Microbial Strain Prioritization Using Metabolomics
Tools for the Discovery of Natural Products

Yanpeng Hou,1 Doug R. Braun,1 Cole R. Michel,1 Jonathan L. Klassen,2 Navid Adnani,1

Thomas P. Wyche,1 Tim S. Bugni1*

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1 Pharmaceutical Sciences Division, School of Pharmacy, University of Wisconsin, Madison, WI 53705, USA

2 Department of Bacteriology, University of Wisconsin, Madison, WI 53705, USA

* To whom correspondence should be addressed. E-mail: tbugni@pharmacy.wisc.edu
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**Additional Experimental Section**

**Bacteria Cultivation.** Ascidian specimens were collected in December 2010 in the Florida Keys (24° 37.487”, 81° 27.443”). For cultivation, a sample of ascidian (1 cm³) was rinsed with sterile seawater, macerated using a sterile pestle in a micro-centrifuge tube, and dilutions were made in sterile seawater, with vortexing between steps to separate bacteria from heavier tissues. Dilutions were separately plated on three distinct media: R2A\(^1\), ISP2 (see recipe in Table S2 in supporting information) supplemented with artificial sea water \(^2\) and M4. \(^3\) Media were supplemented with 50 \(\mu\)g/mL cycloheximide and 25 \(\mu\)g/mL nalidixic acid. Plates were incubated at 30 °C for at least 28 days.

**Fermentation of Micromonosporaceae and Nocardia Strains for LC/MS Profiling.** Seed cultures (10 mL) were grown in 25 × 150 mm culture tubes in ASW-A media (see recipe in Table S2 in supporting information) for seven days at 200 RPM at 28 °C. Erlenmeyer flasks (125 mL) containing 25 mL of ASW-A were inoculated with 2 mL of seed culture and shaken at 200 RPM at 28 °C for seven days.

**Agar-based Media of Streptomyces for LC/MS Profiling.** Each strain was inoculated onto ISP2 media (see recipe in Table S2 in supporting information) supplemented with artificial sea water in Petri dishes and incubated at 30 °C for 10 days or until sporulation was observed.

**16S Ribosomal DNA Sequencing.** Genomic DNA was extracted using the UltraClean Microbial DNA Isolation kit (Mo Bio Laboratories, Inc.). 16S rDNA genes were amplified using 100-200 ng genomic DNA template with the primers 8-27F (5’ to 3’ GAGTTTGATCCTGGCTCAG) and 1492R (5’ to 3’ GGTTACCTTGTTACGACTT). The following PCR conditions were used: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1.5 min, with a
final step of 72 °C for 5 min. The PCR bands were excised from the gel and purified using the QIAquick Gel Extraction kit (QIAGEN). One μL purified product was sequenced. Sequencing reactions were performed by the Biotechnology Center at University of Wisconsin-Madison and reactions were sequenced with an ABI 3730xl DNA Analyzer. The phylogenetic tree was constructed using Tree Builder at the Ribosomal Database Project site (http://rdp.cme.msu.edu/) using the default settings.

**UHPLC/HRMS Analysis.**

**Supplementary table 1** Isolation and fragmentation list for auto MS/MS

| Mass value | Isolation width | Collision energy | Charge state |
|------------|----------------|------------------|--------------|
| 500        | 8              | 40               | 1            |
| 500        | 8              | 25               | 2            |
| 500        | 8              | 20               | 3            |
| 1000       | 10             | 50               | 1            |
| 1000       | 10             | 40               | 2            |
| 1000       | 10             | 35               | 3            |
| 2000       | 15             | 70               | 1            |
| 2000       | 15             | 50               | 2            |
| 2000       | 15             | 45               | 3            |

If an isolation mass does not correspond to the criteria shown in the list, the parameters are determined by interpolating the adjacent peaks. For example, the isolation width for a singly charged m/z 750 would be 9.

