The Preussomerins: Novel Antifungal Metabolites from the Coprophilous Fungus *Preussia isomera* Cain

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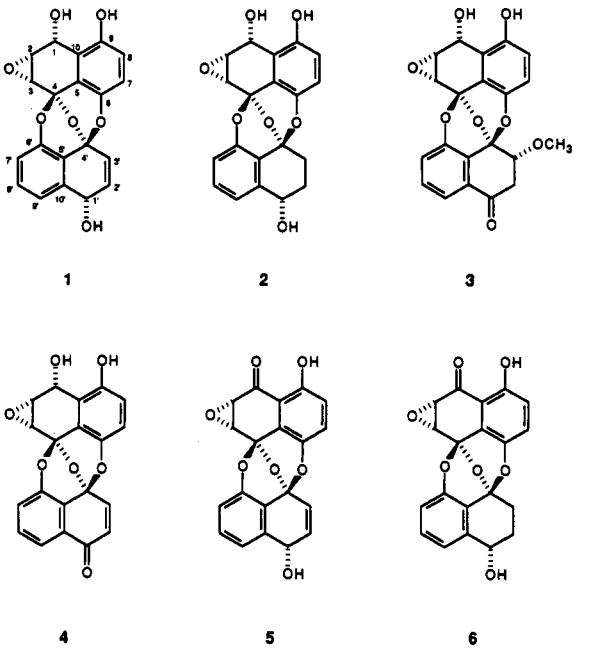
Preussomerins A–F (1–6), a series of new aromatic bis-ketals with antifungal and antibacterial activities, have been isolated from the coprophilous fungus *Preussia isomera* (CBS 415.82). Preussomerins A–F were obtained from ethyl acetate extracts of liquid cultures of *P. isomera* by silica gel chromatography and reversed-phase HPLC. The structures of preussomerins B–F (2–6) were proposed on the basis of extensive NMR experiments and by comparison with the data for preussomerin A (1), whose structure was confirmed by single-crystal X-ray diffraction analysis. The isolation process was guided by in vitro bioassays for antifungal antagonism toward other coprophilous fungi.

Interference competition between species of fungi has been frequently observed and may play an important role in the patterns of colonization and successional changes within fungal ecosystems. However, there have been few reports pertaining to the chemistry involved in these interactions. Reports of interspecies competition among coprophilous (dung-colonizing) fungi[1,2] have led us to investigate such species as potential sources of natural antifungal agents.[3-6] Chemical studies of coprophilous fungi as a group have been limited, partly because many such organisms are not commonly encountered unless they are specifically sought. Investigation of the chemistry of the antagonistic coprophilous fungus *Preussia isomera* Cain (CBS 415.82) has resulted in the discovery of an unusual series of antifungal metabolites. The isolation of these metabolites was guided by bioassays for antifungal activity toward the early successional coprophilous fungus *Sordaria fimicola* (NRRL 6459). We recently communicated the X-ray crystal structure of the most abundant antifungal metabolite preussomerin A (1).[6] We now report further details of these studies, including the structures and biological activities of five additional related compounds, which we have called preussomerins B–F. The structures of preussomerins B–F (2–6) were assigned by analysis of NMR and mass spectral data and by comparison with the data for preussomerin A (Chart I).

Chart I



Results and Discussion

Preussomerins A–F were obtained from ethyl extracts of liquid cultures of *P. isomera* that were fractionated by silica gel chromatography and reversed-phase HPLC. Preussomerin A was isolated in yields ranging from 2 to 6 mg/L of culture medium. Analysis of preussomerin A by HREIMS and ^{13}C NMR suggested the molecular formula $C_{20}H_{14}O_7$ (14 unsaturations). Examination of ^1H NMR and ^{13}C NMR data (Tables I and II), decoupling experiments, and data for the acetylation product revealed the presence of oxygenated 1,2,3-trisubstituted and 1,2,3,4-tetrasubstituted aromatic rings, one phenolic OH, a secondary hydroxyl group adjacent to a cis-disubstituted or annular double bond, and another secondary hydroxyl group adjacent to a 1,2-disubstituted epoxide. Changes in the ^{13}C shifts for the tetrasubstituted aromatic ring upon acetylation indicated that the phenolic OH group is associated with this unit. The only ^{13}C NMR signals not accounted for by the above units were two singlets at 96.00 and 91.13 ppm, which were consistent with the presence of two ketal carbons connected to sp^2 carbons.[7]

A one-bond heteronuclear C–H shift correlation experiment permitted assignment of all protonated carbons. Selective INEPT experiments[8,9] (Table III) were conducted to afford long-range C–H correlations and to help assign nonprotonated carbons. These data permitted establishment of the two partial structures **a** and **b**, although unequivocal assignments were hindered by the observation

