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## Macrotermycins A-D, glycosylated macrolactams from a termite-associated *Amycolatopsis* sp. M39

Christine Beemelmans<sup>§,1,\*</sup>, Timothy R. Ramadhar<sup>§,2</sup>, Ki Hyun Kim<sup>§,3</sup>, Jonathan L. Klassen<sup>4</sup>, Shugeng Cao<sup>5</sup>, Thomas P. Wyche<sup>2,6</sup>, Yanpeng Hou<sup>6</sup>, Michael Poulsen<sup>7</sup>, Tim S. Bugni<sup>6</sup>, Cameron R. Currie<sup>8</sup>, and Jon Clardy<sup>2,\*</sup>

<sup>1</sup>Leibniz Institute for Natural Product Research and Infection Biology – Hans-Knöll-Institute, 07745 Jena, Germany

<sup>2</sup>Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, 02115, USA

<sup>3</sup>Natural Product Research Laboratory, School of Pharmacy, Sungkyunkwan University, Suwon 440-746, Republic of Korea

<sup>4</sup>Department of Molecular & Cell Biology, University of Connecticut, Storrs, CT, 06269, USA

<sup>5</sup>Daniel K Inouye College of Pharmacy, University of Hawaii at Hilo, HI, 96720, USA

<sup>6</sup>Pharmaceutical Sciences Division, School of Pharmacy, University of Wisconsin–Madison, Madison, WI, 53705, USA

<sup>7</sup>Centre for Social Evolution, Section for Ecology and Evolution, Department of Biology, University of Copenhagen, 2100 Copenhagen East, Denmark

<sup>8</sup>Department of Bacteriology, University of Wisconsin–Madison, Madison, WI, 53705, USA

### 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 *S. aureus* and of greater ecological relevance, they also had selective antifungal activity against a fungal parasite of the termite fungal garden.

### Graphical Abstract

\*Corresponding Authors: christine.beemelmans@hki-jena.de, jon\_clardy@hms.harvard.edu.

#### §Author Contributions

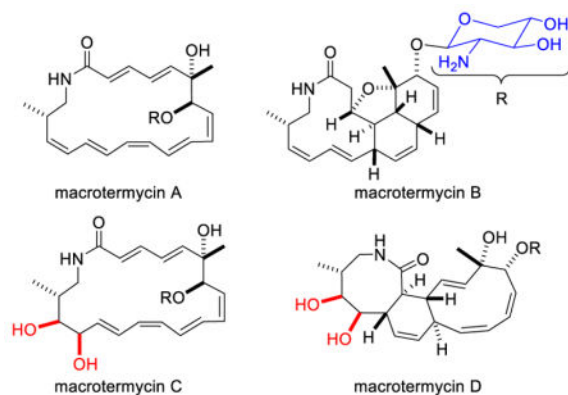
C.B., T.R.R., and K.H.K. contributed equally to this work.

#### Notes

The authors declare no competing financial interest.

#### Supporting Information

Procedures for isolation and characterization of *Amycolatopsis* sp. M39, dereplication methods, challenge assays, compound purification, HRMS and NMR spectra, detailed NMR and computational analysis, activity assays, computational procedures, and Cartesian coordinates. The Supporting Information is available free of charge on the ACS Publications website.