**Data Processing and PCA.** Molecular formulas were predicted using Bruker SmartFormula™ algorithm under these parameters: ratio of element H/C: 0~3; rings plus double bonds: -0.5~40; the nitrogen rule and ions of even electron configuration needed to be fulfilled. PCA was performed using Bruker ProfileAnalysis 2.0 software. Finding molecular features was applied to LC/MS data under these parameters: S/N threshold: 5; correlation coefficient threshold: 0.7; minimum compound length: 10 spectra; smoothing width: 1. MS peak finder used the following
parameters: using the same width as used in the acquisition; S/N threshold: 5; relative intensity threshold (base peak): 0.1%; absolute intensity threshold: 100. The bucket generation was performed under the following parameters. The LC/MS data sets were evaluated in a time range from 120 s to 840 s and in a mass range from \( m/z \) 200 to 1500. Advanced bucketing was employed using \( \Delta RT = 20 \text{ s} \) and \( \Delta m/z = 0.02 \text{ Da} \) as parameters. Sum of bucket values was applied for normalization in this study, and pareto scaling algorithm\(^4\) was applied.

Heat Map Generation. The heatmap was constructed in R v1.14.1 using the "heatmap.2" function in the gplots package v2.7.4, according to its default parameters, and RColorBrewer v1.0. All values were log transformed prior to plotting.

**Finding Unique Natural Products by Dynamically Linked PCA Scores Plot and Loadings Plot.** The PC planes were adjusted using the PC selection tool in the work panel of Profile Analysis. The distribution of a compound in each strain was visualized in a corresponding bucket statistic plot.

**Scale-up Fermentation, Extraction and Purification of Natural Products.**

Strains of interest (Which strains were selected; did you have starter cultures?) were cultured in ASW-A with Diaion\textsuperscript{®} HP20 (Supelco) as an adsorbent (60 g/L) This doesn’t seem correct?. The culture was filtered, and HP20 adsorbent was collected and extracted in acetone. The dried acetone extract was suspended in MeOH/H\textsubscript{2}O (9:1) and extracted using hexanes followed by further adjusting to MeOH/H\textsubscript{2}O (6:4), which was then extracted using CHCl\textsubscript{3}. Purification was completed by flash chromatography, HPLC, and an LC/MS-SPE-NMR system equipped with an
Agilent 1200 HPLC system, a Bruker microTOF Q II mass spectrometer, a Spark SPE system and a Bruker Avance 600 equipped with a $^1\text{H}{}^{13}\text{C},{}^{15}\text{N}$ 1.7 mm cryogenic probe.
**Supplementary Table 2** The 47 strains of *Verrucosispora*, *Micromonospora*, and *Nocardia* isolated from ascidians was used in this proof-of-concept study.

