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Macrotermycins A–D, Glycosylated Macrolactams from a Termite-Associated *Amycolatopsis* sp. M39

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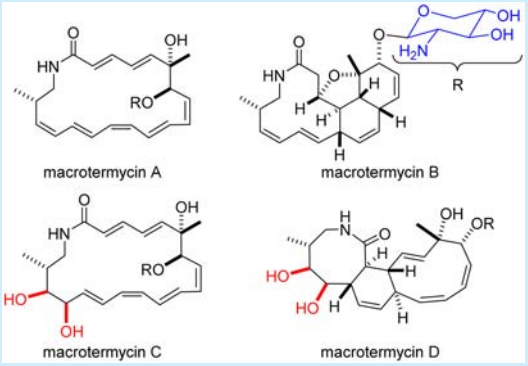
Supporting Information

ABSTRACT: Bioassay-guided metabolomic analyses led to the characterization of four new 20-membered glycosylated polyketide macrolactams, macrotermycins A–D, from a termite-associated actinomycete, *Amycolatopsis* sp. M39. M39's sequenced genome revealed the macrotermycin's putative biosynthetic gene cluster. Macrotermycins A and C had antibacterial activity against human-pathogenic *Staphylococcus aureus* and, of greater ecological relevance, they also had selective antifungal activity against a fungal parasite of the termite fungal garden.



Explorations of the bacterial symbionts of phylogenetically diverse insects have repeatedly led to biologically active natural products with interesting chemical scaffolds.[1] The bacteria often provide small molecule chemical defenses that selectively inhibit the insects' microbial competitors and pathogens. The small molecule defenses found in current studies reflect the numerous rounds of mutation, selection, and amplification between the insects' defenders and antagonists.

Our earlier studies investigated symbiotic bacteria from ants, beetles, and termites that live in large colonies and grow fungi in specialized gardens. The bacteria provide chemical defenses against microbial threats, especially against fungal parasites that specialize in consuming the fungal crop. These studies led to selective antifungal agents such as the depsipeptide dentigerumycin from a fungus-growing ant (*Apterostigma dentigerum*) symbiont[2] and the polyene peroxide mycangimycin from a Southern pine beetle (*Dendroctonus frontalis*) symbiont.[3] While our initial studies of bacteria associated with a species of fungus-growing termites (*Macrotermes natalensis*)[4] led to the discovery of chemically intriguing compounds like the microtermolides[5] and natalamycin A,[6] they did not identify any *selective* antifungal agents. We focused our efforts on finding selective agents from bacteria associated with *M. natalensis* through a systematic combination of LC–HRMS-based dereplication and bioactivity assays against the basidiomycete cultivar (*Termitomyces* spp.) and an ascomycete competitor (*Pseudoxylaria* spp.).[7] As a result of these studies, the actinomycete *Amycolatopsis* sp. M39 was found to exhibit both a unique metabolomic profile and promising anti-*Pseudoxylaria* activity. We now report the isolation and structural characterization, biosynthetic analysis, and biological activity of four new macrolactams isolated from *Amycolatopsis* sp. M39. Two of the four exhibited selective inhibition of the *Pseudoxylaria* pathogen over the *Termitomyces* cultivar.

We started our systematic analysis with 41 termite-associated Actinobacteria isolated from a South African *M. natalensis* colony.[8] Principal component analysis (PCA) on preprocessed

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LC−HRMS traces identified *Amycolatopsis* sp. M39 as having a unique metabolomic profile. The putative new natural products responsible for the variance observed in the PCA were quasimolecular ions at m/z 473.2678 and 489.2622. Analysis of the metabolomic profiles indicated a strong time and medium dependence. Whereas the first group of putative new compounds (m/z 473.2678) was mainly detected after 7 days of cultivation, the second group of compounds (m/z 489.2622) increased with longer growth times. We also tested *Amycolatopsis* sp. M39 in ecologically relevant plate challenge assays and found that it suppressed the growth of *Pseudoxylaria* sp. (strain X802) and insect entomopathogenic fungi such as *Beauveria bassiana* and *Metarhizium anisopliae*. The cultivar's (*Termitomyces* sp.) relative resistance to M39 was especially noteworthy (Figure S3).