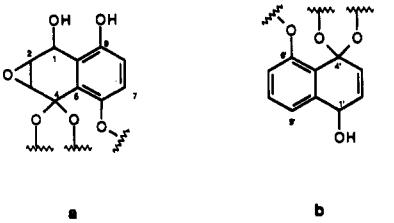


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Table I. ^{1}H NMR Data (360 MHz) for Preussomerins A–F (1–6)

pos.	1[a,c]	2[a]	3[a]	4[b]	5[a]	6[b]
1	5.61 (br s)	5.53 (br s)	5.63 (br s)	5.39 (br s)		
2	3.80 (dd, 4.6, 1.5)	3.83 (dd, 4.4, 1.8)	3.91 (dd, 4.2, 0.5)	3.72 (dd, 4.5, 1.5)	3.89 (d, 4.2)	3.84 (d, 3.9)
3	3.88 (d, 4.6)	3.87 (d, 4.3)	4.03 (d, 4.2)	3.89 (d, 4.4)	4.43 (d, 3.9)	4.27 (d, 4.2)
4						
5						
6						
7	6.60 (d, 9.2)	6.64 (d, 8.9)	6.75 (d, 9.0)	6.66 (d, 9.2)	7.10 (d, 9.0)	6.92 (d, 9.0)
8	6.70 (d, 9.2)	6.70 (d, 8.5)	6.75 (d, 9.2)	6.58 (d, 9.2)	6.97 (d, 9.0)	7.04 (d, 9.3)
9						
10						
1′	5.28 (br s)	4.91 (dd, 5.8, 5.9)			5.30 (br s)	4.90 (dd, 5.7, 5.5)
2′a	6.30 (dd, 10.8, 2.9)	2.62 (m)	2.98 (dd, 18.1, 2.7)	6.46 (d, 10.2)	6.37 (dd, 9.8, 2.5)	2.68 (m)
2′b		1.90 (dddd, 13.9, 8.0, 6.2, 4.7)	3.35 (dd, 18.1, 3.2)			1.97 (m)
3′a	6.63 (d, 10.8)	2.39 (ddd, 13.5, 7.0, 4.7)	4.32 (dd, 2.9, 2.9)	7.23 (d, 9.9)	6.70 (d, 9.5)	2.42 (m)
3′b		2.18 (m)				2.31 (m)
4′						
5′						
6′						
7′	6.73 (d, 8.1)	6.68 (d, 8.1)	7.12 (dd, 8.1, 1.0)	6.98 (dd, 8.1, 1.1)	6.80 (dd, 7.8, 1.0)	6.72 (dd, 7.6, 1.1)
8′	7.27 (dd, 8.1, 8.1)	7.14 (dd, 8.8, 8.1)	7.43 (dd, 7.8, 7.8)	7.32 (dd, 7.9, 7.8)	7.33 (dd, 7.6, 8.1)	7.27 (dd, 8.1, 7.8)
9′	7.36 (d, 8.1)	7.24 (d, 8.7)	7.53 (dd, 7.6, 1.0)	7.44 (dd, 7.8, 1.1)	7.38 (dd, 8.3, 1.0)	7.14 (dd, 8.1, 1.1)
10′						
3′-OCH$_3$			3.91 (s)			

[a] Spectrum taken in acetone-d_6. [b] Spectrum taken in MeOH-d_4. [c] J values are in hertz.

Table II. ^{13}C NMR Data (90.7 MHz) for Preussomerins A–F (1–6)

pos.	1[a]	2[a]	3[a]	4[b]	5[a]	6[b]
1	69.2 (d)	69.4 (d)	69.3 (d)	68.9 (d)	197.0 (s)	197.2 (s)
2	53.2 (d)	53.1 (d)	53.2 (d)	53.6 (d)	52.8 (d)	54.8 (d)
3	53.1 (d)	53.1 (d)	53.0 (d)	53.4 (d)	54.4 (d)	53.2 (d)
4	96.2 (s)	96.0 (s)	96.3 (s)	96.9 (s)	94.6 (s)	96.6 (s)
5	114.8 (s)	115.1 (s)	114.3 (s)	114.7 (s)	116.8 (s)	111.7 (s)
6	144.5 (s)	144.5 (s)	143.4 (s)	144.3 (s)	145.0 (s)	145.2 (s)
7	117.3 (d)	120.2 (d)	117.4 (d)	117.6 (d)	127.2 (d)	121.7 (d)
8	120.3 (d)	114.8 (d)	120.7 (d)	121.0 (d)	121.6 (d)	126.9 (d)
9	151.8 (s)	151.9 (s)	152.3 (s)	152.4 (s)	156.0 (s)	156.1 (s)
10	117.1 (s)	117.3 (s)	117.3 (s)	117.8 (s)	111.0 (s)	116.8 (s)
1′	66.0 (d)	65.4 (d)	193.8 (s)	185.5 (s)	65.9 (d)	65.9 (d)
2′	125.7 (d)	28.8 (t)	41.5 (t)	133.9 (d)	124.9 (d)	29.8 (t)
3′	143.0 (d)	31.2 (t)	80.3 (d)	143.3 (d)	143.6 (d)	28.8 (t)
4′	91.1 (s)	95.0 (s)	94.9 (s)	90.2 (s)	92.2 (s)	94.8 (s)
5′	117.4 (s)	120.3 (s)	121.6 (s)	122.3 (s)	116.9 (s)	118.7 (s)
6′	150.2 (s)	150.1 (s)	151.8 (s)	150.9 (s)	149.3 (s)	149.7 (s)
7′	114.6 (d)	117.1 (d)	122.0 (d)	121.7 (d)	114.7 (d)	115.3 (d)
8′	130.5 (d)	130.9 (d)	131.6 (d)	132.0 (d)	131.0 (d)	131.7 (d)
9′	118.7 (d)	120.3 (d)	120.3 (d)	120.8 (d)	119.4 (d)	118.6 (d)
10′	143.3 (s)	142.6 (s)	132.0 (s)	131.6 (s)	143.3 (s)	141.8 (s)
3′-OCH$_3$			59.5 (q)			

