Ravenic Acid, a New Tetramic Acid Isolated from a Cultured Microfungus, *Penicillium* sp.

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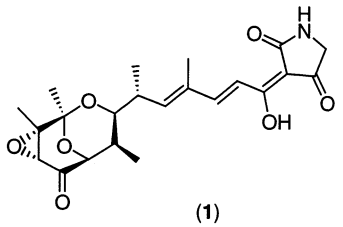
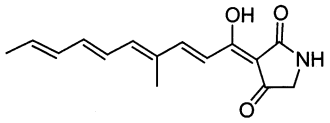
A new antibiotic polyene tetramic acid, ravenic acid (**2**), has been isolated from the mycelia of a cultured fungus, *Penicillium* sp. The structure of ravenic acid was determined by detailed spectroscopic analysis and the major isomer identified as possessing (3*Z*, 7*E*, 9*E*, 11*E*, 13*E*) stereochemistry.

Natural products containing the tetramic acid moiety continue to receive interest due to the range of biological activities they display, including antibiotic and antiviral activity, cytotoxicity, and phospholipase A_2 inhibition.[1] One of the earliest examples of a naturally occurring tetramic acid was the isolation and structural elucidation of tirandomycic acid (**1**), an antibiotic isolated from a *Streptomyces* sp. and characterized in part by X-ray diffraction techniques in 1973.[2]

Several related tetramic acid derivatives have since been isolated.[3] Of these, the majority are present as the *exo*-enol tautomer of the substituted 3-acyl derivative, as seen in **1** and **2**. In addition, some of the compounds isolated exist as *N*-Me derivatives, for example, Bu 2313 A,[4] and/or as 5-alkyl derivatives, such as physorubrinic acid.[3a,5]



(**1**)



(**2**)

In a search for new antibiotics derived from cultured microfungi our attention was focused toward a *Penicillium* sp. (MINAP9902) that exhibited activity against methicillin-resistant *Staphylococcus aureus* (MRSA), resulting in the isolation of the new polyene tetramic acid that we have named ravenic acid (**2**).[6]

The fungus *Penicillium* sp. (MINAP9902) was grown in liquid culture on malt extract broth. The concentrated ethanolic extract of the mycelium demonstrated antibiotic activity against several of the nosocomial pathogens employed in the screening program including methicillin-resistant *S. aureus* (MRSA). Solvent partitioning followed by gel permeation chromatography returned a major bioactive component.

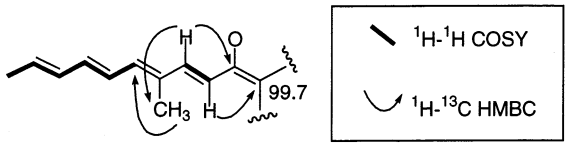


Figure 1. Major structural fragment derived from 2D correlations.

Ravenic acid (**2**) was isolated as an orange-yellow, amorphous solid. While both electron impact and FAB mass spectrometry failed to return useful information, electrospray ionization mass spectrometry operating in positive ion mode revealed significant ions at *m*/*z* 260, 519, 541, and 557. This strongly suggested the parent ion possessed a mass of 259 with the quasi-molecular ion (M + H) at 260 and 2 M peaks at 519 (+H), 541 (+Na), and 557 (+K). This was supported when an electrospray mass spectrum operating in negative ion mode revealed an intense quasi-molecular ion at *m*/*z* 258, while high-resolution mass measurement of this ion supported a molecular formula of $C_{15}H_{17}NO_3$.

Careful analysis of the ^{1}H NMR spectrum revealed the presence of 16 protons, indicating that an exchangeable proton was not observed. In addition a D_2O exchange experiment performed in $CDCl_3$ revealed a further slowly exchanging proton resonance at δ 5.85. The slow exchange rate of this resonance, along with its chemical shift, suggested it to be an amide NH, which was consistent with the observation of a carbonyl resonance at δ 176.6 and IR absorptions at 3288 and 1663 cm^{-1}. Examination of the ^{1}H−^{1}H COSY spectrum allowed a trienyl fragment to be established, and this in turn could be connected through HMBC correlations to a methyl resonance and an isolated *trans* coupled doublet. Additional correlations to two quaternary carbons, one of which was oxygenated (δ 174.8) from the *trans* coupled doublet, led to the partial structure depicted in Figure 1.

