Alkaloids from the Mushroom *Pseudobaeospora pyrifera*, Pyriferines A−C[⊥]

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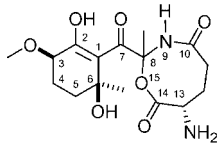
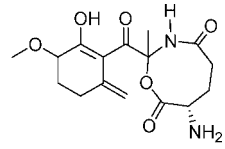
Three novel alkaloids (**1**−**3**), named pyriferines A−C, were isolated from fruiting bodies of *Pseudobaeospora pyrifera*. They possess an unusual eight-membered N/O-acetal ring, derived from L-glutamic acid, that is connected to an enolized 1,3-diketo moiety. The structures were determined by spectroscopic methods, and the absolute configuration of the glutamic acid moiety was established using GC-MS after Mosher-type derivatization.

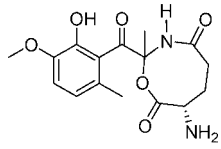
The fungal genus *Pseudobaeospora* (Tricholomataceae) is very rare and only described for Europe and North America. Thirteen species are known, but their chemical constituents are unknown.[1] The genus was first established by Singer[2,3] and recently generically circumscribed by Bas.[1,4] The species *Pseudobaeospora pyrifera* Bas & L. Krieglst. (Agaricales) investigated herein was described in 1998.[5] *P. pyrifera* is characterized by a 0.5−2.5 cm wide cap and a 1−3.5 cm long and 0.3−0.5 cm broad stipe. Cap and stem are pruinose sordid pink to pruinose vinaceous; the lamellae are rather dark reddish to violaceous pink colored. This little species grows on the ground in more or less moist areas (*Pruno-Fraxinetum* woods with *Alnus, Prunus padus, Fraxinus*). *P. pyrifera* is characterized by a greenish-blue to brownish-green reaction of the pileipellis with KOH solution. In this work, we describe the isolation and structural characterization of three novel unusual alkaloids that possess unprecedented skeletons (**1**−**3**).



Pyriferine A (**1**)



Pyriferine B (**2**)



Pyriferine C (**3**)

Compound **1** was obtained as a colorless oil. Its positive ion ESIMS gave an $[M + Na]^+$ ion at *m/z* 379.1467, corresponding to the molecular formula $C_{16}H_{24}O_7N_2Na$, which was revealed by ESI-FTICR-MS, indicating the presence of six double-bond equivalents. Analysis of its IR spectrum suggested that it contained carbonyl groups (1681 cm^{-1}) and a double bond (1615 cm^{-1}). The ^1H NMR spectrum of **1** indicated the presence of two methyl, four methylene, two methine, and one methoxy group. Its ^{13}C NMR exhibited signals for 16 carbon atoms, in agreement with the molecular formula

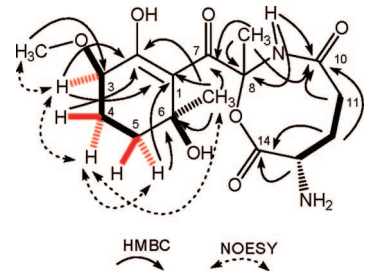


Figure 1. Important $^1H-^1H$ COSY (bold lines), HMBC, and NOESY correlations of pyriferine A (**1**).

$C_{16}H_{24}O_7N_2$. According to the DEPT spectrum, one conjugated ketone (δ_C 201.3), one enol carbon (δ_C 183.1), two carbonyl groups, and other signals as described in the Experimental Section were present. The $^1H-^1H$ COSY spectrum of **1** (Figure 1) provided evidence for partial structures CH_2-CH_2-CH-O and $CH-CH_2-CH_2-$. The proton connected to the oxygen-bearing carbon (δ_H 4.03) had HMBC correlations with C-2, C-4, C-5, and 3-OMe; therefore, the signal should belong to H-3. The methyl group at δ_H 1.56 showed HMBC correlations with C-1, C-2, C-5, C-6, and C-7; thus, it should be located at C-6. The second methyl group is attached to C-8 due to its HMBC correlations with C-7 and C-8. Since C-6 (δ_C 68.2) was observed at relatively low field, it is likely connected to an oxygen atom. The presence of a glutamic acid moiety was also revealed by HMBC correlations between H-13/C-11, C-12, and C-14 and between H-11/C-10 and C-12 (Figure 1) and by comparison with data in a previous publication.[6] The C-14 carbonyl group (δ_C 173.7) coupled to H-12 and H-13 in the HMBC spectrum and together with the low-field shift of C-8 indicated that C-14 is connected to C-8 via an oxygen atom. Furthermore, only one NH signal was observed when the HMBC spectrum was recorded in DMSO-d_6. The signal at δ 9.22 showed correlations with C-7, C-8, and C-10; thus it was assigned as NH-9. The other end of the glutamic acid moiety must be attached to N-9 to generate an eight-membered-ring partial structure in **1**.

