts for C-6 (δ_C 154.4) and C-8 (δ_C 143.6), which suggested the connection of them via an oxygen atom. The observed HMBC correlations of H-10 with C-12 and of H-11 with C-6 provided further information to confirm the presence of the 3*H*-oxepine ring.

The fused 3,6,8-trioxabicyclo[3.2.1]octane moiety was deduced from the relevant HMBC correlations of the oxymethine proton H-21 with two oxygenated quaternary carbons C-13 and C-22 as well as with the geminal methyl groups CH_3-23 and CH_3-24. Furthermore, H-11 correlated with the oxy-carbon C-13, while H-20 correlated with the oxygenated carbons C-12 and C-22, indicating the part of the oxa-cage unit, ring C, which was condensed with the pyrimidine moiety via C-12 and C-13. However, the connection of C-13 to C-22 via an oxygen atom, which is expected to form ring B as another part of the oxa-cage unit, was not confidently assigned, since the characteristic HMBC correlation could not be detected. The remaining heteroatom (N-5) was assigned to connect with C-4 and C-6 to form the tetrahydro-pyrimidine ring, which accounted for the remaining unsaturation equivalents. However, again no useful HMBCs could be observed for the determination of the pyrimidine core rendering the assignment of this unusual planar structure somewhat ambiguous.

The lack of some key definitive 2D NMR correlations that could be used to unequivocally elucidate the structure of **1** prompted us to make efforts toward a single crystal X-ray analysis. With many attempts, some small but suitable crystals were fortunately obtained by slow evaporation and slow diffusion of H_2O into a saturated mixture of $CHCl_3$−MeOH (1:1) after eight months.[5] Once the X-ray structure could be resolved, the planar structure and the (12*R**,13*S**,15*S**,21*R**) relative configuration of **1** were confidently assigned (Figure 3).

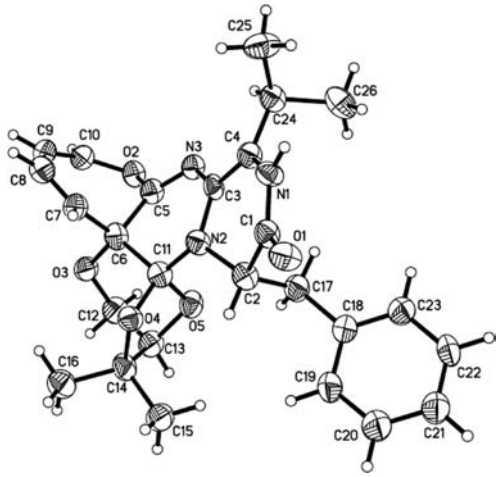

Figure 3. X-ray crystallographic structure of compound **1**. (Note: A different numbering system is used for the structure in the text.)

The X-ray structure of **1** confirmed the unique ring system including the unusual oxa-cage moiety (Figure S1 in the Supporting Information), which has not been reported among natural products yet.

The relative configuration of **1** was also studied by 2D ROESY measurements simultaneously with the X-ray analysis. ROE correlations observed between H-15, H-21, and CH_3-24 suggested the same spatial orientation of these groups. The weak correlations observed from H-11 to H-15 and H_3-24 indicated a *cis* relationship between H-11 and H-15. This suggested that the pyrimidine ring is *cis*-fused with the 1,4-dioxane ring (ring C) and *trans*-fused with the 1,4-dioxepine ring (rings B + C).[6] The phenyl protons showed ROE correlations with protons of the C-3 isopropyl methyls as well as with CH_3-24, and these correlations must derive from different conformers suggesting the axial orientation of the C-15 benzyl group (*vide infra*). Next, 1D selective gradient ROESY experiments were performed (Figure S2 in the Supporting Information), and the proton signal of H-21 at δ_H 3.97 was irradiated (Table 1). The results corroborated the previous finding of the 2D ROESY experiment, but they did not bring further information.

In order to determine the absolute configuration of **1**, conformational analysis and TDDFT-ECD calculations were performed on the arbitrarily chosen (12*R*,13*S*,15*S*,21*R*) enantiomer of **1**, which is an approach that has proved to be an efficient tool for studying the stereochemistry of condensed natural products.[7] The ECD spectrum of **1** in acetonitrile showed negative bands at 346 and 242 nm and positive ones at 298 and 213 nm (Figure 4). The DFT reoptimization of the initial MMFF conformers of (12*R*,13*S*,15*S*,21*R*)-**1** at the B3LYP/6-31G(d) level in gas phase afforded three major conformers (37.4%, 32.0%, and 26.4%) above 2% population