Six of the eight available degrees of unsaturation were accounted for between the structural unit depicted in Figure 1 and the amide carbonyl. ^{13}C NMR data indicated a further point of unsaturation could be attributed to a carbonyl resonance at δ 192.4. With a single carbon resonance (δ 51.5) unaccounted for, the remaining point of unsaturation could only be a heterocycle incorporating the amide functionality as a lactam. Detailed analyses of ^{1}H−^{13}C HMBC data were consistent with the 2,4-pyrrolidinedione moiety (Figure 2a), while a correlation from the

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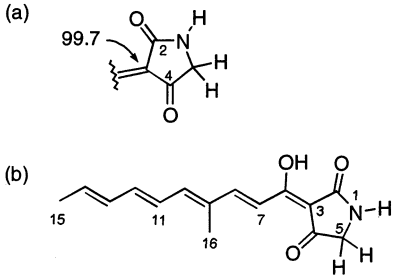

Figure 2. (a) Pyrrolidine-2,4-dione fragment derived from 2D correlations. (b) Deduced structure of 3*Z*-ravenic acid (**2**) including stereochemistry.

Table 1. ^{13}C NMR Shift Differences for the 3-*exo*-Enol Acyltetramic Acid Tautomers (**3a**,**b**) and (**2a**,**b**)

carbon no.	Δ (ppm) (**3a** major−**3b** minor)	Δ (ppm) (**2a** major−**2b** minor)
2	6.0	6.6
3	−3.3	−2.6
4	−5.3	−8.9
5	3.3	2.2

carbon resonance at δ 99.7, common to both structural fragments, secured the assignment of ravenic acid (**2**) as an enolized 3-acyltetramic acid (Figure 2b).

The $\Delta^{7,8}$, $\Delta^{11,12}$, and $\Delta^{13,14}$ bonds were determined to possess *E*-geometry in each case by the magnitudes of the vicinal coupling constants of 15.3, 14.0 (in DMSO-d_6), and 15.0 Hz, respectively. The geometry about the $\Delta^{9,10}$ bond was determined to be *E* by the observation of NOEs to 10-H and 16-H_3 upon irradiation of 8-H and 7-H, respectively. Only the final element of stereochemistry about the $\Delta^{3,6}$ bond remained. The ability of 3-acyltetramic acids to isomerize via two pairs of tautomers has been well documented;[7] however, both 1D and 2D NMR data suggested that ravenic acid existed solely as the pyrrolidine-2,4-dione, that is, as the *exo*-enol tautomer. It was equally apparent from analysis of the spectra that ravenic acid existed as an equilibrium mixture of double-bond isomers. Table 1 shows a comparison of selected ^{13}C NMR resonances between a model 3-acetyl tetramic acid, tenuazonic acid (**3a**,**b**),[7] and ravenic acid (**2a**,**b**). The clear parallels between the direction and magnitude of the carbon shifts of the major and minor isomers in each case strongly suggest that the dominant (*Z*) isomer in tenuazonic acid is also the dominant isomer in ravenic acid. Thus ravenic acid (**2**) was shown to exist as a tautomeric mixture (ratio ≈ 4:1 at room temperature) in which the major isomer was assigned as 3*Z* (Figure 3). This finding is consistent with modeling studies that suggest the most stable tautomer for 3-acyltetramic acids lacking an *N*-acyl substituent is the *Z*-*exo*-enol tautomer.[8]

Ravenic acid was found to inhibit the growth of a methicillin-resistant *S. aureus* strain down to 25 μg/mL. Further biological testing is underway to assess the spectrum of activity and determine the mode of action of this antibiotic.

Experimental Section

General Experimental Procedures. NMR spectra (δ) in ppm were recorded on a Varian Inova 500 spectrometer operating at 500 MHz (^{1}H) or 125 MHz (^{13}C). IR spectra were recorded on a Perkin-Elmer 1600 Series FTIR as a thin film on NaCl plates. UV spectra were recorded on a Cary 4G UV−vis spectrophotometer as a solution in methanol. ESIMS spectra were recorded on a Fisons VG Quattro II mass spectrometer, operating at a cone voltage between 15 and 70 V, with both positive and negative ion detection, as indicated.

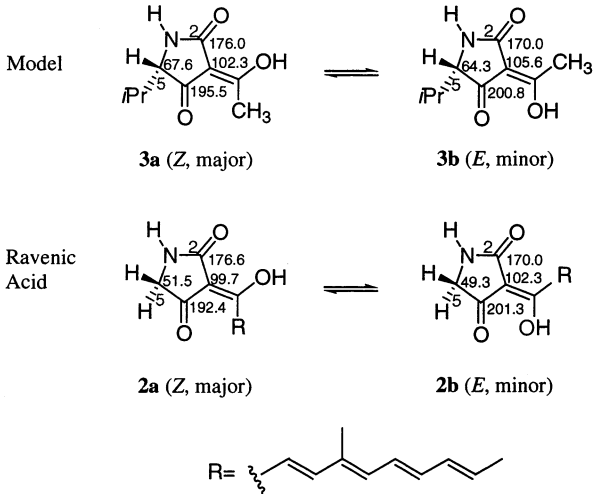

Figure 3. ^{13}C ($CDCl_3$, 125 MHz) shift comparisons for the predominant isomers of **2** and **3**.

