Rugulotrosins A and B: Two New Antibacterial Metabolites from an Australian Isolate of a *Penicillium* sp.

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Two new antibacterial agents, rugulotrosin A (**1**) and B (**2**), were obtained from cultures of a *Penicillium* sp. isolated from soil samples acquired near Sussex Inlet, New South Wales, Australia. Rugulotrosin A (**1**) is a chiral symmetric dimer, and its relative stereostructure was determined by spectroscopic and X-ray crystallographic analysis. Rugulotrosin B (**2**) is a chiral asymmetric dimer isomeric with **1**. Its structure was determined by spectroscopic analysis with comparison to the co-metabolite **1** and previously reported fungal metabolites. Both rugulotrosins A and B displayed significant antibacterial activity against *Bacillus subtilis*, while rugulotrosin A was also strongly active against *Enterococcus faecalis* and *B. cereus*.

Natural products derived from microbes have long been an inspiration for the development of drugs with application in the fields of human and animal health and crop protection. Combating microbial infection and disease with metabolites extracted from bacterial and fungal isolates has been a cornerstone of modern pharmacy. A seemingly unavoidable corollary to such practice has been the buildup of resistance by pathogenic microbes, such that many former (and current) drugs are either no longer effective or their efficacy is seriously compromised. Multiple drug-resistant microbes are proving to be an exceptionally serious challenge to healthcare organizations across the developing and developed world.

In the search for new generation antibacterials that are both safe to the patient and effective against drug-resistant microbes, we have elected to investigate the relatively unexplored Australian microbial biodiversity. We have screened several hundred thousand bacterial and fungal isolates sourced from soil and plant samples across Australia against a suite of biological activities, including antibacterial. Many of the more promising leads have progressed to scale-up culture and are the subject of bioassay-directed chemical fractionation. In this report, we describe the isolation and structure elucidation of two new antibacterials, rugulotrosins A (**1**) and B (**2**), obtained from one such microbial isolate, *Penicillium* sp., MST-F8741.

The solvent extract of a solid phase culture of *Penicillium* sp. was found to exhibit significant activity against *Bacillus subtilis*. Chromatography of this extract yielded two new antibacterial agents, rugulotrosin A (**1**) (*B. subtilis*, LD_{99} 5.5 μg/mL) and rugulotrosin B (**2**) (*B. subtilis*, LD_{99} 25.0 μg/mL). Full details of the isolation procedure are in the Experimental Section.

High-resolution ESI(+)MS analysis of **1** revealed a pseudo-molecular ion (M + Na, Δ mmu = −0.3) consistent with a molecular formula ($C_{32}H_{30}O_{14}$) requiring 18 double-bond equivalents (DBE). The ^{1}H and ^{13}C NMR spectra of **1** clearly indicated a highly substituted aromatic compound possessing a single plane or point of symmetry. Recrystallization of **1** from EtOH returned crystals suitable for X-ray crystallographic analysis,[1] which in turn led to recognition of the relative stereostructure for **1** as shown in Figure 1. Data were collected at room temperature, and the structure contains two EtOH molecules of crystallization, which are disordered. For the purposes of the discussion on the crystal structure a separate nomenclature is used that corresponds to atom positions in Figure 1. The molecules of rugulotrosin A (**1**) lie on a 2-fold axis of symmetry, and the biaryl linkage is essentially orthogonal with a dihedral angle (C1−C2−C2′−C1′ 87.4(5)°). Although the molecule is chiral, the X-ray crystallographic analysis was unable to assign absolute configuration (Flack parameter −0.5(5)).

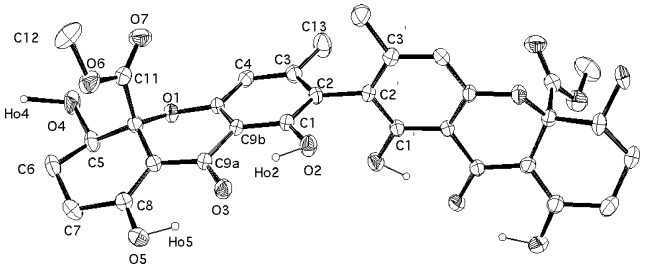

Figure 1. Thermal ellipsoid plot of rugulotrosin A (**1**). Ellipsoids are at the 20% probability level. Some atom labels have been omitted for clarity.

