Anserinones A and B: New Antifungal and Antibacterial Benzoquinones from the Coprophilous Fungus *Podospora anserina*

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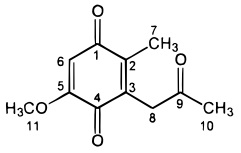
Two new benzoquinones with antifungal, antibacterial, and cytotoxic activities have been isolated from liquid cultures of the coprophilous fungus *Podospora anserina*. The structures of anserinones A (**1**) and B (**2**) were assigned on the basis of MS and NMR results, and the absolute stereochemistry of **2** was deduced by analysis of ^{1}H-NMR data for its (*R*)- and (*S*)-2-phenylbutyryl ester derivatives.

Antagonistic interactions among coprophilous (dung-colonizing) fungi often involve the production of a chemical agent by one species that inhibits the growth of another.[1] Our studies of these fungi have led to the discovery of a variety of new antifungal agents.[1,2] As part of a continuing study of coprophilous fungi, we investigated the chemistry of an isolate of *Podospora anserina* (Cesati) Niessl (Lasiosphaeriaceae) that displayed antifungal activity. This investigation led to the isolation of two new benzoquinones, which we named anserinones A (**1**) and B (**2**). This report describes the isolation, structure elucidation, and biological activities of these metabolites.



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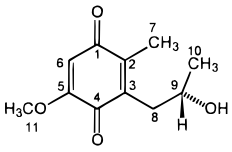

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A subculture of an isolate of *P. anserina* (JS 162), originally obtained from mouse dung, inhibited the growth of coprophilous fungal competitors in direct competition assays, and the EtOAc extract of the filtered culture broth also exhibited antifungal effects. This extract was fractionated by radial chromatography on Si gel, and the resulting fractions were separated further by reversed-phase HPLC or preparative TLC to afford anserinones A (**1**) and B (**2**).

Analysis of ^{1}H- and ^{13}C-NMR data for anserinone A (Table 1) revealed the presence of one aliphatic ketone carbonyl, two conjugated ketone units, one oxygenated olefinic carbon, three non-oxygenated olefinic carbons, one methylene unit, one OCH_3 group, and two additional methyl groups. The three carbonyls, together with the oxygenated sp^2 carbon and the OCH_3 group, required at least four oxygen atoms. The ^{1}H-NMR spectrum (Table 1) contained five signals that integrated for a total of 12 protons. On the basis of these data, and on HREIMS analysis, anserinone A was assigned the formula $C_{11}H_{12}O_4$.

Table 1. NMR Data for Anserinones A (**1**) and B (**2**)

	anserinone A (**1**)			anserinone B (**2**)	
position	δ_H (mult.)[a]	δ_C[b]	selective INEPT (C no.)[b]	δ_H (mult., J_{HH})[a,c]	δ_C[b]
1		186.9			187.3
2		144.1			143.4
3		136.2			139.6
4		181.4			182.9
5		158.3			158.3
6	5.92 (s)	107.5	1, 2, 4, 5	5.90 (s)	107.3
7	1.97 (s)	12.8	1, 2, 3	2.08 (s)	12.8
8	3.65 (s)	40.9	2, 3, 4, 9	2.68 (d, 6.6)	35.9
9		203.0		3.96 (m)	67.6
10	2.26 (s)	30.2	9	1.25 (d, 6.2)	24.0
11	3.79 (s)	56.2	5	3.78 (s)	56.1

[a] Recorded at 300 MHz. [b] Recorded at 75 MHz. [c] J_{HH} = Hz.

The NMR data suggested a trisubstituted benzoquinone-type structure for **1**, and the quinone unit, together with the aliphatic ketone group, accounted for all six degrees of unsaturation. Irradiation of the methyl singlet at δ 2.26 (H_3-10) and the methylene singlet at δ 3.65 (H_2-8) in selective INEPT experiments (Table 1) revealed the connection of both of these groups to the aliphatic ketone carbonyl to give an acetonyl group. On the basis of chemical shift and selective INEPT data, the olefinic carbon at δ 158.3 must be linked to the OCH_3 group and to the upfield-shifted olefinic methine (δ 107.5/5.92). The substituted olefinic quinone carbons at δ 136.2 and δ 144.1 must, therefore, bear the remaining methyl and acetonyl substituents. Selective INEPT correlations of the vinyl methyl signal at δ 1.97 to C-1 (δ 186.9) and of the acetonyl methylene signal at δ 3.65 to C-4 (δ 181.4) required a 1,4-benzoquinone structure. Although additional selective INEPT correlations (Table 1) supported the assignment of structure **1** for anserinone A, the position of the OCH_3 group could not be unambiguously determined. Because of its expected upfield-shift effect on β-carbon signals,[3] however, this group was assigned the position adjacent to C-4 (δ 181.4) rather than to C-1 (δ 186.9).