| Strains | Closest match in NCBI (16S) | Ascidians | GeneBank Accession # (16S) |
|---------|-----------------------------|-----------|---------------------------|
| WMMB-202 | *Verrucosispora* sp. FIM 0657 | *Trididemnum orbiculatum* | JQ716941 |
| WMMB-203 | *Nocardia araoensis* W9705 | *Trididemnum orbiculatum* | JQ716942 |
| WMMB-204 | *Verrucosispora* sp. 234606 | *Trididemnum orbiculatum* | JQ716943 |
| WMMB-205 | *Verrucosispora* sp. FIM 0657 | *Trididemnum orbiculatum* | JQ716944 |
| WMMB-206 | *Verrucosispora* sp. FIM 0657 | *Trididemnum orbiculatum* | JQ716945 |
| WMMB-207 | *Nocardia araoensis* W9705 | *Trididemnum orbiculatum* | JQ716946 |
| WMMB-208 | *Verrucosispora* sp. 0657 | *Trididemnum orbiculatum* | JQ716947 |
| WMMB-209 | *Verrucosispora* sp. 0657 | *Trididemnum orbiculatum* | JQ716948 |
| WMMB-210 | *Verrucosispora* sp. 0657 | *Trididemnum orbiculatum* | JQ716949 |
| WMMB-211 | *Verrucosispora* sp. FIM 0657 | *Trididemnum orbiculatum* | JQ716950 |
| WMMB-212 | *Verrucosispora* sp. 0657 | *Trididemnum orbiculatum* | JQ716951 |
| WMMB-213 | *Nocardia* sp. W9241 | *Trididemnum orbiculatum* | JQ716952 |
| WMMB-214 | *Verrucosispora* sp. FIM 0657 | *Trididemnum orbiculatum* | JQ716953 |
| WMMB-215 | *Nocardia araoensis* W9705 | *Trididemnum orbiculatum* | JN638997 |
| WMMB-216 | *Verrucosispora* sp. FIM 0657 | *Trididemnum orbiculatum* | JQ716954 |
| WMMB-217 | *Verrucosispora* sp. 0657 | *Trididemnum orbiculatum* | JQ716955 |
| WMMB-218 | *Verrucosispora* sp. 0657 | *Trididemnum orbiculatum* | JQ716956 |
| WMMB-219 | *Verrucosispora* sp. 0657 | *Trididemnum orbiculatum* | JQ716957 |
| WMMB-220 | *Verrucosispora* sp. FIM 0657 | *Trididemnum orbiculatum* | JQ716958 |
| WMMB-221 | *Nocardia araoensis* W9705 | *Didemnum psammathode* | JQ716959 |
| WMMB-222 | *Nocardia araoensis* W9705 | *Didemnum psammathode* | JQ716960 |
| WMMB-223 | *Verrucosispora* sp. 234606 | *Didemnum psammathode* | JQ716961 |
| WMMB-224 | *Verrucosispora* sp. 234606 | *Didemnum psammathode* | JQ716962 |
| WMMB-225 | *Micromonospora* sp. SR15 | *Didemnum psammathode* | JQ716963 |
| WMMB-226 | *Verrucosispora* sp. 234606 | *Didemnum psammathode* | JQ716964 |
| WMMB-227 | *Verrucosispora* sp. 234606 | *Didemnum psammathode* | JQ716965 |
| WMMB-228 | *Nocardia araoensis* W9705 | *Didemnum psammathode* | JQ716966 |
| WMMB-229 | *Nocardia araoensis* W9705 | *Didemnum orbiculatum* | JQ716967 |
| WMMB-230 | *Micromonospora* sp. CNS-633 SD | *Ecteinascidia turbinata* | JQ716968 |
| WMMB-231 | *Verrucosispora* sp. 234606 | *Didemnum psammathode* | JQ716969 |
| WMMB-232 | *Nocardia araoensis* W9705 | *Didemnum psammathode* | JQ716970 |
| WMMB-233 | *Nocardia araoensis* W9705 | *Didemnum psammathode* | JQ716971 |
| WMMB-234 | *Verrucosispora* sp. 234606 | *Ecteinascidia turbinata* | JQ716972 |
| WMMB-235 | *Micromonospora* sp. 106 | *Ecteinascidia turbinata* | JQ716973 |
| WMMB-236 | *Verrucosispora* sp. 234606 | *Ecteinascidia turbinata* | JQ716974 |
| WMMB-237 | *Verrucosispora* sp. 234606 | *Ecteinascidia turbinata* | JQ716975 |
| WMMB-238 | *Verrucosispora* sp. 234606 | *Ecteinascidia turbinata* | JQ716976 |
| WMMB-239 | *Nocardia* sp. CNS044 PL04 | *Didemnum psammathode* | JQ716977 |
| WMMB-240 | *Verrucosispora* sp. 234606 | *Ecteinascidia turbinata* | JQ716978 |
| WMMB-241 | *Verrucosispora* sp. 234606 | *Ecteinascidia turbinata* | JQ716979 |
| WMMB-242 | *Verrucosispora* sp. 234606 | *Ecteinascidia turbinata* | JQ716980 |
| WMMB-243 | *Verrucosispora* sp. 234606 | *Ecteinascidia turbinata* | JQ716981 |
| WMMB-244 | *Verrucosispora* sp. 234606 | *Ecteinascidia turbinata* | JQ716982 |
| WMMB-245 | *Verrucosispora* sp. 234606 | *Ecteinascidia turbinata* | JQ716983 |
| WMMB-247 | *Micromonospora* sp. JSM5-1 | *Ecteinascidia turbinata* | JQ716984 |
| WMMB-248 | *Micromonospora* sp. 399K7-1 | *Ecteinascidia turbinata* | JQ716985 |
| WMMB-249 | *Verrucosispora* sp. 234606 | *Ecteinascidia turbinata* | JQ716986 |
**Supplementary Table 3** Media recipes used in this study.