Assignments of all ^{1}H and ^{13}C NMR data for rugulotrosin A (**1**) were achieved (see Table 1) by comparison with the published monomers α-diversonolic ester (**3**) and *β*-diversonolic ester (**4**)[2] and the related asymmetric dimer neosartorin (**5**).[3] In an attempt to secure the absolute stereochemistry rugulotrosin A (**1**) was converted to the dibromo derivative **6**. The structure of the dibromo analogue **6** was confirmed by ESI(+)MS, which indicated the inclusion of two bromine atoms, as well as ^{1}H NMR spectroscopy, which revealed substitution at C-4 and C-4′. Unfortunately, the dibromo derivative **6** did not yield crystals suitable for X-ray analysis. It is interesting to note that the antibacterial activity of the dibromo derivative **6** (*B. subtilis*, LD_{99} 3.1 μg/mL) was comparable to **1**.

High-resolution ESI(+)MS analysis of rugulotrosin B (**2**) revealed a pseudo-molecular ion (M + Na, Δ mmu = 0.3) consistent with a molecular formula isomeric with **1**. Likewise, the ^{1}H and ^{13}C NMR spectra for **2** were very

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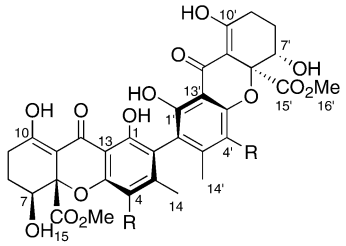
Table 1. NMR ($CDCl_3$, 400 MHz) Data for Rugulotrosin A (**1**)[a,b]

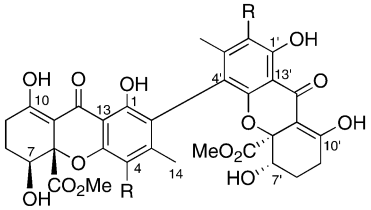
no.	^{13}C δ	^{1}H δ [m, J (Hz)]
1/1′	159.3	
1/1′-OH		11.52 (s)
2/2′	116.7	
3/3′	150.2	
4/4′	109.2	6.53 (s)
5/5′	157.9	
6/6′	84.3	
7/7′	71.9	4.23 (dd, 4.7, 4.7)
8/8′	23.8	2.20 (m)
9/9′	27.5	2.63 (m)
10/10′	177.7	
10/10′-OH		13.86 (s)
11/11′	101.0	
12/12′	186.6	
13/13′	104.8	
14/14′	20.9	2.03 (s)
CO_2CH_3	170.2	
CO_2CH_3	53.1	3.73 (s)

[a] ^{13}C NMR assignments are supported by a DEPT 135 experiment. [b] $^{1}H-^{1}H$ COSY correlations observed: H-8/H-8′ to H7/H-7′ and H-9/H-9′; H-7/H-7′ to H-8/H-8′; H-9/H-9′ to H-8/H-8′.

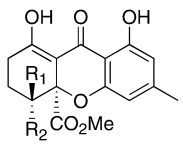
similar to **1** (see Table 2); however, the doubling of each resonance revealed an asymmetric rather than symmetric dimer. Careful analysis of the gHMBC NMR data for **2** revealed diagnostic correlations between H-4 and C-5 and again between H-2′ and C-1′. These correlations unambiguously confirmed an asymmetric C-2 to C-4′ bridge between



1 R = H
6 R = Br



2 R = H
7 R = Br



3 R_1 = H, R_2 = OH
4 R_1 = OH, R_2 = H

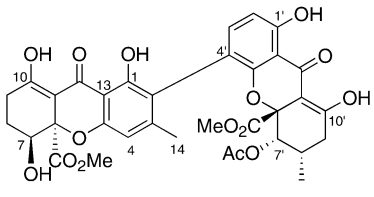


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Table 2. NMR ($CDCl_3$, 400 MHz) Data for Rugulotrosin B (**2**)[a]

