Isolation of Pyrrolocins A–C: cis- and trans-Decalin Tetramic Acid Antibiotics from an Endophytic Fungal-Derived Pathway

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ABSTRACT: Three new decalin-type tetramic acid analogues, pyrrolocins A (1), B (2), and C (3), were defined as products of a metabolic pathway from a fern endophyte, NRRL 50135, from Papua New Guinea. NRRL 50135 initially produced 1 but ceased its production before chemical or biological evaluation could be completed. Upon transfer of the biosynthetic pathway to a model host, 1–3 were produced. All three compounds are structurally related to equeisetin-type compounds, with 1 and 3 having a trans-decalin ring system, while 2 has a cis-fused decalin. All were active against Mycobacterium tuberculosis, with the trans-decalin analogues 1 and 3 exhibiting lower MICs than the cis-decalin analogue 2. Here we report the isolation, structure elucidation, and antimycobacterial activities of 1–3 from the recombinant expression as well as the isolation of 1 from the wild-type fungus NRRL 50135.

Papua New Guinea is a hot spot for plant biodiversity, harboring an estimated 20,000 individual species of vascular plants. An estimated 60% of these are endemic to Papua, one of the highest rates of endemism in the world. Further, it is thought that most tropical plants contain multiple endophytic fungi, providing an immense unexplored reservoir of secondary metabolites.

As part of our International Cooperative Biodiversity Group (ICBG) program, we screened extracts of endophytic fungi isolated from Papua New Guinea terrestrial and marine sources for antibacterial activities. A methanol extract of a phylogenetically novel strain (NRRL accession number 50135) isolated from the stem of an Asplenium sp. fern growing in Watunou, Milne Bay Province, Papua New Guinea, was identified as selectively active against Gram-positive bacteria including Mycobacterium tuberculosis H37Ra (ATCC 25177), Staphylococcus aureus, Streptococcus pneumoniae, and Bacillus subtilis ATCC6633. We employed an antimycobacterial bioassay-guided isolation to obtain the TB-active metabolite pyrrolocin A (1), a trans-fused decalin-containing tetramic acid analogue related to equeisetin. When NRRL 50135 stopped producing 1, we used a recombinant expression platform to induce its production. This heterologous expression platform successfully produced 1, matching the initial molecular weight, UV, NMR, and CD spectra, as well as two additional desmethyl analogues, pyrrolomin B (2) and pyrrolocin C (3). The three compounds have different configurations in the decalin ring system, with 2 having the cis-fused decalin ring and 1 and 3 having the trans-decalin configuration. To our knowledge, this is the first report of both cis and trans configurations being isolated from a single biological source and may be valuable in understanding the biosynthesis of this group of tetramic acid analogues. Furthermore, transplanting, or indeed rescuing, synthetic pathways from one organism into more tractable backgrounds unquestionably furthers drug discovery by magnifying the number and amount of rare natural chemicals with potential pharmacological activities, i.e., accessing dormant biosynthetic potential with a concomitant increase in production. Skillful manipulation of these pathways could theoretically also steer biosynthesis of undesirable intermediates or end products to pharmacologically desirable entities in quantities useful for experimentation or production.

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Tetramic acids are characterized by the presence of a 2,4-pyrrolidinedione moiety in their structures. Tetramic acids that are related to equisetin are particularly important because of their reported antimicrobial activities, HIV-1 integrase inhibition, and microtubule assembly inhibition. The mechanism of their antimicrobial activities has been purported to be due to inhibition of SecA, inhibition of undecaprenyl pyrophosphate synthase, and inhibition of the histidine kinase WalK. Due to their biological activities, the biosynthesis of this group of compounds and related polyketides was also the subject of interest in several reviews.