| Media   | Recipe                                      |
|---------|---------------------------------------------|
| ASW-A   | Soluble starch 20 g/L, glucose 10 g/L, peptone 5 g/L, yeast extract 5 g/L, and CaCO₃ 5 g/L in artificial seawater. |
| ASW-K   | Same as ASW-A, but without potassium.        |
| DI-A    | Same as ASW-A, but in deionized water instead of artificial seawater. |
| ISP2    | Yeast extract 4 g/L, malt extract 10 g/L, dextrose 4 g/L, and Agar 15 g/L in artificial seawater. |

**Supplementary Table 4** Media conditions used for regulation of natural product production in WMMB-224.

| Media   | Iron added                                      |
|---------|-------------------------------------------------|
| ASW-A   | 10 μM FeCl₃<br>100 μM FeCl₃<br>500 μM FeCl₃<br>10 μM EDTA-Fe(II) (EDTA + FeSO₄)<br>50 μM EDTA-Fe(II) (EDTA + FeSO₄)<br>100 μM EDTA-Fe(II) (EDTA + FeSO₄)<br>10 μM EDTA-Fe(III) (EDTA + FeCl₃)<br>50 μM EDTA-Fe(III) (EDTA + FeCl₃)<br>100 μM EDTA-Fe(III) (EDTA + FeCl₃)<br>Control (no iron added) |
| ASW-K   | 10 μM FeCl₃<br>100 μM FeCl₃<br>500 μM FeCl₃<br>10 μM EDTA-Fe(II) (EDTA + FeSO₄)<br>50 μM EDTA-Fe(II) (EDTA + FeSO₄)<br>100 μM EDTA-Fe(II) (EDTA + FeSO₄)<br>10 μM EDTA-Fe(III) (EDTA + FeCl₃)<br>50 μM EDTA-Fe(III) (EDTA + FeCl₃)<br>100 μM EDTA-Fe(III) (EDTA + FeCl₃)<br>Control (no iron added) |
| DI-A    | 10 μM FeCl₃<br>100 μM FeCl₃<br>500 μM FeCl₃<br>10 μM EDTA-Fe(II) (EDTA + FeSO₄)<br>50 μM EDTA-Fe(II) (EDTA + FeSO₄)<br>100 μM EDTA-Fe(II) (EDTA + FeSO₄)<br>10 μM EDTA-Fe(III) (EDTA + FeCl₃)<br>50 μM EDTA-Fe(III) (EDTA + FeCl₃)<br>100 μM EDTA-Fe(III) (EDTA + FeCl₃)<br>Control (no iron added) |
Supplementary Figure 1 Assessment of microbial natural product production (part 1). (a) PCA scores plot. Samples were color-coded based on concentrations of added iron (blue circle: no iron added; red triangle: 10 μM; green cross: 50 μM; blue cross: 100 μM; orange diamond: 500 μM). Iron concentrations are the key factor for clustering behavior. (b) PCA loadings plot. A putative new compound (7, RT 10.5 min-m/z 431.2767, [M+H]+) was detected in Group 3. Major desferrioxamines detected in Group 1 are circled in black on the loadings plot. Most of desferrioxamines in group 1 were eliminated in Group 3 although desferrioxamine B and D1 (8-9) were transformed to Fe³⁺-chelated forms.
**Supplementary Figure 2** Assessment of microbial natural product production (part 2). (a) PCA scores and loadings plot. Samples were color and shape coded based on different iron forms (blue circle: no iron added; red triangle: FeCl₃; green cross: EDTA-Fe(II); blue cross: EDTA-Fe(III)). Clustering behavior is primarily based on iron concentrations (see main text) not on different iron forms. (b) PCA scores and loadings plot. Samples were color and shape coded based on different media (blue circle: ASW-A; red triangle: ASW-K; green cross: DI-A). Clustering behavior is primarily based on iron concentrations (see main text) not on different media.
Supplementary Figure 3 LC/MS and LC/MS/MS chromatograms of WMMB-247
**Supplementary Figure 4** MS and MS/MS spectra for 2 and 3
Structure Elucidation of 2–6.