[a] Spectrum taken in acetone-d_6. [b] Spectrum taken in MeOH-d_4.

of numerous four-bond and five-bond correlations in the selective INEPT data.[10] Most of the four- and five-bond correlations were observed in experiments optimized for 4-Hz couplings, but several were still observed in data optimized for J = 7 Hz. Thus, definitive conclusions from these data were limited. The CH-OH methine proton of the α-hydroxy epoxide (H-1) was correlated with all six carbons of the tetrasubstituted aromatic ring, indicating its direct connection to that ring. Irradiation of H-1, H-3, and one of the aromatic protons (H-7) each afforded correlations with a ketal carbon (C-4), indicating connection of C-4 to the epoxide ring and to the aromatic ring at a position ortho to C-1 (C-5). Attachment of the aryl ether oxygen ortho to C-5 (at C-6) was based on ^{13}C NMR shifts and on the geometrical requirement that the ketal and aryl ether oxygens of partial structure **a** must be the atoms through which **a** and **b** are connected (see below).

Other correlations presented in Table III, especially those arising from irradiation of H-2 and H-8, were consistent with the proposed connectivity of **a**. The only ambiguity in the assignment of partial structure **a** by NMR analysis was the location of the phenolic OH group (C-9 vs C-7). The lack of a NOESY correlation between H-7 and H-1 only afforded negative evidence for the location of the OH at C-9, and attachment of the OH at either carbon would require observation of a five-bond selective INEPT correlation upon irradiation of H-1.

A parallel set of results led to formulation of partial structure **b**. Proton NMR decoupling and COSY data demonstrated that the sp^3 methine proton of the α-hydroxy olefin spin system (H-1′) was long-range coupled to a terminal proton of the 1,2,3-trisubstituted aromatic ring spin system (H-9′), thereby indicating attachment of C-1′ to C-10′ of the aromatic ring. Confirmation of this connection was obtained by irradiation of H-1′, which showed polarization transfer to all of the aromatic ring carbons except C-7′. Irradiations of H-1′, H-3′, and one of the aromatic protons (H-7′) all afforded correlations with a

(10) Although such long-range CH couplings in aromatic systems are typically small (ca. 0–1 Hz),[9b,c] they are detectable in selective INEPT experiments.[9a]

Table III. Selective INEPT CH Correlations for Preussomerins A and C–E (1 and 3–5), 90.7 MHz

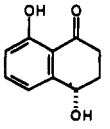
pos.	1[a]	3[a]	4[b]	5[a]
1	4, 5, 6, 7, 8, 9, 10	1, 10	5, 6, 8, 9, 10	
2	1, 3, 5, 9, 10	5	1, 10	1, 3, 9, 10
3	2, 4, 5, 6, 10		1, 4, 5	2, 4, 5, 6
4				
5				
6		5, 6, 9, 10		
7	4, 5, 6, 9	5, 6, 9, 10	4, 6, 9, 10	4, 5, 6, 9
8	1, 6, 9, 10		5, 6, 9, 10	1, 5, 6, 9, 10
9				
10				
1′	2′, 3′, 4′, 5′, 8′, 9′, 10′			2′, 3′, 4′, 5′, 6′, 9′, 10′
2′	1′, 3′, 5′, 10′	1′, 3′, 4′	1′, 4′, 10′	1′, 5′, 10′
3′	4′, 10	1′, 2′, 4′, 5′, 6′, 11′	1′, 2′, 4′, 5′, 6′	4′, 5′, 10′
4′				
5′				
6′				
7′	4′, 5′, 6′, 9′, 10′	5′, 6′, 9′	5′, 6′, 9′	5′,6′,9′,10′
8′	5′,6′,7′	6′, 10′	6′, 9′	5′, 6′, 7′, 9′, 10′
9′	1′, 5′, 6′, 7′, 8′, 10′	1′, 5′, 7′	1′, 5′, 6′, 7′	6′, 8′, 10′
10′				
11′		3′		

[a] Spectrum taken in acetone-d_6. [b] Spectrum taken in MeOH-d_4.

ketal carbon (C-4′), indicating connection of C-4′ to C-3′ and to the aromatic ring at a position ortho to C-1′ (C-5′). These connections require the aryl ether oxygen to be located at C-6′ to give partial structure **b**. The additional correlations provided in Table III are consistent with this assignment.

There were no long-range correlations between the two partial structures **a** and **b**, and two unsaturations were not accounted for by the known structural units. Thus, the two fused ring systems must be connected via the three oxygens as shown in the proposed structure 1. This structure, including relative stereochemistry, was confirmed by X-ray diffraction analysis of a crystal obtained through slow evaporation of a methanol solution of 1.[6] The X-ray model reveals that both partially saturated rings are required to adopt boat conformations and that the planes of the two aromatic rings are 102.6° apart. Despite the apparent ring strain inherent in the structure, preussomerin A is quite stable at room temperature. In retrospect, the rigidity of preussomerin A suggested by molecular models is consistent with the observation of many long-range couplings, although the crystal structure indicates that there is no consistent correlation with W-type structural relationships.