no.	^{13}C δ	^{1}H δ [m, J (Hz)]	gHMBC
1/1′	159.4, 159.3		
1/1′-OH		11.61 (s), 11.51 (s)	C-1/C-1′, C-2/C-2′, C-13/C-13′
2/2′	117.4, 109.2	6.55 (s)	C-1′, C-4′, C-12′, C-13′, C-14′
3/3′	150.2, 149.7		
4/4′	109.3, 116.7	6.51 (s)	C-2, C-5, C-12, C-13, C-14
5/5′	157.9,156.8		
6/6′	84.3, 83.8		
7/7′	71.9, 67.2	4.33 (d, 1.9), 4.32 (d, 1.7)	C-6/C-6′, C-9/C-9′, C-11/C-11′, CO_2CH_3
8/8′	23.8, 23.0	2.70 (m), 2.35 (m)	C-7/C-7′, C-9/C-9′, C-10/C-10′
9/9′	27.5, 24.4	2.66 (m)	C-7/C-7′, C-8/C-8′, C-10/C-10′
10/10′	179.2, 177.6		
10/10′-OH		14.06 (s), 13.88 (s)	C-9/C-9′, C-10/C-10′, C-11/C-11′
11/11′	99.9, 101.0		
12/12′	186.9, 186.2		
13/13′	104.9, 104.9		
14/14′	20.9, 20.8	2.03 (s), 2.01 (s)	C-2/C-2′, C-3/C-3′, C-4/C-4′
CO_2CH_3	171.2, 170.2		
CO_2CH_3	53.6, 53.1	3.74 (s), 3.74 (s)	CO_2CH_3

[a] ^{13}C NMR assignments are supported by HMQC and gHMBC experiments.

the monomeric units in rugulotrosin B (**2**), in contrast to the symmetric C-2 to C-2′ bridge evident in rugulotrosin A (**1**). Unfortunately, rugulotrosin B (**2**) did not yield crystals suitable for X-ray analysis.

Bromination of rugulotrosin B (**2**), as described earlier for rugulotrosin A (**1**), yielded the dibromo derivative **7**, which was an oil and so unsuitable for X-ray analysis. On biogenetic grounds, the relative stereochemistry of **2** can be assigned as for **1**. Rugulotrosin B (**2**) can exist as two atropisomers around the intra-unit bridge, as for rugulotrosin A (**1**). Only one atropisomer was observed; however, which one has not been elucidated. The structure diagrams shown for **1** and **2** are not intended to convey absolute stereochemistry. The antibacterial activity of the dibromo analogue **7** (*B. subtilis*, LD_{99} 6.3 μg/mL) improved 4-fold compared to rugulotrosin B (**2**) (*B. subtilis*, LD_{99} 25.0 μg/mL).

The rugulotrosins are new examples of a known fungal metabolite molecular motif, which possess the less common *cis* stereochemistry for substituents about C-6 and C-7 and an aromatic methyl on ring C as opposed to the more common secondary methyl on ring A (for example neosartorin (**5**)).[3] Rugulotrosin A (**1**) was subsequently tested against a series of bacteria and proved to be antibacterial against Gram-positive (*Enterococcus faecalis*, LD_{99} 1.6 μg/mL; *B. cereus*, 3.1 μg/mL; *Staphylococcus aureus* 200 μg/mL) but not Gram-negative organisms.

Experimental Section

General Experimental Procedures. General experimental procedures are as for previous work.[4] In addition, solid phase extraction was carried out by using either Varian HF C_{18} (10 g) or Alltech Maxi-clean C_{18} (1 g) cartridges.

X-ray crystallographic data for **1** were collected from an Enraf Nonius CAD4f diffractometer. The unit cell dimensions were determined by least-squares refinement of the setting angles of 25 reflections with $20° \leq \theta \leq 25°$; 3915 data were collected at room temperature operating in the $\theta/2\theta$ scan mode of which 3443 were unique [$R_{int} = 0.0442$]. Data were corrected for Lorentz and polarization effects.[5] The intensities of three

standard reflections, measured every 160 min throughout the data collection, showed only small random variations. The structure was solved by direct methods (SHELXS-86)[6] and was refined on F^2 (SHELXL-93).[7] Hydrogen atoms with the exception of the OH hydrogens were fixed in idealized positions.