Supplementary figure 5 Proposed fragmentation mechanism of 2 (RT 7.4 min-\textit{m/z} 679.4026, [M+H]\(^{+}\), calcd. for C\(_{33}\)H\(_{55}\)N\(_{6}\)O\(_{9}\), 679.4026) and 3 (RT 6.0 min-\textit{m/z} 637.3918, [M+H]\(^{+}\), calcd. for C\(_{31}\)H\(_{55}\)N\(_{6}\)O\(_{8}\), 679.4026)
Supplementary Figure 6 $^1$H NMR spectrum of 3 (600 MHz MeOH-$d_4$, peak broadening due to chelated iron)
Supplementary Figure 7 LC/MS and LC/MS/MS chromatograms of WMMB-233 (upper, showing 1) and B-272 (bottom, showing 4, 5 and 6)
### Supplementary Table 5 \(^1\)H NMR and \(^{13}\)C NMR Data of Compounds 4–6\(^c\)

| no. | \(^4\)a \(^1\)H NMR | \(^13\)C NMR | \(^5\)a \(^1\)H NMR | \(^13\)C NMR | \(^6\)b \(^1\)H NMR | \(^13\)C NMR | \(^13\)C NMRd |
|-----|-----------------|-------------|-----------------|-------------|-----------------|-------------|-----------|
| 1   | 165.5           | 165.8       |                 |             |                 |             | 162.4\(^c\) |
| 2   | 101.2           | 101.0       | 5.52 s          | 88.0        |                 |             |           |
| 3   | 167.8           | 167.3       |                 |             |                 |             | 170.8     |
| 4   | 6.57 s          | 96.0        | 6.62 s          | 96.1        | 6.15 s          | 100.7       |           |
| 5   | 157.8           | 158.1       |                 |             |                 |             | 158.7     |
| 6   | 6.39 d          | 122.1       | 6.42 d          | 118.2       | 6.39 d          | 118.6       |           |
|     | \(J = 15.2 \text{ Hz}\) |          | \(J = 15.7 \text{ Hz}\) |             | \(J = 15.6 \text{ Hz}\) |             |           |
| 7   | 7.23 dd         | 135.6       | 7.23 d          | 138.6       | 7.17 d          | 138.6       |           |
|     | \(J = 15.2, 11.0 \text{ Hz}\) |          | \(J = 15.7 \text{ Hz}\) |             | \(J = 15.6 \text{ Hz}\) |             |           |
| 8   | 6.62 dd         | 127.7       |                 | 135.5       |                 | 135.4       |           |
|     | \(J = 15.4, 11.0 \text{ Hz}\) |          |                 |             |                 |             |           |
| 9   | 6.75 d          | 142.4       | 6.58 d          | 136.5       | 6.66 d          | 136.5       |           |
|     | \(J = 15.4 \text{ Hz}\) |          | \(J = 11.8 \text{ Hz}\) |             | \(J = 11.5 \text{ Hz}\) |             |           |
| 10  |                 | 137.0       | 6.94 dd         | 130.8       | 6.94 dd         | 130.5       |           |
|     |                 |             | \(J = 14.5, 11.8 \text{ Hz}\) |             | \(J = 14.7, 11.5 \text{ Hz}\) |             |           |