The absolute stereochemistry was assigned as shown on the basis of the isolation of a known compound as a degradation product. Although the ketal linkages were resistant to acid hydrolysis at room temperature, rigorous ketal cleavage conditions (6 M HCl–acetone, 1:1, 100 °C, 12 h) afforded a number of decomposition products, most of which were present in very small quantities. The major isolated product had properties virtually identical with those reported for (–)-regiolone (7),[11] which closely resem-



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bles partial structure **b** with loss of the C2′–C3′ double bond. Maintenance of the stereochemistry at position 1′ could be rationalized by a mechanism involving protonation at C-2′ during the decomposition process. Protonation at C-2′ and an appropriate sequence of electronic rearrangements culminating in loss of the 9-OH proton (Figure 1) could result in formation of enol ether and partially saturated epoxy naphthoquinone moieties. Hydrolysis of the remaining ketal linkage and tautomerization would then account for the formation of regiolone as a product without loss of stereochemical integrity at the hydroxylated methine carbon. The absolute stereochemistry of (–)-regiolone was originally assigned through CD studies.[11] Since the relative stereochemistry of 1 was provided by the X-ray diffraction data, the absolute stereochemistry can be assigned as shown.

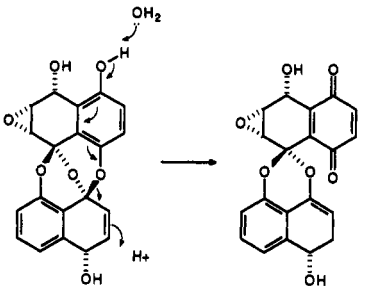

Figure 1.

Five other closely related metabolites with varying degrees of antifungal activity were also isolated from the *P. isomera* extracts. The structures of these compounds were assigned by NMR analysis and by analogy to the data for 1. Preussomerin B (2) was isolated in very small quantities (0 to 0.06 mg/L). Analysis of this compound by ^{13}C NMR and HREIMS suggested the molecular formula $C_{20}H_{16}O_7$, indicating that it has one less degree of unsaturation than compound 1. The ^1H and ^{13}C NMR data (Tables I and II) revealed that the trisubstituted and tetrasubstituted aromatic rings were intact, as well as the α-hydroxy epoxide and ketal functionalities. However, the ^1H NMR spectrum contained four mutually coupled upfield multiplets, two of which were also coupled to the methine signal of a secondary hydroxyl group. The ^{13}C NMR spectrum contained signals for two upfield methylene carbons and only 12 sp^2-hybridized carbons. These data disclosed that the only difference between preussomerin

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B and 1 was saturation of the C-2′, C-3′ double bond, revealing the structure of preussomerin B as 2. The proton and carbon assignments for 2 were determined through HMQC[12] and HMBC[13] experiments.

Preussomerin C (3) was isolated in yields ranging from 0.2 to 0.8 mg/L. Preussomerin C has a molecular formula of $C_{21}H_{16}O_8$ (14 unsaturations) as determined by HREIMS and ^{13}C NMR data (Table II). The ^1H NMR spectrum (Table I) again contained resonances for the characteristic trisubstituted and tetrasubstituted aromatic rings, as well as an α-hydroxy epoxide moiety as found in preussomerin A (1). The spectrum also contained signals for two diastereotopic methylene protons, and one additional oxygenated methine, which was correlated with a methoxy group in selective INEPT experiments. The ^{13}C NMR spectrum (Table II) of preussomerin C is similar to that of 1. However, the signal at 193.8 ppm suggests the presence of a ketone carbon attached to an aromatic ring. The shifts of the methylene protons (2.98, 3.35 ppm) implied that the methylene carbon is attached to the carbonyl functionality. This conclusion is supported by a selective INEPT experiment wherein irradiation of the methine proton H-3′ caused polarization transfer to the aromatic carbons C-5′ and C-6′, as well as C-1′, C-2′, C-4′, and C-11′. Thus, preussomerin C (3) differs from preussomerin A (1) in the oxidation state at C-1′ and the net addition of a molecule of methanol across the C-2′, C-3′ double bond. Most of the stereochemistry for 3 was assigned by NMR analogy to 1. Unfortunately, NOESY data were not helpful in establishing the stereochemistry at C-3′. Molecular models strongly suggest that the corresponding ring would most likely possess a half-chair conformation, since the only other reasonable (boat) conformation would be much more strained. The methine ^1H NMR signal (4.32 ppm; dd; J = 2.9, 2.9 Hz) lacks a large, trans-diaxial-type coupling. Placing the methoxy group on the α face is consistent with all observations, because the corresponding vicinal H–H angles in the half-chair conformation of this isomer are virtually identical and are consistent with the J values. If the methoxy group were on the β face, the compound would be required to adopt a strained boat conformation in order to avoid exhibiting a trans-diaxial J value.

Since preussomerin D (4) is not converted to preussomerin C (3) upon addition of methanol in the presence of silica gel, it appears that 3 is not an artifact of the isolation process. Moreover, preussomerins A–F are all detected in the crude extract of *P. isomera* by TLC analysis prior to any exposure to methanol.