LC−MS analysis extracts from the zone of inhibition (ZOI) revealed a set of metabolites matching the molecular weight of the putative new compounds (m/z 473.3 and 489.3) identified in the metabolomic analysis. To identify these compounds, we performed a preparative-scale fermentation of *Amycolatopsis* sp. M39 on solid ISP-2 agar for 14 d at 30 °C. Agar plates densely covered with *Amycolatopsis* sp. M39 were extracted using *i*-PrOH. The crude extract was purified using an activated prepacked C18 cartridge, followed by preparative and semi-preparative reversed-phase HPLC. LC−MS analysis revealed the HPLC fractions that contained compounds with m/z 473.3 and 489.3. The first isolated compound, macrotermycin A (**1**), was obtained as a colorless solid with a quasimolecular ion of 473.2678 $[M + H]^+$ consistent with **1** exhibiting a molecular formula of $C_{26}H_{36}N_2O_6$ (Figure 1A).[9] The ^{1}H NMR spectrum in DMSO-d_6 indicated a complex overlap of 14 olefinic protons from δ_H 5.10 to δ_H 6.60, four oxygenated methine protons between δ_H 3.00 and δ_H 4.40, two oxygenated methylene protons at δ_H 3.01 and 3.68, one amine-bearing methine proton at δ_H 2.50, an NH signal at δ_H 7.66, two N-amidated methylene protons at δ_H 2.72 and 3.10, and seven aliphatic protons that include two methyl groups at δ_H 0.91 and 1.33. Analysis of the HSQC and HMBC spectra allowed assigning the 14 olefinic carbons between δ_C 120 and δ_C 145, one amide carbonyl carbon at δ_C 165.3, six oxygen-bearing carbons (δ_C 65−105) that include one quaternary carbon at δ_C 75.3 and an anomeric carbon at δ_C 104.8, one amine-bearing carbon at δ_C 57.3, and four aliphatic carbon signals (δ_C 17−45). 2D NMR (COSY, TOCSY, HMBC) analysis revealed a macrolactam core structure coupled with a deoxypentopyranose aminosugar group. The long-range correlation of H-1′ (anomeric proton) to C-7 in the HMBC experiment showed the linkage of the aminosugar unit to the macrocyclic lactam. The stereochemistry of the double bonds was determined as 2*E*,4*E*,8*Z*,10*E*,12*Z*,14*E*,16*Z* on the basis of the characteristic coupling constants observed in the homo *J*-resolved ^{1}H NMR spectrum and corresponding NOESY correlations. The stereochemistry of the three chiral centers of the macrotermycin aglycone was established by NOESY correlations and biosynthetic relation. A strong NOE correlation between H-7/H-10 was observed in a similar fashion to that seen for macrotermycin D (**4**) (vide infra), which sets the stereoconfiguration of C-7 as *R** (Figure 1 and Figure S6). The configuration of C-6 and C-18 cannot unambiguously be resolved by NOE data alone; instead, C-6 and C-18 were assigned on the basis of the configurational analysis of macrotermycin B (**2**) and **4**. The aminosugar moiety was found to be 2-deoxy-2-amino-β-L-xylopyranose using a

