Cortamidine Oxide, a Novel Disulfide Metabolite from the New Zealand Basidiomycete (Mushroom) Cortinarius Species

Gillian M. Nicholas,† John W. Blunt,* and Murray H. G. Munro*
Department of Chemistry, University of Canterbury, PB 4800, Christchurch, New Zealand

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Three disulfide metabolites were isolated from the fruiting bodies of the basidiomycete (mushroom) Cortinarius sp., collected in the Catlins, New Zealand. The structures of these compounds were determined as the unsymmetrical disulfide cortamidine oxide (1), 2,2′-dithiobis(pyridine N-oxide) (2), and the symmetrical disulfide 3. Both 1 and 2 showed significant antimicrobial activity and cytotoxicity, 2,2′-Dithiobis(pyridine N-oxide) (2) and the symmetrical disulfide 3 are assumed to be artifacts of the isolation procedure.

Many species in the Cortinarius genus are known to produce biologically active natural products, including pigments and toxins. Orellanine, a bipyridyl N-oxide-containing compound, is a potent toxin affecting renal epithelial cells that has been isolated from a number of European species in this genus, including C. orelanus and C. speciosissimus.1,2 N-oxide- and N-hydroxy-containing natural products have been isolated from a variety of other sources including plants, bacteria,3 marine sponges,4,5 and dinoflagellates.6 The assignment of these functionalities can be hindered due to lack of direct evidence. Comparison of NMR data with those of known compounds that lack the N-oxidation, or the synthesis of the fragment in question, is often required. More recently a proton-detected 2D NMR experiment, GHNMQC, which shows correlations to nitrogen in the second dimension, has been useful in the assignment of aliphatic N-oxide functionalities.7

During our continued investigation of fungi collected in New Zealand,8 a Cortinarius sp. collected in the Catlins region of New Zealand was targeted for investigation after initial crude extracts (both organic and aqueous) showed significant antimicrobial activity and cytotoxicity against the P388 murine leukaemia cell line. Fractionation of further extracts yielded cortamidine oxide (1), an unsymmetrical disulfide containing both a 2-thiopyridyl N-oxide and an N-substituted cysteine amino acid residue, 2,2′-Dithiobis(pyridine N-oxide) (2)8 and compound 3 were also isolated and characterized as the symmetrical disulfides of each half of cortamidine oxide (1). As 1 was observed to be spontaneously converting to 2,2′-dithiobis(pyridine N-oxide) (2) and compound 3 during isolation, it is assumed that compounds 1 and 3 are artifacts of the isolation procedure and not true metabolites of the Cortinarius sp.

Results and Discussion

Bioassay-guided fractionation of methanolic extracts of Cortinarius sp. utilizing repeated reverse-phase column chromatography yielded compounds 1–3. A major difficulty encountered in this isolation work was the observed conversion of compound 1 into compounds 2 and 3.

HERIIMS on compound 2 gave an apparent molecular formula C13H17O4N3S2. Four aromatic hydrogens, δ 7.56, 7.46, 7.29, 8.24 in D2O, were observed in the 1H NMR spectrum, which was consistent with a 2-substituted pyridine system. The UV absorbance maximum at 240 nm was also consistent with a pyridine ring. The 1H and 13C NMR data of compound 2 were not consistent with 2,2′-dithiobispyridine, but were, however, consistent with 2,2′-dithiobis(pyridine N-oxide) (2).9 The presence of the N-oxides was confirmed with the observation of a molecular ion at m/z 253 in FABMS and the fragmentation pattern observed under EIIMS.10