**RESULTS AND DISCUSSION**

Initially, growth of the fungus on semisolid media led to the robust production of at approximately 50 mg L\(^{-1}\). The compound was purified on the basis of promising activity against \(M.\) tuberculosi. The methanol extract from NRRL 50135 was purified using a preparative C18 column to isolate 1. High-resolution ESIMS determined its molecular formula to be \(C_{27}H_{40}NO_5\). The 13C NMR spectra (Table 1) showed 27 carbon signals, while its\(^1\)H NMR spectra presented five olefinic protons, five methyls, four methylenes, and six methines. The carbon connectivities in the decalin ring and the olefinic side chain were established by COSY, HMBC, and HSQC. Their association with equisetin-type tetramic acid was evident from a review of literature data that showed that 1 is related to phomasetin, with the addition of two carbons in the olefinic side chain and with the presence of an \(N\)-methyl group. In order to establish the relative and absolute configuration of 1 and evaluate its antimycobacterial activity, we fermented NRRL 50135 for additional material. Unfortunately, as is often observed in secondary metabolites isolated from cultivated microorganisms, NRRL 50135 ceased production of 1 before completion of this work. In order to achieve production of 1, we identified the biosynthetic locus for its production and Table 1. 1H and 13C NMR Data for Compound 1 (400 and 100 MHz, respectively, \(\delta\) in ppm), 2, and 3 (500 and 125 MHz, respectively)

| pos | \(\delta_{\text{C}}\) type | \(\delta_{\text{H}}\) (J in Hz) | \(\delta_{\text{C}}\) type | \(\delta_{\text{H}}\) (J in Hz) | \(\delta_{\text{C}}\) type | \(\delta_{\text{H}}\) (J in Hz) |
|-----|----------------|----------------|----------------|----------------|----------------|----------------|
| 1   | 195.7, C       |                | 204.5, C       |                | 198.6\(^{\circ}\), C |                |
| 2   | 48.8, C        |                | 52.5, C        |                | 49.0, C        |                |
| 2-Me| 13.7, CH\(_3\) | 1.33, s        | 19.2, CH\(_3\) | 1.30, s        | 13.3, CH\(_3\) | 1.32, s        |
| 3   | 39.3, CH       | 1.57, br dd (10.2, 10.2) | 38.4, CH | 2.72, br d (10.2) | 40.4, CH | 1.57, m        |
| 4   | 27.7, CH\(_2\) | 1.89, m        | 24.6, CH\(_2\) | 1.38, m        | 27.5, CH\(_2\) | 1.93, m        |
| 5   | 35.4, CH\(_2\) | 1.70, m        | 36.6, CH\(_2\) | 1.67, m        | 35.4, CH\(_2\) | 1.71, m        |
| 6   | 32.7, CH       | 1.47, m        | 29.8, CH       | 1.40, d (5.3)  | 32.9, CH       | 1.49, m        |
| 6-Me| 22.2, CH\(_2\) | 0.87, d (6.7)  | 23.2, CH\(_2\) | 0.81, d (6.5)  | 22.1, CH\(_2\) | 0.88, d (6.5)  |
| 7   | 42.0, CH\(_2\) | 1.77, m        | 38.4, CH\(_2\) | 1.56, d (14.0) | 42.0, CH\(_2\) | 1.77, m        |
| 8   | 38.7, CH       | 1.77, m        | 36.7, CH       | 2.09, br s     | 38.7, CH       | 1.79, m        |
| 9   | 126.0, CH      | 5.18, s        | 126.8, CH      | 5.09, s        | 126.0, CH      | 5.19, s        |
| 10  | 130.9, C       |                | 133.7, C       |                | 131.6, C       |                |
| 10-Me| 22.2, CH\(_2\) | 1.52, s        | 23.3, CH\(_2\) | 1.63, s        | 22.4, CH\(_2\) | 1.52, s        |
| 11  | 48.8, CH       | 3.14, m        | 43.5, CH       | 3.20, d (9.8)  | 48.4, CH       | 3.18, m        |
| 12  | 130.7, CH\(_2\) | 5.21, dd (15.4, 9.8) | 134.1, CH | 5.50, dd (14.2, 10.5) | 131.0, CH | 5.21\(^{d}\) |
| 13  | 131.3, CH\(_2\) | 5.69, dd (15.4, 10.4) | 135.9, CH | 6.04, dt (10.3) | 132.1, CH | 5.72, dd (15.6, 10.0) |
| 14  | 130.9, CH\(_2\) | 5.86, dd (15.4, 10.5) | 136.1, CH | 6.09, dt (10.3) | 131.4, CH | 5.90, dd (15.0, 11.2) |
| 15  | 129.5, CH\(_2\) | 5.49, ddd (15.4, 10.7) | 130.3, CH | 5.63, pentet (7.5) | 130.3, CH | 5.51, dt (15.1, 7.0) |
| 16  | 42.1, CH\(_2\) | 2.09, ddd (13.8, 7, 7) | 42.1, CH\(_2\) | 2.24, pentet (6.4) | 42.4, CH\(_2\) | 2.09, m        |
| 17  | 65.6, CH       | 3.56, pentet (6.5) | 68.7, CH | 3.78, m        | 65.9, CH | 3.59, m        |
| 18  | 22.7, CH\(_2\) | 0.98, d (6.5)  | 23.1, CH\(_2\) | 1.14, d (6.20) | 23.1, CH\(_3\) | 0.98, d (6.1)  |
| 2'  | 1760.\(^{c}\) C |                | 180.8, C       |                | 179.2\(^{\circ}\), C |                |
| 3'  | 100.6\(^{c}\) C |                | 100.0\(^{\circ}\), C |                | 100.0\(^{\circ}\), C |                |
| 4'  | 190.6\(^{c}\) C |                | 192.9, C       |                | 190.5, C       |                |
| 5'  | 67.6, CH       | 3.77, m        | 62.4, CH       | 3.87, br s     | 63.3, CH       | 3.79, m        |
| 6'  | 58.0, CH\(_2\) | 3.80, dd (12, 2) | 64.3, CH\(_2\) | 3.83, dd (11.4, 3) | 60.4, CH\(_2\) | 3.62, d (11.0) |
|     | 3.68, dd (12, 1.8) |                | 3.77, m        |                | 3.57, dd (12.2, 5.9) |                |

