Organic LETTERS

Letter

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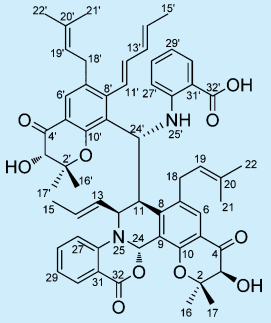
Oxazinin A, a Pseudodimeric Natural Product of Mixed Biosynthetic Origin from a Filamentous Fungus

Zhenjian Lin,[†] Michael Koch,[‡] May Hamdy Abdel Aziz,[‡] Rodrigo Galindo-Murillo,[†] Ma. Diarey Tianero,[†] Thomas E. Cheatham,[†] Louis R. Barrows,[‡] Chris A. Reilly,[‡] and Eric W. Schmidt*[,†]

[†]Department of Medicinal Chemistry and [‡]Department of Pharmacology and Toxicology, L. S. Skaggs Pharmacy Institute, University of Utah, Salt Lake City, Utah 84112 United States

Supporting Information

ABSTRACT: A racemic, prenylated polyketide dimer, oxazinin A (**1**), was isolated from a novel filamentous fungus in the class Eurotiomycetes, and its structure was elucidated spectroscopically. The pentacyclic structure of oxazinin A (**1**) is a unique combination of benzoxazine, isoquinoline, and a pyran ring. Oxazinin A (**1**) exhibited antimycobacterial activity and modestly antagonized transient receptor potential (TRP) channels.



Oxazinin A (**1**)
Racemic

Marine fungal isolates have attracted attention as an important resource for novel natural products. Most natural product-synthesizing marine fungi were isolated from marine sediment, sponges, and algae. Only 3% were isolated from ascidians,[1] marine animals that are rich sources of structurally elegant, pharmaceutically potent secondary metabolites.[2] It has been demonstrated in some cases that symbiotic bacteria produce the compounds isolated from ascidians.[3] Although most of these symbionts have yet to be cultivated, we have aimed to further examine cultivable associates of ascidians as sources of bioactive natural products. Our collection has so far yielded mainly bacterial strains, consistent with metagenomic analyses showing a preponderance of bacterial symbionts in several ascidians.[4,5] However, in one collection we identified a novel filamentous fungus, Eurotiomycetes strain 110162, the crude extract of which exhibited antibacterial activity against *Mycobacterium tuberculosis*.

Strain 110162 was isolated from the ascidian *Lissoclinum patella* collected in Papua New Guinea (10.0370785 S 145.767741 E). Strain analysis using 18S rRNA and internal transcribed spacer (ITS) gene sequences indicated that strain 110162 represents a member of the class Eurotiomycetes (Figure S15, Supporting Information). Using a variety of molecular markers, strain 110162 forms a branch point at the root of order Onygenales,[6] such that it either falls within this order as a relatively novel strain or it may form a related, new order. Gene sequences have been deposited in GenBank, accession nos. KM054976 and KM054977.

Bioassay-guided fractionation led to the isolation of oxazonin A (**1**) as the compound primarily responsible for antimycobacterial activity. The molecular formula $C_{58}H_{62}N_2O_{10}$ was assigned to **1** on the basis of ESI-FT-ICR MS analysis (*m*/*z* 947.4460 $[M + H]^+$). The structure of **1** was elucidated using a combination of NMR experiments observed in CD_3CN, including ^1H, ^{13}C, HSQC, HMBC, COSY, NOESY, and $^1H-^{15}N$-HMBC. Analysis of the ^1H, ^{13}C, and HSQC spectra (Table S1, Supporting Information, and Figure 1) suggested the presence of 10 aromatic protons and 8 double-bond protons, along with 34 olefinic or aromatic carbons and 4 carbonyls. Detailed interpretation of the HMBC and COSY correlations indicated the presence of two 4-chromanone moieties, two prenyl groups, and two anthranilate moieties, suggesting **1** has a dimeric structure.