Explorations of the bacterial symbionts of phylogenetically diverse insects have repeatedly led to biologically active natural products with interesting chemical scaffolds.<sup>1</sup> The bacteria 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 lived in large colonies and grew fungi in specialized gardens. The bacteria provided chemical defenses against microbial threats, especially against fungal parasites that specialized 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,<sup>2</sup> and the polyene peroxide mycangimycin from a Southern pine beetle (*Dendroctonus frontalis*) symbiont.<sup>3</sup> While our initial studies of bacteria associated with a species of fungus-growing termites (*Macrotermes natalensis*)<sup>4</sup> led to the discovery of chemically-intriguing compounds like the microtermolides<sup>5</sup> and natalamycin A,<sup>6</sup> they did not identify any *selective* antifungal agents. We focused our efforts to find 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.).<sup>7</sup> As a result of these studies, the actinomycete *Amycolatopsis* sp. M39 was found to exhibit both 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.<sup>8</sup> Principal component analysis (PCA) on preprocessed 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 quasi-molecular 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 seven 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 (Fig. 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 pre-packed C18 cartridge, followed by preparative and semi-preparative reverse-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 quasi-molecular ion of 473.2678 [M+H]<sup>+</sup> consistent with **1** exhibiting a molecular formula of C<sub>26</sub>H<sub>36</sub>N<sub>2</sub>O<sub>6</sub> (Fig. 1A).<sup>9</sup> The <sup>1</sup>H NMR spectrum in DMSO-*d*<sub>6</sub> indicated a complex overlap of 14 olefinic protons from δ<sub>H</sub> 5.10 to δ<sub>H</sub> 6.60, four oxygenated methine protons between δ<sub>H</sub> 3.00 and δ<sub>H</sub> 4.40, two oxygenated methylene protons at δ<sub>H</sub> 3.01 and 3.68, one amine-bearing methine proton at δ<sub>H</sub> 2.50, an NH signal at δ<sub>H</sub> 7.66, two N-amidated methylene protons at δ<sub>H</sub> 2.72 and 3.10, and nine aliphatic protons that include two methyl groups at δ<sub>H</sub> 0.91 and 1.33. Analysis of the HSQC and HMBC spectra allowed assigning the 14 olefinic carbons between δ<sub>C</sub> 120 and δ<sub>C</sub> 145, one amide carbonyl carbon at δ<sub>C</sub> 165.3, six oxygen-bearing carbons (δ<sub>C</sub> 65–105) that include one quaternary carbon at δ<sub>C</sub> 75.3 and an anomeric carbon at δ<sub>C</sub> 104.8, one amine-bearing carbon at δ<sub>C</sub> 57.3, and four aliphatic carbon signals (δ<sub>C</sub> 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*, and 16*Z* on the basis of the characteristic coupling constants observed in the homo *J*-resolved <sup>1</sup>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. An 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*\* (Fig. 1 and 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 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.<sup>8</sup> 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 quasi-molecular ion of 473.2678 [M+H]<sup>+</sup> that is consistent with a molecular formula of C<sub>26</sub>H<sub>36</sub>N<sub>2</sub>O<sub>6</sub>. Compound **2** exhibited a very different <sup>1</sup>H NMR spectrum with isolated <sup>1</sup>H spin systems and only eight

distinct olefinic protons. Analysis of gCOSY, TOCSY, and HMBC correlations revealed the presence of a macrolactam as part of a fused polycyclic ring system (Fig. 1A). NOE correlations observed between H-3/H-5, H-3/H-13, and H-5/H-13 in conjunction with observing 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 analog of **2** (Fig. 1B).<sup>8</sup> The values indicated that H-3, H-5, H-10, and H-13 exhibit *cis* stereoconfiguration. C-6 configuration was assigned as *S*\* through further computational analysis and observing NOE correlations between H-3/H<sub>3</sub>-20, H-7/H<sub>3</sub>-20, and H-10/H<sub>3</sub>-20. The same strong H-7/H<sub>3</sub>-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 affirmed C-18 as *S*\*.<sup>8</sup>

We then focused on the second set of putative new compounds with quasi-molecular ion 489.2622 [M-H<sub>2</sub>O+H]<sup>+</sup> corresponding to those compounds exhibiting a molecular formula of C<sub>26</sub>H<sub>38</sub>N<sub>2</sub>O<sub>8</sub>. A comparative NMR analysis of macrotermycin C (**3**) vs. **1** revealed that **3** contains 12 olefinic protons and two additional hydroxyl groups ( $\delta_C$  66.0 and  $\delta_C$  79.0), which accounted for the higher polarity of **3**, and led to a proposed structure (Fig. 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.<sup>8</sup> 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 (Fig. 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 comparing 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 analog of **4** (Fig. 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<sub>3</sub>-20/H-5 and H<sub>3</sub>-20/H-7 along with relation to other macrotermycins allows predicting 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 *cis* diol stereoconfiguration (C-16, C-17) (Fig. 1C). An ROE correlation between H-16/H<sub>3</sub>-21 allowed defining C-18 as *R*\* (vs. *S*\* in **1** and **2** due to a Cahn-Ingold-Prelog priority change arising from the C-17 hydroxyl).<sup>8</sup>