\(^{a}\)DMSO-\(_d_6\). \(^{b}\)CD\(_3\)OD. \(^{c}\)Broad signal. \(^{d}\)Overlapped with H-9.
moved it into the expression host, Fusarium heterosporum, as reported elsewhere. In this context, the major compounds produced were not those found in NRRL 50135, but instead comprised approximately 800 mg of desmethyl pyrrolocin analogues B (2) and C (3), along with a smaller amount of 1, per kilogram of fermentation.

Compound 2, which was isolated as an off-white, amorphous solid, was the major metabolite found in the extract. High-resolution ESIMS determined its molecular formula to be C_{26}H_{38}NO_{5}. Likewise 3, which was also isolated as an off-white, amorphous solid, has a molecular formula of C_{26}H_{38}NO_{5} as established by high-resolution ESIMS. Comparison of the UV spectra and ^1H, ^13C, COSY, HSQC, and HMBC data of 2 and 3 revealed that the two have the same carbon structure and that they differ only in their stereochemistry. Furthermore, the HRESIMS and NMR data showed that 2 and 3 are the N-desmethyl analogues of 1. However, the proton and carbon chemical shift values of 1 were more similar to 3 than to 2 (Table 1).

Relative Configuration of the Decalin Ring Moiety. NMR data showed that compound 2 has a cis-fused decalin structure. In the ^1H NMR spectrum of 2, the decalin ring junction signals H-3 (δ 2.72 ppm) and H-8 (δ 2.09 ppm) were deshielded compared to the same protons in 1 and 3. This is consistent with other cis-decalin tetramic acid analogues when compared to their trans-fused counterparts. The NMR data also indicated that the fused cyclohexyl ring assumes a chair conformation where H-3 is axial while H-8 is equatorial (Figure 1). The absence of a diaxial coupling between these two protons caused H-8 to be seen as a broad singlet in the ^1H NMR spectrum, while H-3 was seen as a broad doublet with J = 10.2 Hz due to its coupling with H-4_{ax} (δ 1.38 ppm). The ROESY spectrum of 2 also showed a correlation between H-3 and H-8, which confirmed that the two are not in a diaxial configuration (Figure 1). H-3 was further observed to correlate with H-5_{ax} (δ 0.87 ppm) and H-7_{ax} (δ 1.11 ppm), while H-8 was also seen to correlate with both H-7_{eq} and H-7_{eq} (δ 1.56 ppm). This was also the case with the equatorial C-6 methyl (δ 0.81 ppm), which exhibited same intensity ROESY correlations with both H-7_{eq} and H-7_{eq} and with H-5_{ax} and H-5_{ax} (δ 1.67 ppm). On the other hand, H-6_{ax} was seen to correlate with H-4_{ax} (δ 1.45 ppm), H-7_{eq} and H-7_{eq}. The fused cyclohexenyl ring also seems to assume a pseudochair configuration with H-3, C-2 methyl, and H-8 in equatorial positions, which was deduced from their ROESY correlations. Correlations were also observed between H-4_{ax} and H-12 since C-4 and C-12 are in pseudoaxial positions. Likewise, the pseudoaxial H-9 (δ 5.09 ppm) also correlated with H-6_{eq}.