Fungus Isolation and Fermentation. The fungus identified as a *Penicillium* sp. was isolated from the interior of a surface-sterilized fruiting body of the myxomycete *Lycogala epidendrum* collected at Ravensbourne National Park, south east Queensland in June 1998. The fungus was amended to the culture collection maintained in the Department of Chemistry at the Australian National University and has been allocated the accession number MINAP9902. The fungus was grown on malt extract agar (Difco) plates and vegetative mycelia used to inoculate 2 × 250 mL flasks each containing 100 mL of malt extract broth. Fermentation at 25 °C (3 days, 125 rpm) produced seed cultures used to inoculate 4 × 1 L flasks each containing 400 mL of a sterile solution of malt extract broth (Difco). These cultures were grown at 25 °C (15 days, 125 rpm) before the yellow mycelia (27 g wet weight) were mechanically separated from the broth by filtration and the mycelia homogenized in a solution of CH_2Cl_2/EtOH (1:4, 150 mL). The crude extract was concentrated, then suspended in 250 mL of deionized water, and extracted successively with hexane (2 × 100 mL), CH_2Cl_2 (2 × 100 mL), and EtOAc (2 × 100 mL). The combined CH_2Cl_2 extracts, containing 100% of the bioactivity, were dried ($MgSO_4$), filtered, and concentrated to return a red-brown solid that was subjected to Sephadex LH-20 chromatography (320 mm × 25 mm i.d.), eluting with MeOH. A distinct yellow-orange band eluting between 450 and 500 mL was concentrated to yield ravenic acid (8.1 mg), as an amorphous orange powder.

Ravenic acid (2): UV (EtOH) λ_{max} (ϵ) 240 (9900), 366 (11 000); (+1 drop, 1 N NaOH) 241 (17 000), 350 (18 000); (+1 drop, 1 N HCl): 241 (8900), 419 (14 600); IR (KBr disk) ν_{max} 3288, 2961, 1663, 1614, 1557, 1438, 1259, 1094, 1019, 798 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) 7.59 (1H, d, J = 15.3 Hz, H-8), 7.20 (1H, d, J = 15.3 Hz, H-7), 6.57 (1H, d, J = 10.2 Hz, H-10), 6.49 (1H, m, H-11), 6.47 (1H, m, H-12), 6.23 (1H, ddd, J = 15.0, 9.2, 1.2, H-13), 5.92 (1H, dq, J = 15.0, 6.9 Hz, H-14), 5.85 (1H, br s, 1-NH), 3.82 (2H, s, H-5), 2.01 (3H, s, H-16), 1.83 (3H, dd, J = 6.9, 1.2 Hz, H-15); ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.70 (1H, br s, NH), 7.51 (1H, d, J = 15.0 Hz, H-8), 7.06 (1H, d, J = 15.0 Hz, H-7), 6.75 (1H, d, J = 11.0 Hz, H-10), 6.63 (1H, dd, J = 14.0, 11.0 Hz, H-11), 6.55 (1H, dd, J = 14.0, 10.5 Hz, H-12), 6.29 (1H, ddq, J = 15.0, 10.5, 2.0 Hz, H-13), 5.78 (1H, dq, J = 15.0, 7.0 Hz, H-14), 3.75 (2H, s, H-5), 1.92 (3H, s, H-16), 1.80 (3H, br d, J = 7.0 Hz, H-15); ^{13}C NMR (125 MHz, $CDCl_3$) 192.4 (s, C-4), 176.6 (s, C-2), 174.8 (s, C-6), 149.6 (d, C-8), 142.6 (d, C-10), 139.5 (d, C-12), 134.5 (d, C-14), 134.2 (d, C-9), 132.0 (d, C-13), 126.4 (d, C-11), 116.4 (d, C-7), 99.7 (s, C-3), 51.5 (t, C-5), 18.7 (q, C-15), 12.5 (q, C-16); ESIMS (+ve ion, CV = 50 V) 260 (M + H), 282 (M + Na), 519 (2M + H), 541 (2M + Na), 557 (2M + K); ESIMS (−ve ion, CV = 60 V) 258 (M − H, 100%); HRESIMS 258.1132 ($C_{15}H_{16}NO_3$ requires 258.1138).

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Supporting Information Available: Figure 4 showing ^{1}H (500 MHz) and Figure 5 showing ^{13}C (APT, 125 MHz) NMR spectra for **2**. These data are available free of charge via the Internet at http://pubs.acs.org.

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