From HRFABMS a molecular formula of C13H17O4N3S2 was determined for cortamidine oxide (1). Ionization by EI gave no parent ion, but the fragmentation pattern was similar to that observed for 2. The 1H NMR spectrum of compound 1 also contained four aromatic proton signals (δ 7.26, 7.56, 7.96, 8.16), at chemical shifts similar to those observed for 2, but included one methine (δ 3.95) and five methylenes between δ 1.5 and 3.2. The 13C NMR spectrum contained a carbonyl resonance at δ 177.7 and resonances from two methylenes (δ 42.3 and 54.2) and one methine (δ 57.1) that were consistent with proximity to heteroatoms. NMR correlation data for 1 allowed the identification of three isolated spin systems (see Table 1, Supporting Information). The first fragment, a 2-substituted pyridine ring (H3′′′−H6′′′), was confirmed with correlations in the
COSY, HMQC, and HMBC NMR spectra. The UV absorbance maximum observed for 1 (240 nm) was consistent with a pyridine ring, as in 2. The N-oxidation of the pyridine ring was deduced from comparison of NMR chemical shift data for compounds 1 and 2 and also the fragmentation pattern in the mass spectrum of compound 1. HREIMS on the fragment ion at m/z 127.0115 gave a molecular formula of C₆H₅N₂O₂S, which was also seen in the spectrum of 2. The second spin system assigned from the COSY spectrum was that of a cysteine residue (H-2–H-3). A COSY correlation was observed from the methine proton (δ 3.95, H-2) to the methylene protons at 3.05 and 3.19 (H-3a/b). An HMBC correlation from the methine proton (δ 3.95, H-2) to the carbonyl (δ 177.7, C-1) and the methylene carbons (δ 42.3, C-3) completed the assignment of the cysteine residue. The chemical shift of C-3 (δ 42.3) was consistent with placement adjacent to a disulfide bond. As the molecular formula of compound 1 contained two sulfur atoms, a disulfide bond was assigned between the 2-substituted pyridine ring and the cysteine residue. This was consistent with the observed disproportionation of 1 to the symmetrical disulfides 2 and 3. The third fragment that was assigned contained four consecutive methylenes (H-3’–H-6’), assigned from the COSY spectrum. These methylenes showed correlations from H-3’ to H-4’, then to H-5’, and finally to H-6’. The methylene at δ 54.2 was consistent with being adjacent to a nitrogen atom, even if the carbon was slightly downfield from that expected for the proposed amidine structure. This sequence of four carbons (C-3’–C-6’) was assigned as part of a 3,4,5,6-tetrahydropyridine ring based on HMBC correlations from H-3’, H-4’, and H-6’ to the quaternary carbon (δ 153.3, C-2’). A weak correlation in the HMBC spectrum from H-2 (δ 3.95) to a quaternary carbon (δ 153.3) suggested that the cysteine residue might be connected to the 3,4,5,6-tetrahydropyridine through nitrogen. Unfortunately, the closeness in chemical shift of the quaternary carbon of the tetrahydropyridine ring (δ 153.3) to that of the pyridine N-oxide (δ 153.4) did not allow a definitive assignment. This connection was confirmed during the structure elucidation of the related compound 3 (vide infra) from observation of a correlation in the HMBC spectrum of compound 3 from the equivalent H-2 (now at δ 4.16) to the quaternary C-2’ (now at δ 152.9). The connection between the tetrahydropyridine and the cysteine fragment of compound 1 was therefore confirmed from the assignment for compound 3.

At this point all the elements of unsaturation from the molecular formula had been accounted for, but one oxygen atom remained unassigned. As no further carbon atoms in the molecule were consistent with oxidation, that left only the heteroatoms. The chemical shifts of C-3 and C-6’ were not consistent with oxidation of either of the sulfur atoms of the disulfide bond. The isolation of 2,2’-dithiobis-(pyridine N-oxide) (2) and compound 3, from disulfide exchange, supported this conclusion. That therefore left the two nitrogen atoms, that of the cysteine residue or that of the 3,4,5,6-tetrahydropyridine ring, as the potential sites of oxidation. There were no fragments in the mass spectra obtained on compound 1 (EI, FAB, ES) that could discriminate between these two possible sites of oxygenation. Oxidation on nitrogen shifts adjacent quaternary imine or amidine ¹³C NMR resonances upfield, while the other adjacent carbon (and proton) resonances shift downfield. These differences in carbon chemical shifts are characteristic, as oxidation can move the carbon resonance by ΔδC 10. In contrast, the differences in the proton resonances on adjacent carbons are usually small and shifted downfield by Δδ 0.2–0.4. The protonation state is, of course, also a factor. The ¹³C NMR data for 2,3-dihydrosoquinoline, 2,3-dihydrosoquinoline N-oxide, and the corresponding protonated forms are possibly the best examples to illustrate this. For both the protonated and free-base forms an increase of Δδ ≈ 10–11 was observed in the chemical shift of the α-carbon on oxidation. Another example is the addition of an N-hydroxyl function to the guanidinium group of saxitoxin to form neosaxitoxin. This moved the methine carbon adjacent to nitrogen downfield by Δδ11.2,6 Unfortunately, the functional group proposed for 1 is not a guanidinium, nor a nitroene, but rather an amidine N-oxide. Therefore, the direct comparison of chemical shift data is inappropriate, but the chemical shifts observed in these examples can be used as a guide. The only example of an amidine N-oxide in the literature with ¹³C and ¹H NMR data was for 2-amino-5,5-dimethylpyrroline N-oxide. Regrettably, the ¹³C NMR data were not complete. In 1 the chemical shift of the cysteine α-carbon was slightly downfield compared to that of cystine, but only by Δδ 2.4,14 If no oxygen were present on the adjacent nitrogen, the chemical shift of C-6’ in 1 would be expected at around δ 45.12 Therefore, with the chemical shift of C-6’ being assigned as δ 54.2 the final oxygen from the molecular formula was assigned to the nitrogen of the 3,4,5,6-tetrahydropyridine ring.