Analysis of the ^{1}H-, ^{13}C-, and DEPT NMR spectra for anserinone B (**2**) indicated the molecular formula $C_{11}H_{14}O_4$. This formula was confirmed by HREIMS, and differs from that of anserinone A (**1**) by addition of two hydrogen atoms. The EIMS also contained a

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fragment corresponding to a loss of H_2O. The aliphatic ketone signal at δ 203.0 in the ^{13}C-NMR spectrum of **1** was replaced by an oxygenated methine carbon resonance (at δ 67.6) in **2**, and the ^{1}H-NMR spectrum of **2** contained a new oxygenated methine multiplet at δ 3.96. In addition, the ^{1}H-NMR signals for H_3-10 (δ 1.25) and H_2-8 (δ 2.68) of anserinone B appeared as upfield-shifted doublets, as would be expected if the ketone carbonyl present in anserinone A were reduced to a CHOH group. These observations, together with NMR data otherwise closely paralleling those for **1**, led to the assignment of structure **2** for anserinone B.

In order to determine the absolute configuration at C-9, a sample of anserinone B (**2**) was treated with (*R*/*S*)-2-phenylbutyryl chloride[4] to afford a monoacylated compound (a pair of diastereomers) at the 9-OH group. Similar treatment of **2** with (−)-(*R*)-2-phenylbutyryl chloride afforded the (*R*)-phenylbutyryl ester. Upfield shifts of the H-6 (Δ_{R-S} −0.14 ppm), H_3-7 (Δ_{R-S} −0.26 ppm), and H_3-11 (Δ_{R-S} −0.04 ppm) signals, and a downfield shift of the H_3-10 (Δ_{R-S} +0.13 ppm) signal, for the (*R*)-phenylbutyryl ester relative to those of the (*S*)-derivative were observed. Based on Helmchen's rules,[5] the configuration at C-9 was assigned as *S*.

Anserinone A (**1**) was active in centerpoint inoculation disk assays[6] against the coprophilous fungi *Sordaria fimicola* (NRRL 6459) and *Ascobolus furfuraceus* (NRRL 6460), causing respective 60% and 100% reductions in radial growth rates of these fungal competitors at 200 μg/disk. At the same level, anserinone B (**2**) caused radial growth reductions of 50% and 37% against *S. fimicola* and *A. furfuraceus*, respectively. Both compounds also afforded ca. 40-mm zones of inhibition in disk assays against *B. subtilis* (ATCC 6051) and *S. aureus* (ATCC 29213); however, neither compound showed activity against *C. albicans* (ATCC 90029) at 200 μg/disk. Anserinones A and B also displayed moderate cytotoxicity in the NCI's 60 human tumor cell line panel.[7] The average GI_{50} values for **1** and **2** were 9.1×10^{-6} M (1.9 μg/mL) and 2.1×10^{-5} M (4.4 μg/mL), respectively.

Benzoquinone derivatives are commonly encountered as bioactive metabolites of fungi and plants.[8−10] Anserinones A and B differ from naturally occurring precedents in the identity of the substituents and their substitution patterns. These compounds, however, bear close biogenetic resemblance to a variety of fungal metabolites, and can be classified as pentaketides. Examples of biogenetically similar fungal metabolites include 2,4-dihydroxy-6-propylbenzoic acid and 6,8-dihydroxy-3-methylisocoumarin.[8]

Experimental Section

General Experimental Procedures. NMR spectra were recorded using $CDCl_3$ solutions, and chemical shifts were referenced relative to the corresponding solvent signals (δ 7.24/77.0). The specific rotation was measured on a JASCO model DIP-1000 digital polarimeter. Descriptions of NMR parameters, as well as NMR, MS, UV, IR, and mp instrumentation have been provided elsewhere.[11] Chemical reagents were used as purchased without further purification.

Fungal Material. The culture of *Podospora anserina* (Cesati) Niessl (Lasiosphaeriaceae) was a subculture of an isolate originally obtained from a sample of mouse dung collected by D. Malloch at a site 1 km east of Soquel, Santa Cruz, CA, on May 11, 1990. This isolate was identified by J. A. Scott and assigned the accession number JS 162 in the D. Malloch culture collection at the University of Toronto. The subculture of *P. anserina* was used to inoculate two 2-L Erlenmeyer flasks, each containing 450 mL of autoclaved potato dextrose broth in distilled H_2O. Three 0.25-cm^2 agar plugs were aseptically transferred into each flask, and the cultures were incubated at room temperature on rotary shakers at 150 rpm for 30 days.