Antibacterial activity was determined in an agar-based, microtiter plate assay. Briefly, an aliquot of an overnight fermentation of *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), or *Staphylococcus aureus* (ATCC 25923) was applied to the surface of an agar matrix that contained the test compound, then incubated at 28 °C. A qualitative assessment of bacterial growth was made at 24 h, with the LD_{99} determined as the lowest concentration of the test compound at which no growth of bacteria was observed.

Biological Material. This organism was isolated from a road-side soil sample collected near Sussex Inlet on the southern coast of New South Wales, Australia. The morphological characteristics of the strain place it in the Biverticillium subgroup of the genus *Penicillium*, close to *P. rugulosum*. Although the strain has no outstanding features, it does not fit any of the described *Penicillium* species and has been assigned a nominated code, MST-F8741.

Extraction and Isolation. A fermentation (200 mL, malt agar, 21 days) was extracted with MeOH (~800 mL). This extract was concentrated in vacuo to an aqueous residue that was diluted with H_2O to a final volume of 1000 mL. Passage through two parallel C_{18} SPE cartridges (2 × 10 g, Varian HF C_{18}), eluting with MeOH (2 × 40 mL each), afforded an antibacterially active fraction. This material was partitioned between n-BuOH and H_2O. The n-BuOH-soluble material (973 mg) was recrystallized from MeOH to yield rugulotrosin A (**1**) (191 mg, 13.5%, LD_{99} 5.5 μg/mL). The supernatant (782 mg) was subjected to further partitions between petroleum spirits and 20% H_2O/MeOH, with the aqueous MeOH layer (208 mg) dried and further partitioned between 50% H_2O/MeOH and CH_2Cl_2. The resulting CH_2Cl_2-soluble material (162 mg) was then subjected to preparative C_{18} HPLC (3 injections, 7.5 mL/min with gradient elution of 65% MeOH/H_2O (0.05% TFA) to 80% MeOH/H_2O (0.05% TFA) over 20 min, through a 5 μm Phenomenex LUNA C_{18} 150 × 21.2 mm column). This produced more of rugulotrosin A (**1**) (21.5 mg, 1.5%) plus a mixture of rugulotrosin A (**1**) and rugulotrosin B (**2**) (58.6 mg), which was then rechromatographed on the same C_{18} HPLC column, with 6 injections under identical elution conditions. The compound eluting at between 11.5 and 13 min was rugulotrosin B (**2**) (7.9 mg, 0.6%, *B. subtilis*, LD_{99} 25 μg/mL).

Rugulotrosin A (1): yellow crystals; mp 189−190 °C; $[\alpha]^{25}_D$ +260° (*c* 1.30, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3009, 2936, 2858, 1732, 1611, 1583, 1460, 1437, 1238, 1194, 1175, 1115 cm^{-1}; UV (MeOH) λ_{max} (ϵ) 205 (27900), 222 (sh), 281 (5500), 337 (33200), 372 (sh) nm; ^{1}H NMR ($CDCl_3$, 400 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; ESI(+)MS (30 kV) *m/z* 661 (M + Na)$^+$, *m/z* 639 (M + H)$^+$; HRESI(+)MS *m/z* 661.1530 [(M + Na)$^+$, $C_{32}H_{30}O_{14}Na$ requires 661.1533].

Crystallographic Data for 1. A yellow rod was obtained from EtOH of size 0.4 × 0.15 × 0.1 mm, $C_{32}H_{30}O_6 \cdot (EtOH)_2$, MW = 730.7. The crystal system was orthorhombic with cell parameters a = 13.486(2) Å, b = 11.929(2) Å, c = 11.525 Å, V = 1854.1(5) Å^3, space group $P2_12_12$, μ(Cu Kα) = 0.877 mm^{-1}, θ range for data collection 3.8−69.8°, index ranges $-16 \le h \le 16$, $-14 \le k \le 14$, $-14 \le l \le 11$, data/restraints/parameters 3443/1/255, goodness-of-fit on F^2 = 1.010, final R indices [$I > 2\sigma(I)$] R_1 = 0.0707, wR_2 = 0.1354, R indices (all data) R_1 = 0.1622, wR_2 = 0.1745, largest difference peak and hole 0.209 and −0.192 e Å^{-3}, respectively.