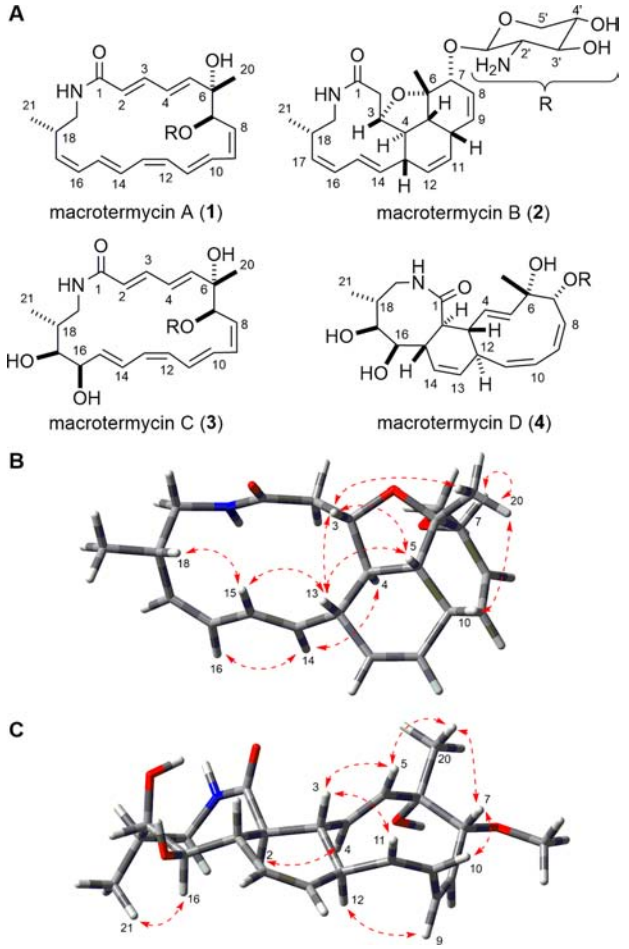

Figure 1. (A) Structures of macrotermycins A−D. Computational models of (B) a macrotermycin B derivative and (C) a macrotermycin D derivative calculated at the DFT B3LYP/6-31+G(d,p) level of theory without geometric constraints and supported by key NOE/ROE correlations (amino sugar moiety was replaced by a methoxy group to simplify calculations).

combination of 1D and 2D NMR analysis, coupling constant analysis, and Snatzke's method and was found to be identical for all isolated macrotermycin derivatives.[8] In total, the data indicate **1** as the most probable structure for macrotermycin A.

Macrotermycin B (**2**), which is less polar than **1**, has the same quasimolecular ion of 473.2678 $[M + H]^+$ that is consistent with **2** having a molecular formula of $C_{26}H_{36}N_2O_6$. Compound **2** exhibited a very different ^{1}H NMR spectrum with isolated ^{1}H spin systems and only eight distinct olefinic protons. Analysis of COSY, TOCSY, and HMBC correlations revealed the presence of a macrolactam as part of a fused polycyclic ring system (Figure 1A). NOE correlations were observed between H-3/H-5, H-3/H-13, and H-5/H-13 in conjunction with observed good agreement between relevant experimental vicinal scalar coupling constants ($J_{3,4}$ = 9.0, $J_{4,5}$ = 12.5; $J_{5,10}$ = 6.0, $J_{4,13}$ = 10.0 Hz) and estimated values ($J_{3,4}$ = 10.0, $J_{4,5}$ = 12.7; $J_{5,10}$ = 6.7, $J_{4,13}$ = 11.2 Hz), as determined in the Schrödinger software suite using the coupling constant measuring tool on a DFT optimized analogue of **2** (Figure 1B).[8] The values indicated that H-3, H-5, H-10, and H-13 exhibit the *cis* stereoconfiguration. The C-6 configuration was assigned as *S** through further computational analysis and observation of NOE correlations between H-3/H_3-20, H-7/

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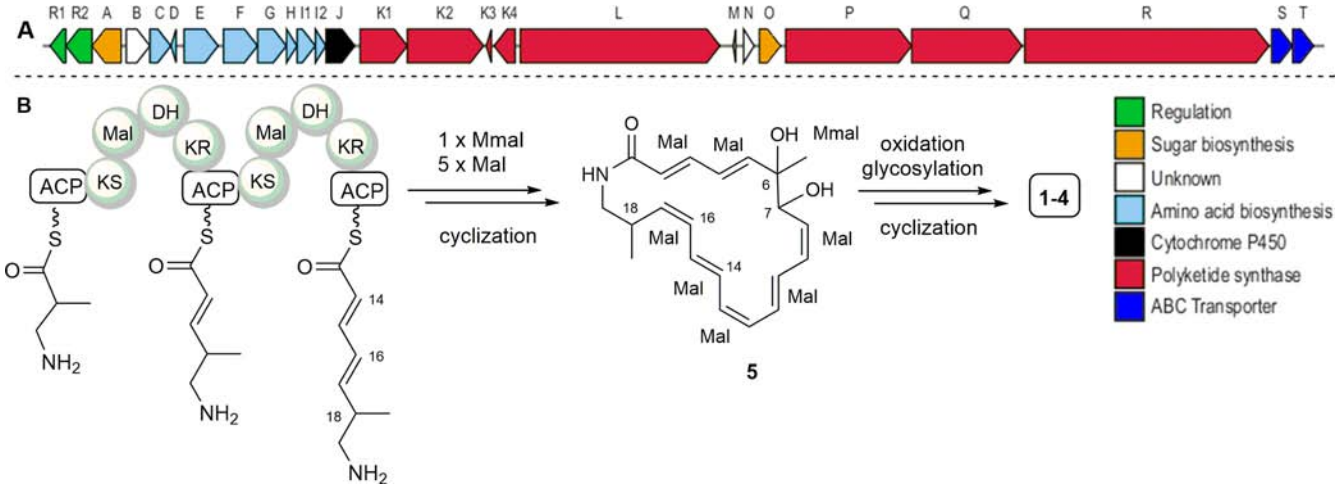