A 4-chromanone moiety was deduced by the HMBC correlations from the aromatic singlet proton at δ_H7.41 (H-6′) to the two nonpronated aromatic carbons (C-8′ and C-10′) and the ketone carbonyl at δ_C194.4 (C-4′), along with HMBC correlations from H-3′ to C-4′ and the oxygenated quaternary carbon C-2′. An upfield chemical shift of C-4′ indicated conjugation with the phenol ring. The chemical shift of C-2′ (δ_C 84.6) and C-10′ (δ_C 157.2) indicated that the phenol ether bond of the chromanone moiety links C-2′ to C-10′. A hydroxyl group at C-3′ was deduced by the chemical shifts of H-3′ and C-3′, and by the COSY correlation between H-3′ and 3′-OH observed in DMSO-d_6 (Supporting Information). The HMBC correlations from the protons of two methyl groups (H_3-16′/17′) to C-2′ and C-3′ indicated a dimethyl substitution at C-2′. Furthermore, the HMBC correlations

Received: July 28, 2014
Published: September 4, 2014

   dx.doi.org/10.1021/ol502227x | *Org. Lett.* 2014, 16, 4774−4777

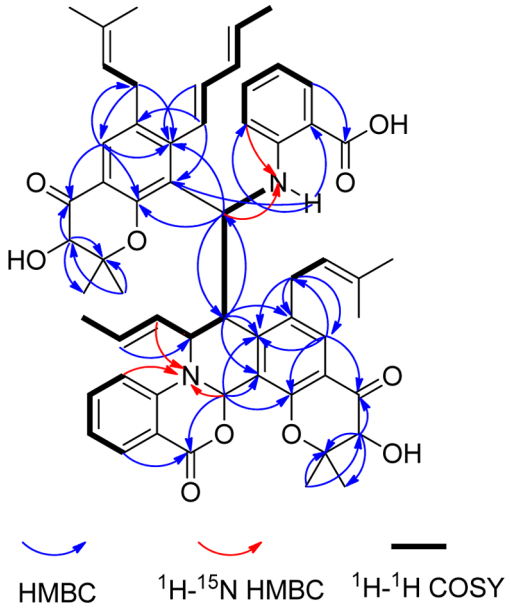


Figure 1. Key HMBC, ^{1}H−^{15}N HMBC, and COSY correlations of compound **1**.

from the methylene protons (H_2-18′) of a prenyl group to C-6′ and C-8′ indicated a prenyl substituent group at C-7′ of the 4-chromanone moiety, which was further confirmed by a NOESY correlation between H_2-18′ and H-6′ and the HMBC correlation from H-6′ to C-18′. A spin system from H-11′ to H_3-15′ was determined to result from a pentadienyl unit by COSY and NOESY correlations. The HMBC correlations from H-11′ to C-7′ and C-9′, and from H-12′ to C-8′, indicated that the pentadienyl group is located at C-8′.

The 4-chromanone moiety thus established was shown to be connected to the nitrogen atom of an anthranilate unit. The COSY correlations of H-27′/28′/29′/30′ and the HMBC correlations from H-30′ to a carboxylic carbonyl (C-32′), and from NH proton H-25′ to C-27′ and C-31′ established an anthranilate moiety. The chemical shift of C-32′ at δ_C170.9, along with the observation of an exchangeable proton at δ_H10.34 indicated a carboxylic acid at C-32′. Furthermore, the HMBC correlations from H-25′ to C-9′ of the 4-chromanone moiety, and from H-24′ to C-8′ and C-10′, as well as a COSY correlation between H-25′ and H-24′ confirmed the connectivity between the 4-chromanone moiety and the anthranilate moiety through the C24′-N25′ bond. A ^{1}H−^{15}N HMBC experiment further reinforced the connectivity: correlations were observed from H-24′ and H-27′ to N-25′.

