An Unusual Oxalylated Tetramic Acid from the New Zealand Basidiomycete *Chamonixia pachydermis*

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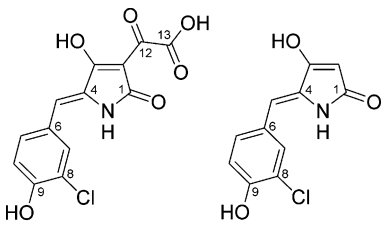
An unusual oxalylated tetramic acid, pachydermin (**1**), has been isolated from the New Zealand basidiomycete *Chamonixia pachydermis*. The full structure, which was not directly accessible by NMR methods, was deduced from that of a degradation product, 5-(3-chloro-4-hydroxybenzylidene)tetramic acid (**2**). The degradation product **2** exhibited mild antibacterial activity against *Bacillus subtilis*.

Within the scope of our ongoing program for the isolation of biologically active natural products from New Zealand filamentous fungi[1] and mushrooms,[2] we investigated the basidiomycete *Chamonixia pachydermis* (Boletaceae).[3] This fungus is a gasteromycete (i.e., its spores are formed inside a closed fruiting body) and has been reported from Australia and New Zealand, where it is often found in association with *Nothofagus* forest.

HPLC-DAD analysis showed that the MeOH extract of this fungus contained gyroporin, which had been described before from another *Chamonixia* species.[4] The only other predominant metabolite, pachydermin (**1**), had a UV absorption maximum at 357 nm, suggesting an extensively conjugated carbonyl chromophore. The molecular mass, 309 Da, was derived from the HPLC-ESIMS, which also displayed an isotopic pattern indicative of monochlorination.

The purification of **1** was complicated by the observed degradation under the acidic conditions (0.05% TFA) used for the preparative HPLC. With a neutral eluent **1** was hardly retained in the reversed-phase chromatography, suggesting the acidic nature of the compound. By consecutive reversed-phase chromatography and gel chromatography on Sephadex LH-20 it was possible to isolate **1** as a water-soluble salt. The ESIMS (pos.) indicated a mixture of Na^+ and K^+ as the counterions.

The ^{1}H NMR spectrum of **1** (as the free acid in DMSO-d_6) showed two doublets, H-7 at δ 7.74 and H-10 at δ 7.05, and one doublet of doublets at δ 7.49 (H-11) with coupling constants characteristic of a 1,3,4-trisubstituted benzene system. One additional singlet (H-5) appeared at δ 6.41 and one broad singlet at δ 9.88. The 13 signals in the ^{13}C NMR spectrum, together with an accurate mass of *m*/*z* 310.0150 for the $[M + H]^+$ ion, supported a molecular formula of $C_{13}H_8ClNO_6$ for **1**. Long-range H,C-couplings of H-5 to C-7 and to C-11 revealed this to be a vinylic proton on a double bond attached to the carbon C-6 of the aromatic ring. The further substituents of this ring were deduced from the ^{13}C chemical shifts to be chlorine at C-8 and a phenolic hydroxyl group at C-9 and confirmed by observed $^2J_{CH}$ and $^3J_{CH}$ couplings in the HMBC spectrum. The chemical shifts of this 3-chloro-4-hydroxyphenyl system are in good agreement with those of 3-chloro-4-hydroxyphenylacetamide, a known metabolite from a plant-associated fungus.[5] This left about half of the molecular formula, a $C_6H_3NO_5$ unit, unaccounted for. For this part of the molecule there were four low-field carbons (δ 179.3, 177.0, 170.3, 165.7) and two sp^2-carbons (δ 130.9, 99.4) in the ^{13}C NMR spectrum, but there were no associated sharp proton signals that could be used for the assembly of a structure by 2D NMR methods. The only HMBC correlation linking the identified substructure with the unknown part was from the vinylic proton H-5 to a carbon at δ 177.0 (C-3), which therefore had to be located in the allylic position of the vinylic double bond.



To get more information about the structure of **1**, it was necessary to identify the structure of the degradation product **2** that was formed, even under weakly acidic conditions, e.g., the 0.05% aqueous TFA used for HPLC. Heating of **1** in 2 M HCl led to complete conversion into **2**. From HRESIMS the molecular formula of **2** was deduced as $C_{11}H_8ClNO_3$, corresponding to a loss of C_2O_3 from the parent structure. The ^{1}H NMR spectrum showed a mixture of two tautomeric forms, **2** and **2′**, in a 2:1 ratio. In both tautomers the 3-chloro-4-hydroxyvinyl system observed in **1** was intact. An additional singlet signal of one olefinic proton (5.04 ppm; H-2) was observed in the subspectrum of **2**, while that of **2′** contained a methylene singlet (δ 3.27; H-2). In contrast to the ^{1}H NMR spectrum of **1**, that of **2** showed exchangeable protons as sharp singlets with HMBC and ROESY correlations useful for the elucidation of structures **2** and **2′** (Figure 1). Chemical shifts and HMBC correlations were consistent with **2′** being a pyrrolidine-2,4-dione,[6] and **2** the corresponding enol, substituted in the 5-position with a 3-chloro-4-hydroxybenzylidene residue. The vinylic double bond was *Z*-configured, as indicated by the ROESY interactions in **2** of the 3-O*H* with H-5 and H-2 and of the amide proton to H-7 and H-11 (Figure 1).