Figure 2. Organization of the putative macrotermycin biosynthetic gene cluster from *Amycolatopsis* sp. M39. (A) Genetic map of the Mte gene cluster; each arrow represents the direction of transcription of open reading frame (ORF). (B) Putative biosynthetic scheme of macrotermycin aglycone (**5**) formation.

H_3-20, and H-10/H_3-20. The same strong H-7/H_3-20 NOE correlation along with the presumed biosynthetic similarity with other macrotermycins facilitated predicting C-7 as *R**. An NOE correlation between H-15/H-18 confirmed C-18 as *S**.[8]

We then focused on the second set of putative new compounds with quasimolecular ion 489.2622 $[M - H_2O + H]^+$ corresponding to those compounds exhibiting a molecular formula of $C_{26}H_{38}N_2O_8$. A comparative NMR analysis of macrotermycin C (**3**) vs **1** revealed that **3** contains 12 olefinic protons and two additional hydroxyl groups (δ_C 66.0 and δ_C 79.0), which accounted for the higher polarity of **3** and led to a proposed structure (Figure 1A). All alkene geometries were confirmed by analyzing *J*–*J* resolved 2D proton spectra, and ROESY correlations between H-2/H-4 and H-3/H-5 were also identified. The stereochemistry of the aglycone chiral centers was determined using ROE correlations and biosynthetic relation and is predicted to be homologous to compounds **1**, **2**, and **4**, respectively.[8] 2D NMR analysis of macrotermycin D (**4**) revealed eight olefinic protons and bonds between C-2/C-15 and C-3/C-12, where the latter yields a tricyclic ring system (Figure 1A). Strong ROE correlations between H-2/H-4, H-3/H-11, H-3/H-5, H-7/H-10, and H-7/H-1′ as well as comparison of experimental coupling constants ($J_{2,3}$ = 11.0; $J_{2,15}$ = 10.0; $J_{3,12}$ = 10.0 Hz) to those estimated for a DFT-optimized analogue of **4** (Figure 1C) ($J_{2,3}$ = 12.2; $J_{2,15}$ = 12.1; $J_{3,12}$ = 10.6 Hz) revealed an all-*trans* substitution pattern for the cyclohexenyl ring and allowed for C-7 stereoconfigurational assignment. A strong ROE correlation between H_3-20/H-5 and H_3-20/H-7 in relation to other macrotermycins allows us to predict that C-6 exhibits *S** stereoconfiguration. Based on comparing experimental coupling constants ($J_{15,16}$ = 9.0 Hz, $J_{16,17}$ = 2.0 Hz, $J_{17,18}$ = 0.5 Hz) with estimated values ($J_{15,16}$ = 9.9 Hz, $J_{16,17}$ = 0.9 Hz, $J_{17,18}$ = 1.7 Hz), the dihedral angle between H-15 and H-16 is calculated to be close to 180°, whereas the dihedral angles between H-16/H-17 and H-17/H-18 are assumed to be close to 90°, defining the *cis* diol stereoconfiguration (C-16, C-17) (Figure 1C). An ROE correlation between H-16/H_3-21 allowed us to define C-18 as *R** (vs *S** in **1** and **2** due to a Cahn–Ingold–Prelog priority change arising from the C-17 hydroxyl).[8]