Preussomerin D (4) is a major component, with isolated yields ranging from 0.5 to 2 mg/L, and is a much more effective antifungal agent than preussomerin A. Analysis of preussomerin D by ^{13}C NMR (Table II) and HREIMS indicated a molecular formula with one more degree of unsaturation than in preussomerin A. The ^1H NMR data (Table I) indicated the presence of only one secondary hydroxyl group, which is in the α-hydroxy epoxide spin system. The ^{13}C NMR data (Table II) were nearly identical with those for 1, except for the presence of a carbonyl signal at 185.5 ppm. A complete set of shift correlations and selective INEPT experiments (Table III) verified that all other structural units appeared identical with those of 1. Thus, the only difference in preussomerin D (4) and 1 is the oxidation state at carbon C-1′.

Preussomerin E (5) was isolated in yields ranging from 0.3 to 0.8 mg/L and is an isomer of component 4. Analysis of the ^1H NMR spectrum and decoupling experiments revealed the presence of an isolated 1,2-disubstituted epoxide and an α-hydroxy cis-disubstituted olefin moiety, as well as the two aromatic rings. These proton spin systems and the presence of a carbonyl signal at 197.0 ppm suggest that preussomerins E and A differ in the oxidation state at C-1. Again, the structure of preussomerin E (5) was confirmed through a parallel set of NMR experiments (Table III).

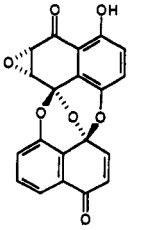
Table IV. Biological Activities for Preussomerins A–F (1–6) toward Early-Successional Coprophilous Fungi *S. fimicola* and *A. furfuraceus* in μg/mL

	S. fimicola		*A. furfuraceus*	
cmpd	MIC[a]	IC_{50}[b]	MIC	IC_{50}
1	>200	200	200	50
2	NA[c]	NA	NA	NA
3	NA	NA	NA	NA
4	50	12.5	10	<5
5	25	10	<5	
6	50	25	25	10

[a] MIC = minimum concentration required to completely prevent fungal growth. [b] IC_{50} = Concentration at which fungal growth is inhibited by 50%. [c] No activity at a concentration of 200 μg/mL.

Preussomerin F, an isomer of 1, was also isolated in very low yields (0 to 0.08 mg/L) and is also a much more potent antifungal agent than 1. Analysis of the ^1H NMR data (Table I) and decoupling experiments led to the elucidation of several spin systems including the two aromatic spin systems found in preussomerin A, an isolated 1,2-disubstituted epoxide, and a secondary hydroxyl group adjacent to an ethylene unit. Thus, preussomerin F (6) is closely related to preussomerin B (2) and differs from 2 only in the oxidation state at C-1. This is further supported by ^{13}C NMR comparison of 6 with preussomerins B (2) and E (5).

The absolute stereochemistry of compounds 2–5 is assumed to be analogous to that of 1 on the basis of NMR and structural similarities. Some supporting evidence for this conclusion was provided by the CD curves of preussomerins A (1) and E (5), which were virtually identical. This relationship was confirmed by PCC oxidation[14] of compound 1, which gave diketone product 8, identical with that obtained upon oxidation of compound 5. It was hoped that NOESY data might provide additional support for other stereochemical assignments, but no relevant conclusions could be drawn from the few NOESY correlations observed.



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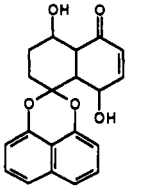

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Neither the novel polycyclic bis-ketal ring system found in the preussomerins nor the unusual α-hydroxy epoxide ketal moiety in preussomerins A–D had been reported prior to the discovery of these compounds. However, a microbial metabolite with apparent biogenetic similarity containing two fewer rings (9) has recently appeared in the

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patent literature.[15] A compound similar to 9 has also recently been reported from a plant source.[16]

The preussomerins inhibit the growth of early successional coprophilous competitors *S. fimicola* and *Ascobolus furfuraceus* (NRRL 6460) (Table IV). Preussomerins A and D–F (1, 4–6) are also active in assays against gram-positive bacteria. In standard disc assays,[17] preussomerin E (5) was the most potent against *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 29213) with zones of inhibition of 20 and 17 mm, respectively, at 10 μg/disc. None of the preussomerins were active in assays against a strain of *Candida albicans* (ATCC 14053) at 250 μg/disc. Preussomerin A did not show activity in assays against herpes viruses at 20–50 μg/mL and exhibited only mild cytotoxicity (14 μg/mL) toward a mammalian cell line.

Although no immediate conclusions can be drawn regarding the significance of these compounds to the ecology and composition of coprophilous fungal communities, the discovery of this set of novel antifungal metabolites does support the hypothesis that relatively slow-growing, late-successional coprophilous fungi comprise a potentially valuable source of antifungal agents.