On the second half of the dimer structure of **1**, prenylated 4-chromanone and anthranilate moieties were similarly confirmed by 2D NMR correlations (Table S1, Supporting Information, and Figure 1). Both halves of the dimer showed similar chemical shifts, with three notable exceptions. (1) No exchangeable NH proton was observed for the second anthranilate moiety, indicating a trisubstituted N atom. The chemical shift of the carbonyl (δ_C 166.1, C-32) was shifted upfield by 4 ppm in comparison to that of C-32′, suggesting an ester bond at C-32 rather than a free carboxylic acid. (2) A methine group (δ_H 6.51, H-24, δ_C 80.8, C-24) was connected to C-9 of the second 4-chromanone moiety, as shown by the HMBC correlations from H-24 to C-8, C-9, and C-10. The methine proton H-24 also showed a HMBC correlation to C-32, indicating that the ester bond is between C-32 and C-24. The methine proton H-24 has an unusual downfield chemical shift of δ_H 6.51, which suggested that C-24 was substituted by both oxygen and nitrogen. ^{1}H−^{15}N HMBC correlations from H-24 to N-25 and from H-27 to N-25 suggested that the second anthranilate moiety and the methine group at C-24 were cyclized to form a hydro-1,3-benzoxazine-4-one substructure (Figure 1). (3) The presence of two more methine carbon signals (δ_H 4.56, H-11, δ_C 46.0, C-11 and δ_H 3.85, H-12, δ_C 59.1, C-12) indicated the pentadienyl group at C-8 of the second 4-chromanone moiety was further substituted at both C-11 and C-12, due to the absence of a double bond, and instead, the presence of two more methine carbon signals was evident (δ_H 4.56, H-11, δ_C 46.0, C-11 and δ_H 3.85, H-12, δ_C 59.1, C-12). This is consistent with the absence of a double bond on the second half of the compound. The ^{1}H−^{15}N HMBC correlation from H-13 to N-25 and the HMBC correlations from H-11 to C-7, C-8, and C-9 established the second monomeric unit of **1** as shown in Figure 1. Finally, the COSY correlation between H-24′ and H-11, along with the HMBC correlations from H-24′ to C-11 and from H-11 to C-24′, indicated the **1** is pseudodimeric and the units are linked through the C-11-C-24′ bond.

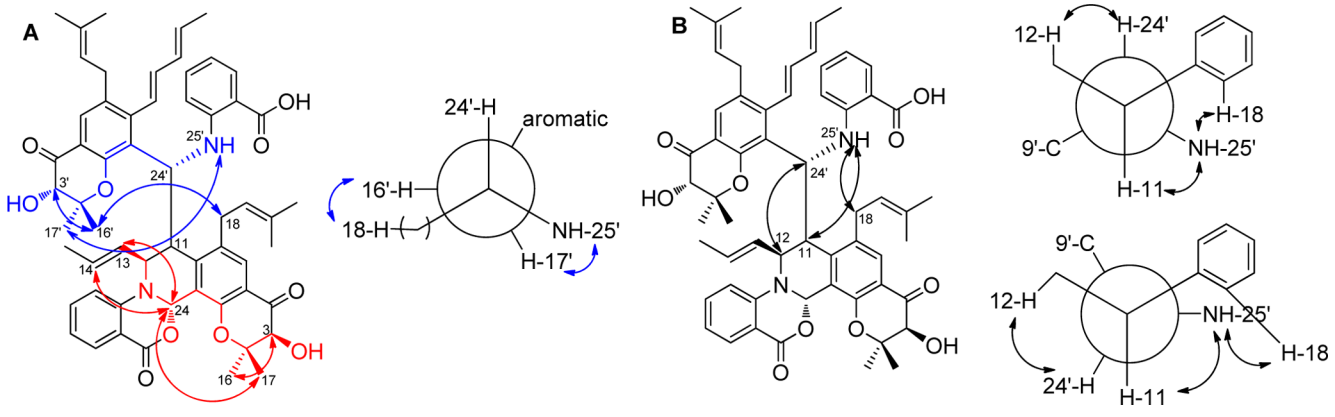


Figure 2. Key ROESY correlations for compound **1**. (A) ROESY correlations in red and blue determine the relative configurations of centers C-3, C-24, C-12 and C-3′, C-24′, respectively. (B) Along with a 10 Hz coupling constant between H-24′ and H-11, the ROESY correlations (black arrows) define the relative configuration of the molecule except at position C-11, for which two possibilities remained. Presented are not true Newman projections, but they clearly indicate the relative orientations of the labeled groups.