The relative configuration of 3 differs from that of 2 at stereocenters C-8 and C-11. The first indication was the upfield shifts of the proton signals H-3 (δ 1.57 ppm) and H-8 (δ 1.79 ppm) as mentioned above. H-8 was also more deshielded than H-3 compared to cis-fused decalins, where H-3 is more deshielded. The ROESY spectrum established the relative configuration of 3 (Figure 2). The chair conformation of the cyclohexyl ring was also deduced from the following ROESY correlations: H-3 correlated with H-5_{ax} (δ 1.71 ppm) and H-7_{ax} (δ 0.82 ppm); H-8 correlated with H-4_{ax} (δ 1.00 ppm) and H-6_{ax} (δ 1.49 ppm). This therefore indicated that the C-6 methyl is equatorial. The equatorial C-6 methyl also correlated equally to H-5_{eq} (δ 1.71 ppm) and H-5_{eq} (δ 1.00 ppm) and to H-7_{eq} (δ 1.77 ppm). The fused cyclohexenyl ring in 3 also seems to adopt a pseudochair configuration where H-3 and the C-2 methyl are anti to each other in pseudodiastereol positions. H-11 and C-10 methyl appeared to be in pseudoequatorial and pseudoaxial positions, respectively, due to the observed correlation between the C-10 methyl and C-2 methyl.

Absolute Configuration: The Tetramic Acid Ring. The absolute configuration of C-5' was determined by oxidative bond cleavage of the tetramic ring followed by acid hydrolysis (Figure 3). The crude extract containing both compounds 2 and 3 was reacted with sodium hypochlorite and sodium hydroxide at room temperature for 8 h. The oxidation products were extracted using ethyl acetate and evaporated in vacuo. The residue was hydrolyzed using 6 M HCl for 15 h at 110 °C to yield serine. Marfey derivatization of the hydrolytic product was performed using FDLA (1-fluoro-2,4-dinitrophenyl-L-serine). The resulting hydrolytic product serine-FDLA (HPS-FDLA) was analyzed by analytical HPLC together with reference standards L- and D-serine, which were derivatized in the same way as the hydrolytic product serine. The HPS-FDLA eluted at 25.00 min, while D-serine-FDLA injected alone and L-serine-FDLA injected alone eluted at 24.83 and 24.63 min, respectively. The HPS-FDLA that was spiked with L-serine-FDLA showed two peaks, at 24.54 and 24.99 min, while the HPS-FDLA that was spiked with D-serine-FDLA showed one peak, at 24.96 min. The HPS-FDLA was also analyzed by LC-MS and showed the expected ion peak at m/z 339 [M + H]^+.
Absolute Configuration: The C-17 Hydroxy Group of the Olefinic Side Chain. Mosher derivatization was performed on 2 and 3 separately. Each compound was reacted with (R)- and (S)-MTPA chloride to form (S)-MTPA and (R)-MTPA esters, respectively. $^1$H NMR analysis of the Mosher esters was performed, and the $\Delta\delta_{S-R}$ calculated (Figure 4). $^{21}$ Both 2 and 3 were found to have the 17R configuration.

Absolute Configuration: The Decalin Ring of 2. Without any derivatization step, the exciton CD method $^{22}$ was used to elucidate the absolute configuration of 2. This was possible because two existing chromophores can possibly interact and exhibit exciton split CD spectra, which can then be observed as a positive or negative Cotton effect. The positive Cotton effect that is predicted from the configuration of the two chromophores of 2 (Figure 5A) was observed in the experimental CD spectra of 2 (Figure 5B). The tetramic acid ring attached at C-2 and the conjugated diene attached at C-11 are two chromophores that coupled to cause the positive Cotton effect that was observed in the experimental CD spectrum of 2. The absolute configuration of 2 was therefore assigned as 2R, 3S, 6S, 8S, 17R, and 5'R. This method has been applied to establish the absolute configuration of natural products with pre-existing chromophores, including abscisic acid, quassin, dendryphiellin F, and arnottin II. $^{22}$

Absolute Configuration: The Decalin Ring of 3. The optical rotation $[\alpha]_D = +130$ and the CD spectrum of 3 (Figure 6) were almost identical to that of phomasetin ($[\alpha]_D = +93.9$) and ent-equisetin (Table 2) and opposite of that of altersetin/coniosetin (Figure 7). $^{6,8}$ The C-S'R configuration of the tetramic ring as determined above is also consistent with that of phomasetin. The absolute configuration of 3 was therefore assigned as 2R, 3S, 6S, 8R, 17S, and 5'S.