Experimental Section

General Procedures. NMR spectra were recorded in acetone-d_6 or MeOH-d_4, and chemical shifts were referenced relative to the corresponding acetone (2.04 ppm/29.8 ppm) or methanol (3.30 ppm/49.0 ppm) signals. Carbon multiplicities were established by DEPT experiments. One-bond C–H correlations were obtained with an XHCORR pulse sequence optimized for $^1J_{CH}$ of 135 Hz. Proton signals studied with the selective INEPT technique were individually subjected to three or four separate experiments, optimizing for $^nJ_{CH}$ of 15, 10, 7, and/or 4 Hz. J values are in hertz. Semipreparative HPLC purifications employed Beckman Ultrasphere C18 columns (5 μm particles, 250 × 10 cm) with a flow rate of 2.0 mL/min. Peaks were detected on a variable wavelength detector monitoring at 215 nm. Procedures used in antifungal assays have been described previously.[4,5]

Cultivation of *P. isomera*. A culture of *P. isomera* (CBS 415.82), originally isolated from cattle dung, was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. Ten 2-L Erlenmeyer flasks, each containing 400 mL of potato dextrose broth (Difco), were individually inoculated with one 1-cm^2 agar plug taken from stock cultures of *P. isomera* (Difco potato dextrose agar). Flask cultures were incubated at 25–28 °C and aerated by agitation on an orbital shaker at 180 rpm. The antagonistic activity of the culture filtrate toward early-successional coprophilous fungi reached a maximum after 24 days. Despite efforts to reproduce conditions as closely as possible, yields and ratios of 1–6 varied significantly. Efforts to produce 1–6 on a soy flour medium were unsuccessful. The results given below provide an example of a typical isolation procedure.

Isolation and Characterization of Preussomerins A–F (1–6). The filtered broth (4000 mL) was extracted with EtOAc (10 × 250 mL), and the organic phase was dried ($MgSO_4$) and concentrated to afford 500 mg of dark brown oil that showed antifungal activity. The dried mycelia were extracted with EtOAc (4 × 500 mL), and the solvent was filtered and evaporated to give 1.5 g of brown-black oil, which exhibited comparable activity. The extracts were combined and chromatographed on a silica gel column (4 × 65 cm) with a stepwise gradient from 0 to 10% (v/v) MeOH in $CHCl_3$, and 7-mL fractions were collected. Fractions of similar composition as determined by TLC analysis were pooled and bioassayed. Combined fractions eluting at 2% MeOH exhibited antifungal activity and were purified further by reversed-phase semipreparative HPLC (70:30 MeOH–H_2O) to afford a mixture of preussomerins A and B (1 and 2; 20 mg), preussomerin C (3; 2.1 mg), preussomerin D (4; 3.8 mg), preussomerin E (5; 2.6 mg), and preussomerin F (6; 0.4 mg). The mixture of preussomerins A and B was rechromatographed, using 60:40 MeOH–H_2O, to afford 16.1 mg of preussomerin A (1) and 0.3 mg of preussomerin B (2). Despite extensive purification efforts to remove trace pigments associated with these compounds, all of the metabolites were isolated as reddish brown gums. Attempts to crystallize a representative compound focused primarily on 1 and ultimately led to the formation of off-white crystals from methanol (mp 235–240 °C). Compound 1 has the following properties: $[\alpha]_D$ −212° (c 0.050 g/dL; 31 °C; MeOH); HPLC t_R 10.8 min (60:40 MeOH–H_2O); UV at pH 7 (MeOH) 334 (ε 788), 310 (3566), 301 (3423), 256 nm (2223); at pH 13 319 (3555), 284 (2513), 275 nm (2506); CD (MeOH) 335 nm (Δε 0), 330 (−3.6), 228 (0), 280 (2.4), 260 (0), 240 (5.4), 232 (0), 210 (−65), 200 (−10); ^{1}H NMR and ^{13}C NMR, Tables I and II; IR 3700, 3306, 2917, 2852, 1700, 1590, 1483, 1280 cm^{-1}; EIMS m/z 366 (M$^+$; rel int 26), 348 (43), 346 (27), 319 (20), 175 (92), 160 (72), 147 (76), 118 (80), 91 (63), 89 (64), 75 (100); HREIMS, obsd 366.0735, calcd for $C_{20}H_{14}O_7$, 366.0739.

Preussomerin B (2): $[\alpha]_D$ −242° (c 0.08; 31 °C; MeOH); HPLC t_R 11.0 min (60:40 MeOH–H_2O); UV (MeOH) 366 (ε 208), 303 (2119), 282 (1800), 254 nm (1181); ^{1}H NMR and ^{13}C NMR, Tables I and II; IR 3306, 2944, 2872, 1700, 1592, 1485, 1283, 964 cm^{-1}; HREIMS obsd 368.0874, calcd for $C_{20}H_{16}O_7$, 368.0896.

Preussomerin C (3): $[\alpha]_D$ −155° (c 0.03; 31 °C; MeOH); HPLC t_R 9.85 min (70:30 MeOH–H_2O); UV (MeOH) 303 (ε 3813), 254 nm (7480); ^{1}H NMR and ^{13}C NMR, Tables I and II; IR 3700, 3650, 3360, 1020, 2860, 1685, 1590, 1520, 1470, 1415, 1330, 1280, 1210 cm^{-1}; HREIMS, obsd 396.0850, calcd for $C_{21}H_{16}O_8$, 396.0845.

Preussomerin D (4): $[\alpha]_D$ −144° (c 0.025; 31 °C; MeOH); HPLC t_R 11.7 min (70:30 MeOH–H_2O); UV at pH 7 (MeOH) 295 (ε 12593), 254 nm (1284); at pH 13 313 (13900), 248 nm (6800); ^{1}H NMR and ^{13}C NMR, Tables I and II; IR 3319, 1700, 1696, 1679, 1631, 1600, 1590, 1483, 1472, 1260 cm^{-1}; EIMS m/z 364 (M$^+$; rel int 46), 346 (40), 318 (46), 289 (24), 261 (28), 161 (37), 46 (100); HREIMS, obsd 364.0602, calcd for $C_{20}H_{12}O_7$, 364.0592.