The relative configuration within the hexene ring of **1** was established by creation of a three-dimensional model and by its correlations to NOESY data (Figure 1). First of all, H-3 showed a NOE correlation with 3-OMe and appeared as a dd (δ_H 4.03, J = 4.4, 4.4); thus H-3 is equatorial. Accordingly 3-OCH_3 must be axial. H-3 coupled strongly with H-4e (δ_H 2.13) and weakly with H-4a (δ_H 1.90). Furthermore, H-4e (δ_H 2.13) coupled with H-5e (δ_H 1.68). Finally, 6-Me was determined to be axial and located on the opposite face relative to 3-OCH_3 due to the NOE correlations between 6-Me and H-4a, H-5e (δ_H 1.68). Since, there was no correlation observed between 8-Me and other protons, the relative

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configuration of 8-CH_3 remains unclear. In addition, LC-ESIMS/MS spectra in both positive and negative modes were recorded. Detailed analyses of all major fragments are shown (see S4 in the Supporting Information). From the above discussion and spectroscopic evidence, compound **1** was determined to be 7-amino-2-(2,6-dihydroxy-3-methoxy-6-methylcyclohex-1-enecarbonyl)-2-methyl[1,3]oxazocane-4,8-dione, and it was named pyriferine A.

Compound **2** was also purified as a colorless oil. Its molecular formula, $C_{16}H_{22}O_6N_2$ (ESI-FTICR-MS), corresponded to a dehydrated compound **1**. Its NMR spectroscopic data resembled those of **1**, except for the presence of one *exo*-methylene group with signals at δ 4.89 and 5.62 in its ^{1}H NMR spectrum. The *exo*-methylene was placed at C-6 due to HMBC correlations between 6-CH_2 and C-1, C-5, C-6. The relative position of H-3 was established as equatorial since it appeared as a dd (J = 5.4, 5.4 Hz); therefore 3-OCH_3 is axial. Consequently, pyriferine B was determined to be 7-amino-2-(2-hydroxy-3-methoxy-6-methylenecyclohex-1-enecarbonyl)-2-methyl[1,3]oxazocane-4,8-dione.

Pyriferine C (**3**) exhibited an $[M + Na]^+$ ion at m/z 359.1214 in the ESI-FTICR-MS, corresponding to the molecular formula $C_{16}H_{20}O_6N_2Na$, two hydrogen atoms less than compound **2**. The spectroscopic data were very similar to those of **2**, except for signals typical for an aromatic ring. The locations of substituents on the aromatic ring were determined by 2D NMR spectra. The 3-OMe group coupled to the aromatic C-3 in the HMBC spectrum. Two proton signals (δ 7.13 and 6.78) appeared as doublets with a large coupling constant (J = 7.8 Hz), correlating in the HMBC to C-2, C-3, C-6 and C-1, C-3, respectively; thus they were assigned as H-4 and H-5. In addition, the methyl group at δ 2.47 is located at C-6 on the basis of HMBC correlations between 6-Me and C-1, C-5, C-6. Their positioning was supported by a NOESY spectrum in which correlations between H-4 and 3-OCH_3, H-5 and between H-5 and H-4, 6-Me were also detected. To confirm the proposed structure, compound **3** was treated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide to give the silylation product (**3a**, see the Supporting Information), which was analyzed by GC-EIMS. The EI mass spectrum exhibited the molecular peak at m/z = 624, indicating the presence of four TMS groups in **3a**.