Rugulotrosin B (2): yellow oil; $[\alpha]^{25}_D$ +139° (*c* 0.52, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3013, 2957, 2930, 1736, 1611, 1583, 1450, 1364, 1285, 1240, 1171 cm^{-1}; UV (MeOH) λ_{max} (ϵ) 207 (26300), 225 (sh), 281 (7700), 338 (22700), 375 (sh) nm; ^{1}H NMR ($CDCl_3$, 400 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; ESI(+)MS (30 kV) *m/z* 661 (M + Na)$^+$, *m/z* 639 (M + H)$^+$; HRESI(+)MS *m/z* 661.1536 [(M + Na)$^+$, $C_{32}H_{30}O_{14}Na$ requires 661.1533].

Bromination of Rugulotrosin A. A sample of **1** (19.8 mg) was dissolved in $CHCl_3$ (20 mL) and $FeCl_3$ (80 mg) added under ice cooling. To this solution was added dropwise 20 mL of bromine water and the reaction left to stir for 90 min. After this time the flask was removed from the ice bath and left to stir for a further 30 min at room temperature. The reaction was quenched by the addition of $Na_2S_2O_4$. The reaction mixture was then partitioned between $CHCl_3$ and H_2O to yield 4,4′-dibromorugulotrosin A (**6**) (29.0 mg, quantitative yield) as a yellow solid: mp 145−146 °C; $[\alpha]^{25}_D$ +31° (*c* 0.1, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3032, 3013, 2957, 2930, 1736, 1611, 1578, 1435, 1362, 1273, 1238 cm^{-1}; UV (MeOH) λ_{max} (ϵ) 205 (36800), 216 (sh), 282 (6900), 338 (26500), 372 (sh) nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 13.72 (2H, s, C-10/C-10′-O*H*), 11.53 (2H, s, C-1/C-1′-O*H*), 4.38 (2H, dd, J = 5.2, 5.2 Hz, H-7/H-7′), 3.75 (6H, s, CO_2CH_3), 2.69−2.20 (8H, m, H-8/H-8′/H-9/H-9′), 2.18 (6H, s, H-14/H-14′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 186.3 (s, C-12/C-12′), 178.7 (s, C-10/C-10′), 169.6 (CO_2CH_3), 158.3 (s, C-1/C-1′), 154.3 (s, C-5/C-5′), 148.9 (s, C-3/C-3′), 118.3 (s, C-2/C-2′), 105.6 (s, C-4/C-4′), 103.7 (s, C-13/C-13′), 100.8 (s, C-11/C-11′), 85.3 (s, C-6/C-6′), 71.8 (d, C-7/C-7′), 53.3 (CO_2CH_3), 27.6 (t, C-9/C-9′), 23.5 (t, C-8/C-8′), 21.6 (q, C-14/C-14′); ESI(+)MS (30 kV) *m/z* 795/797/799 ([M + H]$^+$), *m/z* 817/819/821 ([M + Na]$^+$); ESI(−)MS (30 kv) *m/z* 793/795/797 ([M − H]$^-$).

Bromination of Rugulotrosin B. A sample of **2** (6.0 mg) was brominated in a manner identical to that described above for **1** to afford 4,2′-dibromorugulotrosin B (**7**) (7.5 mg, quantitative yield) as a yellow oil: $[\alpha]^{25}_D$ +61° (*c* 0.2, $CHCl_3$); IR (film) ν_{max} 2955, 2930, 1736, 1610, 1578 cm^{-1}; UV (MeOH) λ_{max} (ϵ) 225 (sh), 275 (8200), 359 (15000) nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 13.93 (1H, s), 13.73 (1H, s), 11.68 (1H, s), 11.52 (1H, s), 4.44 (1H, brs), 4.39 (1H, dd, J = 5.1, 5.1 Hz), 3.75 (3H, s), 3.74 (3H, s), 2.75 (m), 2.40 (obs), 2.16 (3H, s), 2.15 (3H, s), 1.70 (m), 1.40 (m); APCI(−)MS (30 kV) *m/z* 793/795/797 ([M − H]$^-$).

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References and Notes

(1) Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre (CCDC number 197837). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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