Preussomerin E (5): $[\alpha]_D$ −244° (c 0.023; 31 °C; MeOH); HPLC t_R 14.4 min (70:30 MeOH–H_2O); UV (MeOH) 343 (ε 3228), 264 (4906), 223 (14700), 218 (5755), 206 nm (18929); CD (MeOH) 360 nm (Δε 0), 327 (3.3), 300 (−0.7), 270 (−6.6), 254 (−3.6), 240 (−7.4), 216 (−65), 200 (25); ^{1}H and ^{13}C NMR, Tables I and II; IR 3700, 3650, 3020, 2410, 1660, 1590, 1510, 1470, 1415, 1370, 1280, 1080, 1010, 920 cm^{-1}; EIMS m/z 364 (M$^+$; rel int 100), 189 (8), 175 (14); HREIMS, obsd 364.0579, calcd for $C_{20}H_{12}O_7$, 364.0582.

Preussomerin F (6): $[\alpha]_D$ −240° (c 0.045; 31 °C; MeOH); HPLC t_R 14.7 min (70:30 MeOH–H_2O); UV (MeOH) 365 (ε 1188), 289 (1788), 258 nm (3017); ^{1}H and ^{13}C NMR, Tables I and II; IR 3710, 3020, 2420, 1970, 1700, 1530, 1470, 1420, 1290, 1210 cm^{-1}; EIMS m/z 366 (M$^+$; rel int 2.8), 348 (5), 191 (7), 137 (12), 83 (46), 55 (100); HREIMS, obsd 366.0744, calcd for $C_{20}H_{14}O_7$, 366.0739.

Acetylation of Preussomerin A (1). A sample of preussomerin A (1.1 mg) was dissolved in 1 mL of acetone along with 0.5 mg of DMAP (required for complete reaction) and 0.5 mL of acetic anhydride. The mixture was stirred for 18 h at room temperature, evaporated, redissolved in 2 mL of water, and extracted with $CHCl_3$ (3 × 2 mL). The organic layer was collected, dried, and evaporated to afford 1.0 mg of a yellow oil. The oil was purified by reversed-phase HPLC (90:10 MeOH–H_2O) to yield 0.8 mg of triacetylpreussomerin A (54% yield), t_R 17.5 min: ^{1}H NMR ($CDCl_3$) 7.36 (t, J = 7.8, 1 H), 7.07 (dd, J = 7.8, 0.9, 1 H), 7.05 (d, J = 9.3, 1 H), 6.91 (d, J = 7.8, 1 H), 6.89 (d, J = 9.3, 1 H), 6.61 (dd, J = 9.8, 1.6, 1 H), 6.51 (dd, J = 9.8, 2.9, 1 H), 6.48 (m, 1 H), 6.42 (m, 1 H), 4.08 (d, J = 4.4, 1 H), 3.89 (dd, J = 4.4, 2.0, 1 H), 2.26 (s, 3 H), 2.24 (s, 3 H), 2.19 (s, 3 H); EIMS m/z 492 (M$^+$; rel int 5.3), 450 (15), 362 (6.8), 348 (17), 320 (9.7), 208 (16), 191 (15), 150 (12), 147 (23), 133 (20), 97 (19), 81 (34), 73 (100).

Acid-Catalyzed Cleavage of Preussomerin A (1). A 20-mg sample of preussomerin A containing small amounts of preussomerins B–D (>90% 1 by HPLC analysis) was placed in a 1-dram screw-cap vial. Acetone (1.0 mL) and 6 M HCl (1.0 mL) were added. The vial was tightly sealed and the mixture was stirred for 12 h in an oil bath at 100 °C. The solvent was removed and

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the dark black-brown residue was dissolved in MeOH. The residue was purified by using reversed-phase HPLC (70:30 MeOH–H_2O) to afford 3.0 mg of compound 7 (34.3% isolated yield based on the amount of 1 used; t_R 8.5 min), as well as a number of unidentified minor components. Although the starting material employed in this experiment was only >90% pure, the amount of product obtained indicated that most of it must be derived from 1. The ^1H NMR, ^{13}C NMR, and EIMS data for 7 were identical with those reported in the literature for (–)-regiolone,[10] while the $[\alpha]_D$ (–5.6° in EtOH at 31 °C) was comparable to the literature value (–3.3° in EtOH; temperature not reported).

Acetylation of Preussomerin D (4). The procedure described above for compound 1 was repeated, using 1.7 mg of 4. In this case, 1.4 mg (67% yield) of diacetylated product was obtained, and no HPLC purification was necessary: ^1H NMR (acetone-d_6) 7.56 (dd, J = 7.8, 1.2, 1 H), 7.52 (t, J = 7.8, 1 H), 7.45 (d, J = 10.0, 1 H), 7.22 (dd, J = 7.8, 1.2, 1 H), 7.10 (d, J = 9.0, 1 H), 6.93 (d, J = 9.0, 1 H), 6.67 (d, J = 10.0, 1 H), 6.52 (m, 1 H), 4.03 (d, 4.2, 1 H), 3.90 (dd, J = 4.2, 1.6, 1 H), 2.25 (s, 3 H), 2.21 (s, 3 H).