To unambiguously confirm the 3,4,5,6-tetrahydropyridine ring as the site of oxidation, 2-amino-3,4,5,6-tetrahydropyridine-N-oxide (4) was synthesized (vide infra). This compound had previously been synthesized, but no NMR data were reported.16 The ¹H NMR data for 2-amino-3,4,5,6-tetrahydropyridine N-oxide (4) as the TFA salt, in D₂O) were consistent with those of the relevant fragment in compound 1 (as the TFA salt, in D₂O). The ¹³C NMR data, particularly for C-2’ and C-6’ of 1 (as the free base, in D₂O), were consistent with those of 2-amino-3,4,5,6-tetrahydropyridine N-oxide (4, in D₂O with pyridine). The position of oxygenation in 1 was therefore confirmed as the nitrogen of the 3,4,5,6-tetrahydropyridine ring, as assigned from the NMR data.

The amidine N-oxide functionality in 1 could exist as a number of tautomers. There could also be pH-dependent chemical shift changes, as was indicated by the observed ¹H NMR chemical shifts for 1 (in D₂O) as the TFA salt and the free base. The tautomeric behavior of 2-amino-5,5-dimethyl-1-pyrroline and the corresponding N-oxide has been reported using IR spectroscopy in CHCl₃. It was concluded that both these compounds were amino-pyrroline derivatives and not the tautomeric iminopyrrolidines, at least in CHCl₃ solution. The N-hydroxy protons in benzazolines G–I were observed in DMSO-d₆ as a sharp singlet at δ 10.5.14 No such resonance was observed by ¹H NMR spectroscopy for the natural product 1 or 2-amino-3,4,5,6-tetrahydropyridine N-oxide (4), even when DMSO-d₆ was used as the solvent. The fragment ions observed by electrospray ionization (ESI) mass spectrometry were consistent with the oxygen anion because of the loss of oxygen (16) from a number of key fragment ions. A loss of oxygen (16) was also observed in the FAB mass spectrum of 2-amino-3,4,5,6-tetrahydropyridine N-oxide (4), which supports the assignment that cortamidine oxide (1) exists as the N-oxide tautomer.

Although cortamidine oxide (1) is optically active, the stereochemistry of the cysteine residue was not ascertained due to the problems encountered with instability of these compounds and the added complications of disulfide ex-
change. It is depicted in the structural diagram as corresponding to that of C-2 in the naturally occurring enantiomer of cysteine.

The third compound to be isolated from Cortinarius sp. was a symmetrical disulphide (3) of the nonaromatic half of cortamidine oxide (1). The molecular ion for the sodium salt (MNa+) of compound 3 was observed (HRFABMS) at m/z 457.1193, which corresponded to a molecular formula of C20H16O6N2S2Na. The 13C and 1H NMR data for 3 were almost identical (ΔδH = 0.0–1.2; ΔδC = 0.00–0.24) to those of the relevant part of cortamidine oxide (1). 2D NMR experiments confirmed the connectivity through the molecule as for 1 (see Table 1, Supporting Information). The crucial HMBC correlation from the methine proton H-2 (δ 4.16) to the quaternary carbon C-2’ (δ152.9) was observed in an HMBC experiment when optimization for long-range coupling constants (J not) was set at 4 Hz.