The core structure of the macrotermycins (**5**) belongs to a family of macrolactam polyketides that includes incednine,[10] vicenistatin,[11] silvalactam,[12] heronamides,[13] verticilactam,[14] ciromicins,[15] mirilactams, and lobosamides.[16] Both **1** and **3** can be viewed as polyene precursors for intramolecular cycloadditions leading to **2** and **4**, respectively, and the relevant reaction pathways, whether enzymatically catalyzed or occurring spontaneously either in situ or during purification, are being investigated. Formation of the substituted tetrahydrofuranyl ring in **2** can occur through a 1,4-conjugate addition of a C-6 hydroxyl group to proximal $\Delta^{2,3}$ alkenyl bond in an unisolated precursor. To gain further insights into macrotermycin biogenesis, the *Amycolatopsis* sp. M39 genome was sequenced and annotated (accession no. LWSF00000000, Figure 2).[8] The likely macrotermycin biosynthetic gene cluster was identified on the basis of significant homology to the known vicenistatin cluster.[17] The cluster includes the biosynthesis of the 3-amino-2-methylpropionate starter unit (MetC-MteI). It also harbors four PKS genes (MteL, MteP-R), while a fifth PKS (MteK) is fragmented and a likely pseudogene. Only PKS module MteQ is lacking a dehydratase and terminates with a ketoreductase (KR), which was classified as an "A-type" reductase based on comparative alignment of the amino acid sequence of the ketoreductase catalytic site.[18] In this analysis, the polyketide chain is released from the PKS by the termination domains in either MteK or MteL. Oxidation at position C-6, C-16 and C-17 are presumably catalyzed by the encoded cytochrome P-450 (MteJ). O-Glycosylation at position C-7 likely involves the glycosyltransferase MteA or MteO. This preliminary biogenetic analysis is also under investigation.

Macrotermycins A-D were assayed for activity against Gram-negative (*Escherichia coli* ATCC 11775) and Gram-positive (*Bacillus subtilis* ATCC 6051; *Staphylococcus aureus* ATCC 25923) bacteria, and yeasts (*Candida albicans* ATCC 24433, *Saccharomyces cerevisiae* ATCC 9763). While **1** showed moderate to good antimicrobial activity, **3** was only weakly active (Table 1). Both compounds (**1** and **3**) modestly inhibited *Pseudoxylaria* sp. X802 growth. Importantly, growth of the termites' symbiotic fungus *Termitomyces* sp. T112 was not significantly affected by **1** or **3**. In contrast, compounds **2** and **4** showed no activity against all tested strains in the tested concentration range (MIC > 100 μg/mL).

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Table 1. Minimal Inhibitory Concentrations (μg/mL) of 1 and 3 (Positive Controls: Ciprofloxacin and Amphotericin B)

strain	MIC (μg/mL)		
	1	3	positive control
			ciprofloxacin
B. subtilis ATCC 6051	1.0	15	0.3
S. aureus ATCC 25923	1.5	10	0.5
			amphotericin B
C. albicans ATCC 10231	10	25	0.3
S. cerevisiae ATCC 9763	5.0	20	0.2
Termitomyces sp. T112	>100	>100	~ 65
Pseudoxylaria sp. X802	~ 50	~ 80	~ 10

In summary, we have isolated and characterized four previously unreported macrolactam polyketides, macrotermycins A–D, from *Amycolatopsis* sp. M39, proposed their relative structures, and presumptively identified the biosynthetic gene cluster responsible for their production. Macrotermycins A (**1**) and C (**3**) were shown to exhibit selective inhibition of the termite fungal garden competitor *Pseudoxylaria* sp. X802. The identification of these *selective* antifungal compounds demonstrates the potential of using ecologically relevant assays to identify biologically active compounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03831.

Procedures for isolation and characterization of *Amycolatopsis* sp. M39, gene cluster analysis, dereplication methods, challenge assays, compound purification, activity assays, computational procedures, Cartesian coordinates, detailed NMR and computational analysis, HRMS data (PDF)
NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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