With the acid degradation product **2** identified, a structure for the natural product **1** can be proposed. The loss of C_2O_3 during the conversion of **1** to **2** can be interpreted as a combined decarbonylation−decarboxylation reaction.[7] Consequently, the presumed structure of the natural product is that of **2** substituted in the 2-position of the pyrrole ring with an oxalyl residue (**1**). A possible mechanism for the formation of **2** involves the cyclization of the keto tautomer of **1** to the intermediate **1a**, which then by consecutive or concerted decarbonylation−decarboxylation forms **2** (Scheme 1). The tautomers **2** and **2′** are stable enough to form two peaks with very distinct UV spectra in the HPLC. However, initially only the peak corresponding to tautomer **2** was observed. Thus, it can

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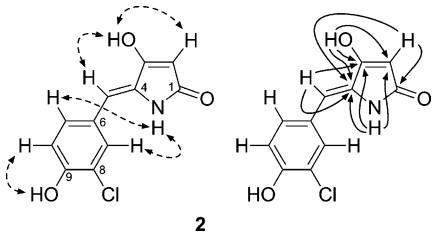

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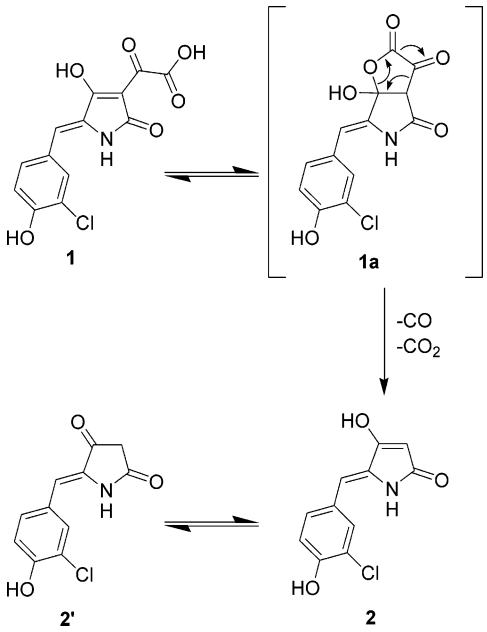
Figure 1. ROESY (left) and HMBC correlations (right) for **2**.

Scheme 1. Decarbonylation−Decarboxylation of **1** to **2**



be assumed that the direct product of the reaction is the enol **2** and not the ketone **2′**. This supports the elimination mechanism proposed.

While the 3-chloro-4-hydroxyphenyl residue is common to various fungal[5,8] and bacterial[9] natural products, the oxalylated tetramic acid moiety is a unique structural feature of **1**. In contrast to pachydermin (**1**) itself, the degradation product **2** exhibits mild antibacterial activity against *Bacillus subtilis*. In an agar diffusion assay it caused an inhibition zone of 1 mm around a disk loaded with 40 μg of substance. It remains to be investigated if **2** is formed also in vivo, e.g., as a response to injury of the fruiting body or predatory attack.

Experimental Section

General Experimental Procedures. UV data were extracted from the diode array detector signal from the HPLC; only relative intensities of absorption maxima are given. NMR spectra were recorded on a Varian INOVA AS-500 spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively), using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 2.60 and δ_C 39.6 ppm for DMSO-d_6). For HRESIMS and LC-MS detection a Micromass LCT TOF mass spectrometer was used. Solvents for extraction and isolation were distilled prior to use. Antimicrobial activity against *Bacillus subtilis* was measured using a standard protocol.[10]

Fungus. Fruiting bodies of *Chamonixia pachydermis* (Boletaceae) were collected in *Nothofagus* forest at Bealey Spur, New Zealand, in May 2005, and unambiguously identified by their spore shape and fruiting body morphology.[3] A voucher specimen (F-5832) has been deposited at the School of Biological Sciences.

Extraction and Isolation. The fresh fruiting bodies (280 g wet wt) were extracted with MeOH (2 × 1 L). The solvent was evaporated to dryness, and the residue was taken up in H_2O (250 mL), extracted first with EtOAc (2 × 250 mL) and then with *n*-BuOH (8 × 150 mL). The combined *n*-BuOH phases were evaporated and subjected to gel chromatography on Sephadex LH-20 eluting with MeOH−H_2O (1:1). The fractions containing **1** were combined and dried in vacuo. The residue was redissolved in H_2O and further purified by vacuum liquid chromatography on an ODS phase eluting with MeOH−H_2O mixtures (from 0% to 20% MeOH). The fractions eluting with 10% MeOH were dried to yield pure **1** in its salt form (37.5 mg). For acquiring NMR spectra the salt of **1** (5.7 mg) was dissolved in H_2O (5 mL), acidified with 2 M HCl, and extracted immediately with EtOAc (2 × 5 mL). The combined EtOAc phases were dried, and the resulting free acid of **1** was dissolved in DMSO-d_6.