dx.doi.org/10.1021/ol502227x | *Org. Lett.* 2014, 16, 4774−4777

The ROESY correlations H-24/H-13 and H-24/H_3-17 indicated that these protons are in the β-orientation (Figure 2A). The ROESY correlations between H-3/H_3-16 and between H_3-17/H-24 indicated that the 3-OH is also in the β-orientation, establishing stereocenters 3*S**,12*S**,24*S**. A ROESY correlation between H-3′ and H_3-16′ indicated that these protons are on the same face (Figure 2A). Moreover, the strong ROESY correlations between H_3-16′/H_2-18 and H_3-17′/H-25′ are only possible if the molecule adopts a preferred conformation orienting the pyrone portion of the 4-chromanone moiety anti (or nearly anti) to H-24′. This allowed the relative configuration between C-24′ and C-3′ to be assigned as 3′*S**,24′*R**. What remained was to define the relative configuration between C-12/C-11/C-24′.

From modeling with Chem3D and molecular dynamics simulation (MD) using AMBER 14,[7] it was clear that rotation about the bonds C-11/C-24′/C-9′ was hindered due to steric constraints, and the molecule should adopt a single conformation. The large coupling constant (10.0 Hz) between H-11 and NH-24′ suggested either a nearly 180° or a 0° dihedral angle between H-11 and H-24′. The ROESY correlations between H-11/H-25′ and H_2-18/NH-25′ were only likely if H-11 was in the eclipsed conformation with H-24′ in the 11*R**,24′*S** configuration or if the bond was in the anti conformation with the 11*R**,24′*R** configuration (Figure 2B).

With that information, the four remaining possible configurations of the molecule were 3*S**,12*S**,24*S** with the remaining centers: (A) 11*R**,3′*R**,24′*S** (*eclipsed* C-11/C-24′); (B) 11*S**,3′*S**,24′*R** (*eclipsed* C-11/C-24′); (C) 11*R**,3′*S**,24′*R** (*anti* C-11/C-24′); and (D) 11*S**,3′*R**,24′*S** (*anti* C-11/C-24′). Possibilities A and D could be readily ruled out because, with these isomers, H_3-16′/H_2-18 are very distantly located and therefore could not lead to the observed ROESY correlation. Finally, molecular modeling was used to determine the configuration of C-11 (Figure 3).

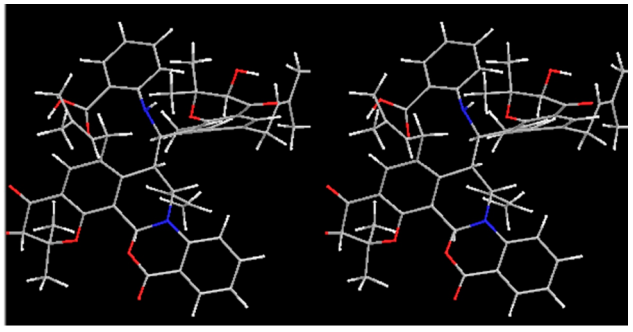


Figure 3. Optimized 3D structure of **1**.

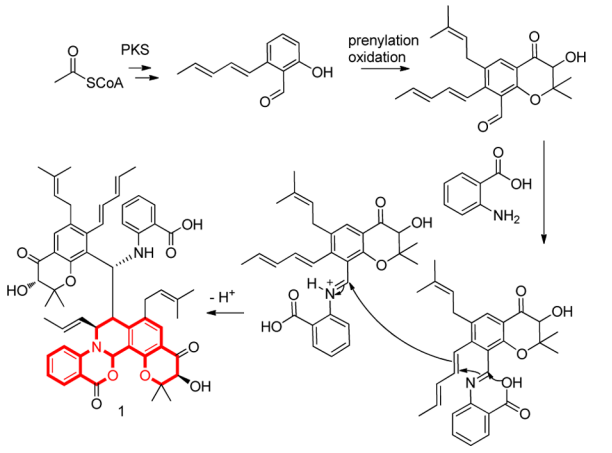
The structures of possibilities B and C were initially optimized using density functional theory with the Truhlar functional M06-2X with the 6-31G(d) basis set as implemented in the D.01 revision of the Gaussian package.[8] The resulting optimized structure was used as the starting structure for further MD optimization using 1000 minimization steps with the steepest descent algorithm and 1000 steps using the conjugated gradients methods. The structure was then heated to 3000 K and slowly reducing the temperature every 5000 steps until a target temperature of 300 K was reached. After the heating stage, distance restraints (Table S2, Supporting Information) were applied for 50000 steps. Results showed that possibility C fit all of the available NOESY data (Figure 3), with the total distance penalty of 0.472 Å, while possibility B had many substantial deviations: higher total distance penalty (2.188 Å) (Table S2, Supporting Information) and a ∼60° dihedral between H-11 and H-24′ (Table S4, Supporting Information). Thus, the relative configuration of **1** is proposed to be 3*S**,11*R**,12*S**,24*S**,3′*R**,24′*R**.