The core structure of the macrotermycins (**5**) belongs to a family of macrolactam polyketides that include incednine,<sup>10</sup> vicenistatin,<sup>11</sup> silvalactam,<sup>12</sup> heronamides,<sup>13</sup> verticilactam,<sup>14</sup> ciromicins,<sup>15</sup> mirilactams and lobosamides.<sup>16</sup> 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 <sup>2,3</sup> alkenyl bond in an unisolated precursor. To gain further insights into macrotermycin biogenesis, the *Amycolatopsis* sp. M39 genome was sequenced and annotated (accession number: LWSF00000000, Fig. 2).<sup>8</sup> The likely macrotermycin biosynthetic gene cluster was identified based on significant homology to the known vicenistatin cluster.<sup>17</sup> 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.<sup>18</sup> 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).

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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

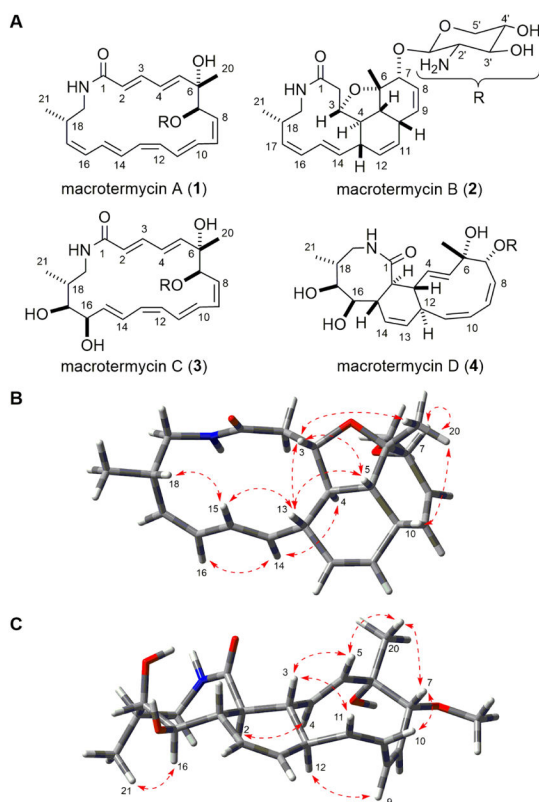
## Acknowledgments

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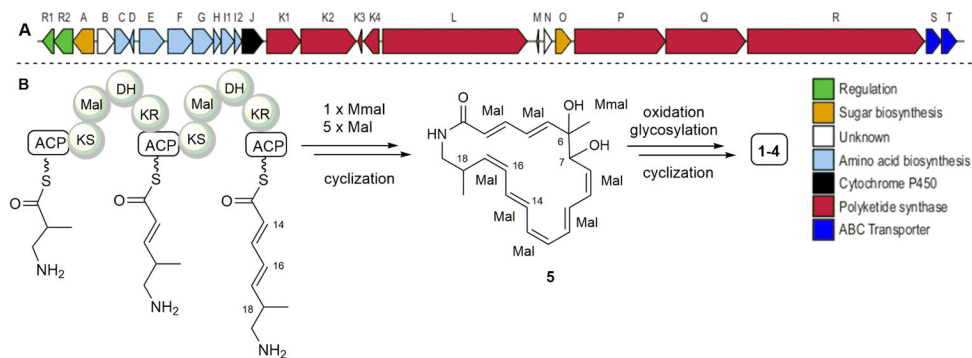
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**Figure 1.**

A) Structures of macrotermycins A-D. B) Computational models of a macrotermycin B derivative, and C) of 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).



**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.



**Table 1**

Minimal inhibitory concentrations ( $\mu\text{g/mL}$ ) of **1** and **3** (positive controls: ciprofloxacin and amphotericin B).

strain	1	3	positive control
	MIC ( $\mu\text{g/mL}$ )		
	ciprofloxacin		
<i>B. subtilis</i> ATCC 6051	1.0	15	0.3
<i>S. aureus</i> ATCC 25923	1.5	10	0.5
	amphotericin B		
<i>C. albicans</i> ATCC 10231	10	25	0.3
<i>S. cerevisiae</i> ATCC9763	5.0	20	0.2
<i>Termitomyces</i> sp. T112	>100	>100	~ 65
<i>Pseudoxyalaria</i> sp. X802	~ 50	~ 80	~ 10

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