Absolute Configuration of 1. Compound 1, which was isolated as an off-white oil, was a minor compound in the recombinant expression. Its HPLC retention time (Figure 8A...
and B), high-resolution ESIMS, UV, \(^1\)H, \(^{13}\)C, COSY, HMBC, and HSQC NMR spectra, and CD data matched those of \(1\) from the wild-type fungus NRRL 50135. Determination of the absolute configuration of \(1\) was achieved by comparison of its ROESY and CD spectra with \(3\). The ROESY spectrum of \(1\) was very similar to that of \(3\), which indicates that \(1\) and \(3\) have the same relative configuration. The similarity of the CD spectra of these two compounds further revealed that \(1\) and \(3\) have the same absolute configuration.

**Biosynthesis of Pyrrolocins and Related Compounds.**

The co-occurrence of \(1\), \(2\), and \(3\) seems to suggest that \(2\) and \(3\) are cis- and trans-adducts originating from an intramolecular Diels–Alder reaction in the biosynthetic process.\(^{16,17}\) Chemically, this reaction usually prefers to go through an endo transition state, which leads to the cis-decalin isomer, while the less favored exo transition state leads to the trans-decalin. In secondary metabolism, the favored stereochemical route is usually controlled by enzymes, rather than by solution chemistry. For example, the fungal polyketide solanapyrone is cyclized by a fairly novel enzyme after release of the intermediate from the polyketide synthase (PKS).\(^{16,17}\) In a more relevant example the fungal decalin lovastatin is produced by a PKS that is similar to the pyrrolocin PKS. In lovastatin biosynthesis, it is the PKS itself that controls decalin stereochemistry, leading to strict production of the trans-decalin. The relevant biosynthetic intermediate is proposed to contain an ene that is activated by conjugation to an enzyme-bound thioester. By contrast, the synthetic analogue of the proposed intermediate leads to a mixture of cis- and trans-decalin compounds, reflecting a mixture of endo- and exo-Diels–Alder reactions, respectively.

Equisetin and its related compounds are thought to be synthesized by a similar mechanism, wherein the PKS itself would dictate whether the Diels–Alder reaction follows the endo or exo pathway. In the present study, \(1\) was found as the major product by NRRL 50135, and only trace amounts of \(3\) and none of \(2\) were detected (Figure 8A), revealing that only trans-decalins were produced in the wild-type fungus. On the other hand, the heterologous expression yielded \(2\) and \(3\) in a \(~2:1\) ratio, which is what one would expect for a nonenzymatic reaction (Figure 8B). Although there are many reasons for this, the result implies that a separate Diels–Alderase domain might be involved in the biosynthesis of equisetin-like compounds, which in the present study might not be present in the heterologous expression. As postulated by other authors,\(^{17}\) it seems that LovB (the lovastatin nonaketide synthase) and EqxS
(equisetin PKS-NRP hybrid), at the very least, contain a binding pocket to direct the stereochemical outcome of the reaction. On the other hand, the direct product of the pyrrolocin PKS still contains a carbonyl in the correct position to activate an ene for the Diels–Alder reaction. By contrast, in lovastatin biosynthesis, more advanced PKS intermediates no longer contain an activated ene. This result implies that perhaps pyrrolocins are enzymatically cyclized in the wild-type host, but not in the recombinant host.

Of additional interest, despite the structural similarities between pyrrolocins and compounds such as equisetin, these compounds are often enantiomers of each other. While this fact is very convenient in terms of assigning the absolute configuration of the compound series as we have done here, it is curious. Why is the D-amino acid always found with one configuration and the L-amino acid with the other? We propose two possibilities. First, the presence of the decalin configuration would then dictate the stereochemically similar to activate an ene for the Diels–Alder reaction. Second, since the α position is relatively labile in the tetramic acid motif, the amino acid may begin as L-configured and equilibrate to the thermodynamically favored configuration based upon the stereochecmy of the decalin ring. These ideas provide a testable hypothesis that will enable understanding of the timing and biochemical basis of the Diels–Alder reaction in pyrrolocins and the many structural analogues.