Pachydermin (1): yellow amorphous solid; UV (H_2O + 0.05% TFA/MeCN (54:46)) λ_{max} (rel int) 203 (86), 254 (42), 357 (100); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 9.88 (1H, bs, NH), 7.74 (1H, d, J = 2.0 Hz, H-7), 7.49 (1H, dd, J = 8.7, 2.0 Hz, H-11), 7.05 (1H, d, J = 8.7 Hz, H-10), 6.41 (1H, s, H-5); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 179.3 (C, C-12), 177.0 (C, C-3), 170.3 (C, C-13), 165.7 (C, C-1), 153.2 (C, C-9), 130.9 (C, C-4), 130.8 (CH, C-7), 129.9 (CH, C-11), 126.3 (C, C-6), 120.4 (C, C-8), 116.8 (CH, C-10), 106.4 (CH, C-5), 99.4 (C, C-2); for HMBC data, see Supporting Information; ESIMS (pos.) *m*/*z* 310.1 [M + H]$^+$, 332.1 [M + Na]$^+$, 348.1 [M + K]$^+$; ESIMS (neg.) *m*/*z* 307.9 [M − H]$^-$; HRESIMS (pos.) *m*/*z* 310.0150 [M + H]$^+$ (calcd for $C_{13}H_9ClNO_6$, 310.0118).

Preparation of 2. Compound **1** (4.5 mg) was dissolved in HCl (2 M; 5 mL) and heated to 80 °C. After 15 min the reaction mixture was cooled and extracted with EtOAc (2 × 5 mL). After evaporating the solvent to dryness the major product was isolated by semipreparative HPLC (Phenomenex Luna C18, 10 × 250 mm, 5 μm; solvents: A H_2O, B MeCN; linear gradient: 0 min 25% B, 15 min 65% B; 5 mL min^{-1}). Compound **2** was eluted at 7 min and yielded a yellow amorphous solid (2.0 mg). NMR and HPLC analysis showed it to be a mixture of two tautomers, **2** and **2′**, in a 2:1 ratio.

Compound 2: UV (H_2O + 0.05% TFA/MeCN (52:48)) λ_{max} (rel int) 204 (82), 243 (48), 341 (100); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 11.74 (1H, s, 3-OH), 10.50 (1H, s, 9-OH), 9.53 (1H, s, NH), 7.67 (1H, bs, H-7), 7.43 (1H, d, J = 8.5 Hz, H-11), 7.03 (1H, d, J = 8.5 Hz, H-10), 6.17 (1H, s, H-5), 5.04 (1H, s, H-2); ^{13}C NMR (DMSO-d_6, 500 MHz; extracted from HSQC and HMBC data) δ 172.7 (C, C-1), 165.6 (C, C-3), 152.4 (C, C-9), 131.6 (C, C-4), 130.1 (CH, C-7), 129.0 (CH, C-11), 126.4 (C, C-6), 119.9 (C, C-8), 116.5 (CH, C-10), 104.1 (CH, C-5), 92.1 (CH, C-2); for HMBC and ROESY data, see Figure 1 and Supporting Information; HRESIMS pos. (mixture of **2** and **2′**) *m*/*z* 238.0220 [M + H]$^+$ (calcd for $C_{11}H_9ClNO_3$, 238.0271).

Compound 2′: UV (H_2O + 0.05% TFA/MeCN (58:42)) λ_{max} (rel int) 202 (100), 222 (36), 280 (6); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 11.04 (1H, s, NH), 10.64 (1H, s, 9-OH), 7.77 (1H, bs, H-7), 7.51 (1H, d, J = 8.5 Hz, H-11), 7.06 (1H, d, J = 8.5 Hz, H-10), 6.29 (1H, s, H-5), 3.27 (2H, s, H-2); ^{13}C NMR (DMSO-d_6, 500 MHz; extracted from HSQC and HMBC data) δ 195.4 (C, C-3), 171.7 (C, C-1), 153.1 (C, C-9), 132.9 (C, C-4), 130.4 (CH, C-7), 129.6 (CH, C-11), 125.2 (C, C-6), 120.2 (C, C-8), 116.5 (CH, C-10), 104.2 (CH, C-5), 39.1 (CH_2, C-2); for HMBC and ROESY data, see Supporting Information.

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Supporting Information Available: Tabulated NMR data of compounds **1**, **2**, and **2′** and photographs of the investigated fungus are available free of charge via the Internet at http://pubs.acs.org.

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