It is suggested that the two symmetrical dimers (2 and 3) arise via disulphide exchange from the unsymmetrical cortamidine oxide (1). Compounds 1 and 2 both contained a 2-thiopyridine N-oxide functionality. This functionality is consistent with the biological activity (cytotoxicity and antimicrobial activity) observed for compounds 1 and 2. The zinc salt of 2-thiopyridine N-oxide is in fact the active ingredient in many antiandruff shampoos.18 Compounds containing a pyridine N-oxide functionality have been reported from species in the Cortinarius genus before, namely, the toxin orellane.1 Both 1 and 3 contained a fragment assigned as an amidine N-oxide. No natural products were found in the literature that contained this functionality, although a number of synthetic compounds have been reported, including 2-amino-6,6-dimethyl-3,4,5,6-tetrahydropyridine N-oxide16 and 2-amino-5,5-dimethylpyrroline N-oxide.11

Experimental Section

General Experimental Procedures. Proton-detected NMR spectra were recorded on a Varian Unity 300 spectrometer using a Nalorac Z spec M1D3000 3 mm indirect detection probe or a pulsed field gradient MLD driver with a 5 mm indirect detection probe, operating at 300 MHz and 23 °C. Chemical shifts were referenced to the appropriate solvent peaks. A Kratos MS80 mass spectrometer operated at 4 kV was used for mass spectrometry. EI was performed at 70 eV. FAB used an Ion Tech ZN11FN ion gun using Xe as the reagent gas, Kratos MS80 mass spectrometer operated at 4 kV was used as a detection probe, operating at 300 MHz and 23 °C. Chemical shifts were referenced to the appropriate solvent peaks. A Kratos MS80 mass spectrometer operated at 4 kV was used for mass spectrometry. EI was performed at 70 eV. FAB used an Ion Tech ZN11FN ion gun using Xe as the reagent gas, operating at 8 kV and 2 mA, with either an Ion Tech ZN11FN ion gun using Xe as the reagent gas, or a pulsed field gradient MLD driver with a 5 mm indirect detection probe, operating at 300 MHz and 23 °C. Chemical shifts were referenced to the appropriate solvent peaks. A Kratos MS80 mass spectrometer operated at 4 kV was used for mass spectrometry. EI was performed at 70 eV. FAB used an Ion Tech ZN11FN ion gun using Xe as the reagent gas, operating at 8 kV and 2 mA, with either an Ion Tech ZN11FN ion gun using Xe as the reagent gas, or a pulsed field gradient MLD driver with a 5 mm indirect detection probe, operating at 300 MHz and 23 °C. Chemical shifts were referenced to the appropriate solvent peaks. A Kratos MS80 mass spectrometer operated at 4 kV was used for mass spectrometry. EI was performed at 70 eV. 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HREIMS, no molecular ion was observed m/z 127.0115 (calcd for C₈H₅NO₂). 15 To 5-nitropentan-1-nitrile (see Supporting Information) (100 mg, 0.78 mmol) and NH₄Cl (33 mg, 0.62 mmol) in MeOH/H₂O (1:1, 10 mL) at 0 °C was added Zn dust (198 mg, 3.15 mmol). The mixture was stirred for 16 h, filtered, and acidified to pH 2 with trifluoroacetic acid. The solvent was removed in vacuo to yield the trifluoroacetate of 2-amino-3,4,5,6-tetrahydropyridine N-oxide (4) as a light orange oil: FTIR (KBr plate, film) νmax 3500–2800 (br), 1720–1620 (br), 1454, 1427, 1201, 1137, 1026, 1001, 839, 800, 723 cm⁻¹; 1H NMR (D₂O, TFA salt) δ 3.54 (2H, t, J = 6.3 Hz, H-6), 2.55 (2H, t, J = 6.4 Hz, H-3), 1.83 (2H, m, H-5), 1.63 (2H, m, H-4); ¹³C NMR (D₂O, TFA salt) δ 152.8 (s, C-2, 53.1 (t, C-6), 27.8 (t, 135.1, 152.9 (s, C-2), 78.7 (t, C-5), 18.9 (t, C-4); FABMS m/z 229 (M + H⁺), 115 M⁺, 99 (M⁺ − 16); HRFABMS 115.0867 (M⁺) (calcd for C₆H₁₃NO₂, 115.0871).

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Supporting Information Available: ¹H NMR, ¹³C NMR spectra, and 2D-correlation data for cortamidine oxide (1), 2,2'-dithiobis(pyridine N-oxide) (2), and the symmetrical disulfide 3. Also available are experimental details for the synthesis of 2-amino-3,4,5,6-tetrahydropyridine N-oxide (4). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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(15) The authors acknowledge the assistance of Dr. Andrew Phillips with the preparation of the compounds in this sequence.