**Biological Activity.** Compounds 1–3 were found to be active against *M. tuberculosis*, with 1 and 3 being more potent than 2 (Table 3). The data seem to indicate that a trans configuration and N-methylation enhance the potency of antimicrobial activity.

**Table 3. Antimycobacterial Activities of 1–3**

| compound | anti-TB IC₅₀ (μM) | cytotoxicity IC₅₀ (μM) |
|----------|-------------------|------------------------|
| 1        | 26.3              | 76.6                   |
| 2        | 112.9             | 167.0                  |
| 3        | 56.4              | 112.9                  |
| rifampicin | 0.152          | not determined         |

**EXPERIMENTAL SECTION**

**General Experimental Procedures.** UV spectra were measured on a Hewlett-Packard 8452A diode array spectrophotometer (Agilent, Santa Clara, CA, USA). IR spectra were recorded using a JASCO (Easton, MD, USA) FT/IR-400 spectrometer. Specific rotations were recorded on a PerkinElmer (Downers Grove, IL, USA) 343 digital polarimeter in MeOH at 22 °C. CD spectra were recorded on an Aviv model 420 CD spectrometer (Aviv Biomedical, Inc., Lakewood, NJ, USA) in methanol at 25 °C. NMR spectra were recorded on a Varian (Palo Alto, CA, USA) INOVA at 500 MHz for 1H and 13C using vendor-supplied pulse sequences.

Accurate mass measurements were performed by HRESIMS on a Micromass Q-TOF Micro (Waters, Milford, MA, USA) using positive ion mode and an FTMS (LTQ-FT, ThermoFisher, Waltham, MA, USA). HPLC was performed on an Agilent 1200 series equipped with a C18 column at a 3.5 mL/min flow rate as follows: 60% ACN/20% 0.1% trifluoroacetic acid (TFA). The peak at 17 min was collected and evaporated under reduced pressure to afford 1 as a colorless oil (7.2 mg). The compound was further chromatographed by HPLC using a different column (YMCDS-A, 10 μμ, 30 × 250 mm) and a gradient solvent of 85–100% acetonitrile in water (20 mL/min, 23 min run), with both solvents containing 0.01% in volume of trifluoroacetic acid (TFA). The peak at 18 min, monitored at 293 nm, was concentrated to give partially purified pyrrolocin. The compound was further chromatographed by HPLC using a different column (YMCDS-A, 10 μμ, 30 × 250 mm) and a gradient solvent of 80–100% acetonitrile in water (20 mL/min, 23 min run), with both solvents containing 0.01% in volume of TFA. The peak at 17 min was collected and evaporated under reduced pressure to afford 1 as a colorless oil (7.2 mg).

**Fermentation Procedure for *F. heterosporum*.** Mutant strains of *F. heterosporum* carrying pyrrolocin biosynthetic genes were maintained and grown as described.

**Isolation of 1–3 from *F. heterosporum*.** Three-week cultures of *F. heterosporum* grown on 500 g of corn grit agar at room temperature in 2.5 L Fernbach flasks were extracted twice with acetone for 12 h. The cells were extracted three times with methanol (0.6 L). The combined extract was evaporated under reduced pressure to dryness. The methanol extract was then purified by reversed-phase HPLC using a C18 column (Phenomenex Luna, 12 μμ, 30 × 250 mm) and a gradient solvent of 85–100% acetonitrile in water (40 mL/min, 0 min run), with both solvents containing 0.01% in volume of trifluoroacetic acid (TFA). The peak at 18 min, monitored at 293 nm, was concentrated to give partially purified pyrrolocin. The compound was further chromatographed by HPLC using a different column (YMCDS-A, 10 μμ, 30 × 250 mm) and a gradient solvent of 80–100% acetonitrile in water (20 mL/min, 23 min run), with both solvents containing 0.01% in volume of TFA. The peak at 17 min was collected and evaporated under reduced pressure to afford 1 as a colorless oil (7.2 mg).