The absolute configuration of **1** was determined by CD spectroscopy. Although a strong dynode voltage was seen corresponding to the UV absorption spectrum of **1**, there was no observed Cotton effect (Figure S17, Supporting Information), indicating that **1** is racemic.

Oxazinin A (**1**) was tested for inhibitory activity against a panel of bacteria, using a previously described method.[9] It showed inhibitory activity only to *M. tuberculosis* (IC_{50} 2.9 μM). We also tested the cytotoxicity of **1** against human CEM-TART T-cell leukemia line (LC_{50} 4.7 μM). Compound **1** exhibited modest potency for inhibition of several human transient receptor potential (TRP) channels. IC_{50} values of 6.6 and 50.8 μM were obtained for TRPM8 and TRPV4, respectively. Compound **1** exhibited inhibition in assays for TRPA1 and TRPV3(∼50% and 36% at 26 μM, respectively), although these activities were not thoroughly explored because of low potency. Compound **1** did not inhibit TRPV1.

A biogenetic hypothesis is proposed for **1** as shown in Scheme 1. First, a polyketide synthase would lead to formation

Scheme 1. Proposed Biogenesis of Compound 1



of an unsaturated aldehyde intermediate, which is subsequently isoprenylated. Precedent for this first intermediate is provided by a series of related polyketides produced by *Magnaporthe*, *Fusarium*, and other fungi.[10−12] Some of these compounds are also prenylated, although not in the same manner as **1**. Attack of the resulting aldehyde by the NH_2 group of the anthranilate unit would lead to a reactive intermediate that could subsequently dimerize to yield **1**. Dimerization could proceed either as shown or through the intermediacy of either one or two aminal units. Because **1** is racemic, it is likely that this final condensation step takes place nonenzymatically within the fungus. Recently, it has become increasingly clear that rare, potently bioactive natural products can be formed non-enzymatically, despite their potential importance to the biology of the producing organism.[13]

Although anthranilate is a very common precursor to a large variety of heterocyclic natural product compounds,[14] this is the first report of anthranilate in a natural benzoxazine ring. The pentacyclic substructure in **1** is also the first example of the

dx.doi.org/10.1021/ol502227x | *Org. Lett.* 2014, 16, 4774−4777

unique combination of benzoxazine, isoquinoline, and a pyran ring.

Oxazinin A (**1**) exhibits many structurally novel features, and it comes from a taxonomic group of fungi for which little chemical data is available. The strain groups most closely with the order Onygenales, a group from which few compounds have been described. However, it may not lie within that order, but instead may represent a fairly novel fungal group. Further work will be needed to clarify the phylogenetic relationships among these strains. Further work is also required to determine whether or not strain 110162 is a true associate of the ascidian from which it was cultivated, as well as whether it is a true marine fungus.

■ ASSOCIATED CONTENT

Supporting Information

Full NMR data and experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: (801) 585-5234. Fax: (801) 585-9119. E-mail: ews1@utah.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was funded by NIH GM107557 and U01TW008163. Strain 110162 was obtained with the appropriate legal permissions and with a revenue-sharing agreement in place. Computer time is gratefully acknowledged from the NSF Extreme Science and Discovery Environment XRAC MCA01S027 and the University of Utah Center for High Performance Computing.

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