| 11  | 6.52 d          | 134.0       | 6.69 dd         | 135.5       | 6.73 dd         | 135.7       |           |
|     | \(J = 11.4 \text{ Hz}\) |          | \(J = 14.5, 10.7 \text{ Hz}\) |             | \(J = 14.7, 10.9 \text{ Hz}\) |             |           |
| 12  | 7.45 dd         | 127.9       | 7.21 dd         | 131.8       | 7.25 dd         | 131.0       |           |
|     | \(J = 15.4, 11.4 \text{ Hz}\) |          | \(J = 15.8, 10.7 \text{ Hz}\) |             | \(J = 15.7, 10.9 \text{ Hz}\) |             |           |
| 13  | 6.75 d          | 129.6       | 6.73 d          | 128.8       | 6.78 dd         | 129.9       |           |
|     | \(J = 15.4 \text{ Hz}\) |          | \(J = 15.8 \text{ Hz}\) |             | \(J = 15.7 \text{ Hz}\) |             |           |
| 2'  | 8.63 br s       | 147.3       | 8.62 br s       | 147.1       | 8.70 br s       | 148.2       |           |
| 3'  |                 | 133.7       |                 | 133.7       |                 | 131.7       |           |
| 4'  | 8.06 br d       | 133.5       | 8.01 br d       | 133.2       | 7.93 br d       | 132.1       |           |
|     | \(J = 8.3 \text{ Hz}\) |          | \(J = 8.1 \text{ Hz}\) |             | \(J = 7.8 \text{ Hz}\) |             |           |
| 5'  | 7.43 dd         | 124.0       | 7.43 dd         | 124.0       | 7.35 dd         | 123.3       |           |
|     | \(J = 8.3, 4.6 \text{ Hz}\) |          | \(J = 8.1, 4.7 \text{ Hz}\) |             | \(J = 7.8, 4.6 \text{ Hz}\) |             |           |
| 6'  | 8.39 d \(J = 4.6 \text{ Hz}\) | 147.3 | 8.39 d \(J = 4.7 \text{ Hz}\) | 147.1 | 8.44 d \(J = 4.6 \text{ Hz}\) | 148.2 |           |
| 2-Me| 1.92 s          | 7.3         | 1.92 s          | 7.3         | n/a             | n/a         |           |
| 3-OMe| 3.98 s         | 55.7        | 3.98 s          | 55.9        | 3.90 s          | 55.6        |           |
| 8-Me| n/a            | 2.06 s      |                 | 11.1        | 2.03 s          | 11.5        |           |
| 10-Me| 2.11           | 10.9        | n/a             | n/a         | n/a             | n/a         |           |

\(^a\) in CD\(_3\)OD. \(^b\) in acetone-\(d_6\). \(^c\) \(\delta\) (ppm) 600 MHz. \(^d\) data were extracted from the HMBC spectrum of 6. 

\(^e\) The \(^{13}\)C chemical shift of C-1 of 6 was estimated by comparison with literature data. 

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Supplementary Figure 8 $^1$H-$^1$H COSY (bold) and key HMBC (arrows) correlations of 6

Supplementary Figure 9 $^1$H NMR spectrum of 4 (600 MHz MeOH-$d_4$)

Supplementary Figure 10 $^1$H NMR spectrum of 5 (600 MHz MeOH-$d_4$)
Supplementary Figure 11 $^1$H NMR spectrum of 6 (600 MHz Acetone-$d_6$)
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