**Isolation of (R)- and (S)-MTPA Ester Derivatives of 2 and 3.** Compounds 2 and 3 (0.5 mg each) were separately dissolved in pyridine-d₅ (150 μL) and transferred into clean NMR tubes. Under a N₂ gas stream, (R)-(+) -α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (5 μL) was added to each sample, and the NMR tubes were then carefully shaken to mix the samples and the MTPA chloride evenly. The NMR tubes were allowed to stand at room temperature and monitored every 2 h by 1H NMR. The reaction was found to be complete after 6 h. 1H NMR data of the (S)-MTPA ester derivative of 2 (2a) (500 MHz, pyridine-d₅, data were assigned on the basis of its 1H–1H COSY correlations): 0.81 (3H, d, J = 6.3 Hz, H3-6-Me), 1.21 (3H, s, H3-2-Me), 1.31 (3H, d, J = 6.3 Hz, H3-7), 2.44 (2H, m, H2-16), 5.12 (1H, s, H-9), 1.64 (3H, s, H5, -10-Me), 5.58 (1H, p, J = 7.3 Hz).
Plant extracts and control drugs were separately dissolved in pyridine-d$_6$ and reacted with (S)-(−)-α-methoxy-α-(trifluoromethyl)phenacyl chloride (5 μL) in NMR tubes at room temperature for 6 h to a was solubilized by the addition of 50 μL of enriched 7H9 medium into 96-well culture clusters at 100 000 cells per 200 μL of 1 N NaHCO$_3$. The mixture was usually agitated for 5 min using a Fisher Scientific MS1 57 plate shaker. A$_{570}$ was determined using a Scientific Multiskan FC (Fisher Scientific) plate reader. The resulting data were processed with a specially made Excel spreadsheet to calculate the percent inhibition as follows: the average of the media-only blank was subtracted from all wells. The average of the test wells for each compound was subtracted from the average of the positive control wells and divided by the average of the positive growth control, resulting in a fraction of inhibition. To determine the percent fraction of inhibition, the fraction of control was subtracted from unity and multiplied by 100. The IC$_{50}$ was determined from the percent inhibition as the concentration of test material that inhibited at least 50% of TARC cell growth.

**Pyrolocin A (1):** brownish oil; [α]$_D$ +56.1 (c 0.03, MeOH); UV (MeOH) $\lambda_{max}$ nm (log ε) 244 (3.48), 295 (3.05); CD $\lambda_{max}$ nm ($\Delta$) 239 (+5.82), 294 (+2.40); IR (NaCl disk) $\nu_{max}$ 3317, 2946, 2832, 2161, 1977, 1679; $^1$H NMR (DMPO-d$_6$, 400 MHz) and $^{13}$C NMR (DMPO-d$_6$, 100 MHz) see Table 1; HRESIMS m/z 458.2906 [M + H]$^+$ (calculated for C$_{26}$H$_{38}$NO$_5$ 458.2906).

**Pyrolocin B (2):** off-white solid; [α]$_D$ = −150 (c 0.025, MeOH); UV (MeOH) $\lambda_{max}$ nm (log ε) 238 (4.156), 286 (4.026); $\lambda_{max}$ NMR (Δc, −16.9), 236 (−16.9), 283 (3.0); IR (NaCl disk) $\nu_{max}$ 3325, 2945, 2834, 2362, 2340, 1683, 1653, 1456; $^1$H NMR (CD$_3$OD, 500 MHz) and $^{13}$C NMR (CD$_3$OD, 125 MHz) see Table 1; HRESIMS m/z 444.2751 [M + H]$^+$ (calculated for C$_{34}$H$_{45}$NO$_5$ 444.2750; Δ +0.2 ppm).

**Pyrolocin C (3):** off-white solid; [α]$_D$ +130 (c 0.025, MeOH); UV (MeOH) $\lambda_{max}$ nm (log ε) 247 (4.776), 286 (4.1049); $\lambda_{max}$ NMR (Δc, −16.9), 236 (−16.9), 283 (3.0); IR (NaCl disk) $\nu_{max}$ 3325, 2945, 2834, 2362, 2340, 1683, 1653, 1456; $^1$H NMR (CD$_3$OD, 500 MHz) and $^{13}$C NMR (CD$_3$OD, 125 MHz) see Table 1; HRESIMS m/z 444.2762 [M + H]$^+$ (calculated for C$_{34}$H$_{45}$NO$_5$, 444.2750; Δ +2.7 ppm).

**ASSOCIATED CONTENT**

1 Supporting Information

1D and 2D NMR spectra for compounds 1–3 and HPLC chromatograms of the FDLA derivatives are available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Author Contributions**

All authors have contributed to the manuscript and have given approval to the final version.

**Notes**

The authors declare no competing financial interest.

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