Extraction and Isolation. The fungal cultures were filtered, and the broth (900 mL) was extracted with EtOAc (4 × 0.5 L). The organic phase was dried over $MgSO_4$, filtered, and evaporated to yield 479 mg of a crude extract. This extract was fractionated by radial chromatography using a Chromatotron (2-mm Si gel 60 PF-254/$CaSO_4$ plate). Before loading the sample, the sorbent was equilibrated with solvent (CH_2Cl_2) for 5 min. The extract was dissolved in 3 mL CH_2Cl_2, pump-loaded onto the plate, and eluted with a solvent gradient (6.5 mL/min; 1%, 3%, 5%, 10%, 30%, 60%, 100% EtOAc in CH_2Cl_2), collecting 9.5-mL fractions. Similar fractions, as judged by TLC, were combined. Combined fractions 84−122 (8 mg) were subjected to semipreparative reversed-phase HPLC (Beckman Ultrasphere 5-μm C_{18}; 1 × 25 cm; 45% CH_3CN in H_2O) to provide anserinone A (**1**, 3.2 mg, t_R 10.3 min). A portion (43 mg) of combined fractions 193−261 (152 mg) was subjected to preparative TLC (Merck, Kieselgel 60 F_{254}) with CH_2Cl_2−MeOH (92:8) to afford anserinone A (**1**, 8.7 mg, R_f 0.48) and anserinone B (**2**, 21.2 mg, R_f 0.69).

Anserinone A (1): yellow needles; mp 122−124 °C; UV (CH_2Cl_2) λ_{max} 359 (ϵ 360), 265 (7100); IR (CH_2Cl_2) ν_{max} 3061, 2939, 1721, 1673, 1652, 1613, 1232 cm^{-1}; ^{1}H-NMR, ^{13}C-NMR, and selective INEPT data, see Table 1; EIMS (70 eV) *m*/*z* 208 (M^+; rel int 5), 166 (100), 151 (4), 138 (34), 123 (34), 109 (7), 95 (9), 77 (6), 69 (40), 53 (24); HREIMS $(M)^+$ 208.0725, calcd for $C_{11}H_{12}O_4$ 208.0736.

Anserinone B (2): yellow oil; $[\alpha]_D$ +43° (*c* 1.0 mg/mL, MeOH); UV (CH_2Cl_2) λ_{max} 369 (ϵ 500), 275 (10 000), 233 (4500); IR (CH_2Cl_2) ν_{max} 3603, 3063, 2975, 2940, 1671, 1648, 1610, 1231 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see Table 1; LREIMS (70 eV) *m*/*z* 210 (M^+; rel int 3), 192 (7), 166 (100), 151 (9), 138 (51), 123 (48), 109 (8), 95 (12), 77 (13), 69 (44), 53 (27); HREIMS $(M)^+$ 210.0898, calcd for $C_{11}H_{14}O_4$ 210.0892.

Formation of (*R*/*S*)- and (*R*)-2-Phenylbutyryl Esters of Anserinone B (2). (*R*/*S*)-2-phenylbutyryl chloride was prepared according to a literature procedure.[4] To a solution of **2** (2.6 mg; 0.012 mmole) in pyridine (40 μL) was added (*R*/*S*)-2-phenylbutyryl chloride (4.6 μL), and the solution was allowed to stand at room temperature for 15 h. 3-Dimethylaminopropylamine (2.8 μL) was then added. After standing for 15 min, the solvent was evaporated under N_2 flow. The residue was partitioned between EtOAc (5 mL) and H_2O (5 mL). The EtOAc layer was dried under N_2 flow to provide the diastereomeric phenylbutyryl esters of anserinone B (3.3 mg, 75%). The (*R*)-2-phenylbutyryl

ester of compound **2** was prepared using (*R*)-reagent under the same conditions.

(*R*)-2-Phenylbutyryl ester of 2: ^{1}H-NMR δ 7.30−7.18 (overlapping m, ϕ), 7.12 (m, ϕ), 5.71 (s, H-6), 5.02 (m, H-9), 3.73 (s, H_3-11), 3.28 (t, 7.7 Hz, H-2′), 2.8−2.6 (overlapping m, H_2-8), 2.2−1.9 (m, H_a-3′), 1.9−1.6 (m, H_b-3′), 1.74 (s, H_3-7), 1.28 (d, 6.2 Hz, H_3-10), 0.79 (t, 7.5 Hz, H_3-4′); EIMS *m*/*z* 356 (M^+; rel int 1%), 312 (7), 228 (4), 210 (11), 194 (34), 166 (18), 146 (10), 119 (67), 91 (100).

(*S*)-2-Phenylbutyryl ester of 2: ^{1}H-NMR δ 7.33−7.22 (m, ϕ), 5.85 (s, H-6), 5.02 (m, H-9), 3.77 (s, H_3-11), 3.30 (t, 7.7 Hz, H-2′), 2.8−2.6 (m, H_2-8), 2.2−1.9 (m, H_a-3′), 2.00 (s, H_3-7), 1.9−1.6 (m, H_b-3′), 1.15 (d, 6.3 Hz, H_3-10), 0.78 (t, 7.5 Hz, H_3-4′).

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