Oxidation of Preussomerins A and E (1 and 5). A solution of 1 (1.8 mg in 50 μL of acetone) was added to 1 mL of CH_2Cl_2 and combined with NaOAc (6.7 mg), PCC (10.6 mg), and Florisil (25 mg) in a 1-dram vial.[14] After stirring at room temperature for 4 h, no starting material remained. At this point, Et_2O (2 mL) was added. Upon stirring for 10 additional min, the supernatant solution was decanted. The residual solids were washed with Et_2O (3 × 2 mL), and the washings were combined with the supernatant solution and passed through a Pasteur pipet column of Florisil, eluting progressively with Et_2O (10 mL), $CHCl_3$ (5 mL), 1:9 MeOH–$CHCl_3$ (5 mL), and MeOH (10 mL). The product 8 (0.9 mg; 50% yield) was obtained upon evaporation of the $CHCl_3$ and MeOH–$CHCl_3$ fractions. Reaction of 5 under identical conditions afforded the same product: HPLC t_R 24.9 min (70:30 MeOH–H_2O); ^1H NMR (acetone d_6; assignments proposed by analogy to those in Table I) 7.59 (dd, J = 7.6, 1.2, H-9′), 7.54 (dd, J = 8.1, 7.6, H-8′), 7.47 (d, J = 10.0, H-2′), 7.22 (d, J = 9.0, H-7), 7.20 (dd, J = 7.8, 1.2, H-7′), 7.05 (d, J = 9.0, H-8), 6.68 (d, J = 10.0, H-3′), 4.53 (d, J = 3.9, H-3), 4.05 (d, J = 4.2, H-2); EIMS (70 eV) m/z 362 (M^+; rel int 100), 333 (6), 289 (2), 174 (53), 165 (16), 125 (45).

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Supplementary Material Available: Selected ^1H NMR spectra (for preussomerins B and F), ^{13}C NMR spectra (for preussomerins A–F), and ORTEP representations of preussomerin A (9 pages). Ordering information is given on any current masthead page.

Structures of Nostocyclophanes A–D

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Nostocyclophanes A–D are the cytotoxins associated with the blue-green alga *Nostoc linckia* (Roth) Bornet ex Bornet & Flahault (UTEX B1932). The gross structures of these [7.7]paracyclophanes have been elucidated by mass and NMR spectral analyses and the relative and absolute stereochemistry of nostocyclophane D determined by X-ray crystallography. Since the CD spectra of the four compounds are essentially identical, nostocyclophanes A–D are proposed to have the same stereochemistry. The sugar unit in nostocyclophanes A and B has been shown to be D-glucose by semisynthesis of nostocyclophane B 9-*O*-(2,3,4,6-tetra-*O*-acetyl)-β-D-glucopyranoside from nostocyclophanes B and D.

In a preliminar paper we reported the isolation and structure determination of the first naturally occurring [*m.n*]paracyclophanes.[1] Cytotoxins associated with two species of terrestrial blue-green algae belonging to the Nostocaceae, e.g., *Nostoc linckia* (Roth) Bornet ex Bornet & Flahault (UTEX B1932) and *Cylindrospermum licheniforme* Kützing (ATCC 29204), were found to be [7.7]-paracyclophanes. The total structure of nostocyclophane D, the major chlorine-containing [7.7]paracyclophane in the *N. linckia*, was established by an X-ray crystallographic study. We describe here the isolation and structure determination of three minor [7.7]paracyclophanes, nostocyclophanes A–C, from this alga.

Results and Discussion

In an evaluation of extracts of blue-green algae for antitumor activity, the lipophilic (1:1 methylene chloride/2-propanol) extract of terrestrial *N. linckia* UTEX B1932 was found to be cytotoxic against KB (human nasopharyngeal carcinoma) and LoVo (human colon adenocarcinoma) cell lines at 13 μg/mL. When a 70% ethanol/water extract of the freeze-dried cyanophyte was subjected to flash reversed-phase chromatography on C-18, a mixture of cytotoxic [7.7]paracyclophanes (MIC 5 μg/mL) was obtained that could be separated by reversed-phase HPLC on C-18 into nostocyclophanes A–D in yields of 0.01, 0.02, 0.01, and 0.13%, respectively. The major compound, nonstocyclophane D, was found to be cytotoxic at 0.5 μg/mL (IC_{50}); the three minor compounds, nostocyclophane A–C, were cytotoxic at 1–2 μg/mL (IC_{50}s).

Nostocyclophane D. The positive-ion fast atom bombardment mass spectrum, which displayed a 10:6.5:1 MH^+ ion cluster at m/z 653/655/657, and the field-desorption mass spectrum, which exhibited a 10:6.5:1 M^+ ion cluster at m/z 652/654/656, indicated that nostocyclophane D had a molecular weight of 652 D and possessed two chlorine atoms. Only 18 carbon signals could be seen in its ^{13}C NMR spectrum (one obscured by solvent signal but clearly visible in DEPT spectrum), however, and this implied that the molecule had a 2-fold axis of symmetry and, therefore, 36 carbon atoms. Of the 54 protons in the molecule, 50 were nonexchangeable (attached to carbon), since DEPT experiments showed the presence of two methyl, seven

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