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Table 1. NMR Data of 1 in $CDCl_3$ (δ in ppm)[a]

no.	δ_H (mult., J [Hz])	δ_C
1		165.5 (s)
2 (NH)	7.80 (s)	
3		120.3 (s)
4		119.5 (s)
6		154.4 (s)
8	6.53 (1H, d, 7.3)	143.6 (d)
9	5.25 (1H, t, 7.3)	102.6 (d)
10	5.96 (1H, dd, 9.9, 7.3)	127.9 (d)
11	5.82 (1H, d, 9.9)	128.3 (d)
12		76.0 (s)
13		111.0 (s)
15	4.56 (1H, dd, 9.6, 4.6)	56.1 (d)
16	3.08 (1H, dd, 13.5, 9.6)	35.8 (t)
	3.24 (1H, m)	
17	3.22 (1H, m)	25.0 (d)
18	0.86 (3H, d, 7.1)	20.1 (q)
19	0.86 (3H, d, 7.1)	20.6 (q)
20	3.74 (1H, d, 11.6)	63.1 (t)
	3.95 (1H, m)	
21	3.97 (1H, m)	79.6 (d)
22		81.4 (s)
23	1.54 (3H, s)	22.0 (q)
24	1.52 (3H, s)	28.6 (q)
1′		136.8 (s)
2′,6′	7.14 (2H, m)	129.4 (d)
3′,5′	7.22 (2H, m)	128.3 (d)
4′	7.15 (1H, m)	126.6 (d)

[a]Recorded at 500 and 125 MHz for ^1H and ^{13}C NMR, respectively. Data were assigned by DEPT and HSQC as well as COSY and HMBC experiments.

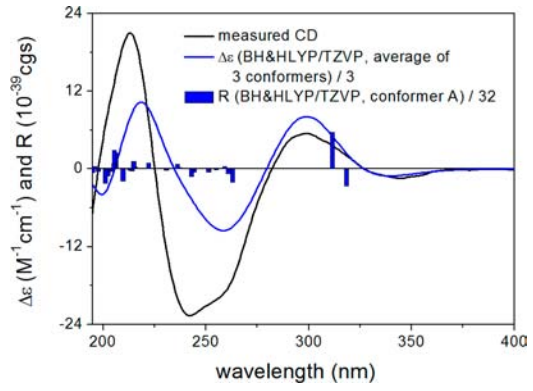

Figure 4. Experimental ECD spectrum of **1** (black curve) in acetonitrile compared with the Boltzmann-weighted BH&HLYP/TZVP spectrum (blue curve) computed for the B3LYP/6-31G(d) conformers of (12*R*13*S*,15*S*,21*R*)-**1**. Bars represent rotational strengths of the lowest-energy conformer.

(Figure 5). In all the conformers, the dihydro-oxepine ring adopted a twist-boat conformation with an axial C-12−C-11 bond, which moved the H-11 to the proximity of Hs-24 and the equatorial H-15. Although the C-15 benzyl group had axial orientation in the three conformers, the alignment of the phenyl group was different. In the lowest-energy conformer (conformer A), the phenyl group was pointing toward the C-3 isopropyl group below the piperazine ring, which was in accordance with the observed NOE correlations between the isopropyl methyl and *para* phenyl proton. In conformer B, the

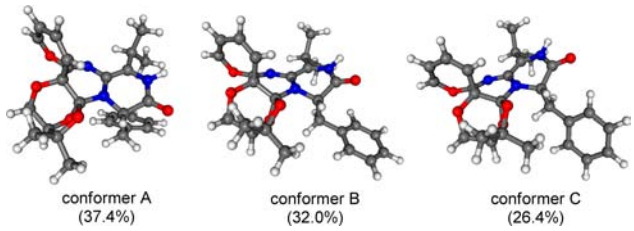

Figure 5. Structures and populations of B3LYP/6-31G(d) conformers of (12*R*,13*S*,15*S*,21*R*)-**1**.

phenyl ring was pointing away from the piperazine ring and this conformer matched the X-ray geometry and it was responsible for the NOE correlation between H_3-24 and *ortho* phenyl protons. The geometry of conformer C was quite similar to that of conformer B, but the plane of the phenyl ring was rotated. The TDDFT-ECD spectra of the conformers were calculated with three functionals (B3LYP, BH&HLYP, PBE0) and the TZVP basis set. The computed ECD spectra of all the conformers reproduced well the experimental solution ECD spectrum and the 346 nm weak positive Cotton effect (CE) derived mostly from conformers B and C. Similarly, the Boltzmann-averaged ECD spectra of (12*R*,13*S*,15*S*,21*R*)-**1** gave good agreement with the experimental curve with BH&HLYP/TZVP showing the best match (Figure 4), which allowed the determination of the absolute configuration as (+)-(12*R*,13*S*,15*S*,21*R*)-**1**.

Compound **1** was hydrolyzed in 6 N HCl (aq.) at 110 °C for 24 h to afford L-phenylalanine with $[\alpha]_D^{20}$ −30.2 (*c* 0.11, H_2O), which was determined by comparison with an authentic standard.[2f] Given the relative configuration, this result suggested (12*R*,13*S*,15*S*,21*R*) absolute configuration, which was in accordance with the result of the TDDFT-ECD calculations. It should be pointed out that the L-configuration of our phenylalanine degradation product is the same as that reported by Lu et al.[2d] and Li et al.[2f] for the related oxepine- or 3*H*-oxepine-containing diketopiperidine metabolites, but opposite to that described by Lee and co-workers.[1]

Oxepine-containing diketopiperazine alkaloids are new members of a rarely observed class of natural products containing both oxepine (or 3*H*-oxepine) and diketopiperazine ring systems. The biosynthesis and total synthesis of these related metabolites have been reported previously.[2c,3,8] Varioxepine A (**1**) may be biosynthesized by the condensation of anthranilic acid I with diketopiperazine II to yield the intermediate III, followed by oxidation of the benzene ring to form the oxepine derivative V via epoxy precursor IV, and subsequent epoxidation of V and simultaneous rearrangement of the double bonds at N-2 and C-4 as well as the opening of the epoxy ring resulted in the formation of VII via VI, as illustrated in Scheme 1.[2d] With the aid of the prenyltransferase, VII is prenylated at the C-12 hydroxyl group to yield the key intermediate VIII, which can be regarded as the precursor of **1**. Finally, cyclization to form the trioxabicyclo[3.2.1]octane subunit presumably occurred from the vicinal diol IX obtained by dihydroxylation of the prenyl group in VIII.

Compound **1** was evaluated for antimicrobial activity against several human- and aqua-pathogenic bacteria (*Aeromonas hydrophila*, *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Vibrio anguillarum*, *V. harveyi*, and *V. parahemolyticus*). The results revealed that compound **1** has diverse antibacterial activities with the MIC values ranging from 16 to 64 μg/mL (Table S1 in the Supporting Information). Moreover, **1**

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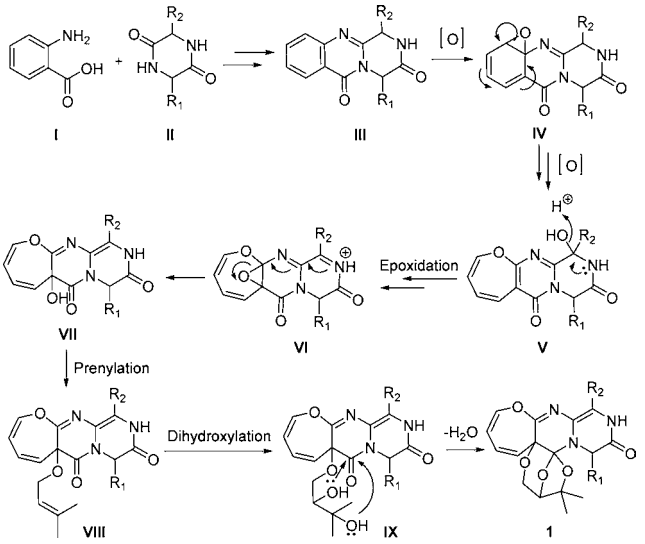
Scheme 1. Plausible Biosynthetic Pathway for Compound 1 (R_1 = Phenyl, R_2 = Isopropyl)



exhibited potent inhibitory activity against the plant-pathogenic fungus *Fusarium graminearum*, with an MIC value of 4 μg/mL.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, fungal material, extraction and isolation, antimicrobial assay, Cartesian coordinates of the computed conformers, details of computational methods and copies of 1D and 2D NMR spectra of **1** are disclosed. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(5) Varioxepine A (**1**): initially obtained as a yellow amorphous solid and then crystals were obtained after eight months from $CHCl_3$−MeOH−H_2O (5:5:1), mp 72−73 °C; $[\alpha]_D^{20}$: +65 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε): 200 (4.18), 326 (3.82) nm; ECD (MeCN, l [nm] ($\Delta\varepsilon$), *c* = 1.51 × 10^{-3} M): 346 (−1.21), 320 sh (1.75), 298 (5.36), 261 sh (−18.37), 242 (−22.88), 213 (21.88), negative CE below 197 nm. For ^{1}H and ^{13}C NMR, see Table 1; HRESIMS: *m*/*z* 464.2184 [M + H]$^+$ (calcd 464.2180).
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