To establish the absolute configuration of the glutamic acid moiety, compound **3** was hydrolyzed to give glutamic acid followed by methylation using diazomethane[7] and esterification with (*S*)-α-methoxy-α-trifluoromethylphenylacetylchloride [(*S*)-MTPA-Cl] to form compound **4**. Compound **4** was analyzed by GC-MS using a mass selective detector[8] and compared to **5** and **6** as standards, which were derived from D- and L-glutamic acid, respectively. From the GC chromatograms (see the Supporting Information for preparation and structures of compounds **4**, **5**, and **6**), it was established that the glutamic acid moiety of compound **3** is *S*-configured (L-glutamic acid). Accordingly, the glutamic acid moieties of **1** and **2** are assumed to have L-configurations by analogy. Therefore, pyriferine C (**3**) is 7*S*-amino-2-(2-hydroxy-3-methoxy-6-methylbenzoyl)-2-methyl[1,3]oxazocane-4,8-dione (please note that the IUPAC 7*S*-carbon corresponds to the stereocenter at C-13).

Unfortunately, the flexible eight-membered ring does not allow an unequivocal stereochemical correlation of the defined stereocenter at C-13 to the center at C-8, and thus the absolute stereochemistry at C-8 and in the six-membered ring in relation to C-13 is not defined. Neither extensive molecular modeling calculations nor NMR studies provided any hint at a preferred orientation. Thus, it cannot be excluded that the *cis*-related *O*-substituents at C-3 and C-6 may both have opposite absolute configuration. *P. pyrifera* is such a rare species that the amount of **1** available was too small to determine the absolute configuration of the C_{1-8} moiety through chemical derivatization/degradation experiments. Microcrystallization was unsuccessful. At this point, only chemical synthesis can eventually provide a final proof of the absolute configuration of these stereocenters in the pyriferines.

This is the first report on chemical constituents of *Pseudobaeospora* sp., and the pyriferines A−C (**1**−**3**) possess unprecedented carbon skeletons. The interconversion of compounds **1**−**3** is presumably by biosynthetic conversions starting from **1**. A dehydration reaction could form compound **2**. Compound **3** could then be obtained from compound **2** by oxidation (see S5 in the Supporting Information). Labeling experiments are needed to clarify whether pyriferines A−C (**1**−**3**) originate from shikimic acid and either the two amino acids alanine and glutamic acid or from pyruvate and glutamine. Although most biosynthetic steps are usually enzyme-catalyzed, for the last step(s) nonenzymatic conversions cannot be excluded and such reactions are not rare in fungi, e.g., by oxidative activation of defense compounds in wounded tissue.

Preliminary tests[9,10] show that pyriferines A and B (**1**, **2**) inhibit acetylcholinesterase, an important medicinal target, e.g., in Alzheimer's disease, to a minor extent (20% and 7% at 100 μM, respectively).

Experimental Section

General Experimental Procedures. Column chromatography was carried out on Sephadex LH-20 (Amersham Pharmacia Biotech). Preparative HPLC was performed with a Varian ProStar 218 system and an ODS C-18 column (5 μm, 150 × 20 mm i.d., YMC). Optical rotations were measured on a JASCO DIP-1000 polarimeter with MeOH as solvent. UV spectra were obtained on a Jasco V-560 spectrophotometer in MeOH. IR spectra were measured on a Thermo Nicolet 5700 FT-IR spectrometer, on an ATR crystal (diamond). The ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity Inova 500 spectrometer and Bruker AMX-600 using CD_3OD as solvent. Chemical shifts are given relative to TMS as internal standard (^{1}H) and 49.0 ppm from CD_3OD as standard (^{13}C). High-resolution ESI mass spectra were recorded on a Bruker Apex III Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. LC-ESIMS/MS were recorded on a Finnigan MAT TSQ Quantum Ultra AM system. The GC-EIMS measurements of the trimethylsilyl derivatives of **3** were performed on a Voyager/Trace GC 2000 (Thermo Quest CE Instruments) under the following conditions: 70 eV EI, source temperature 200 °C, column DB-5MS (J&W, 30 m × 0.25 mm, 0.25 μm film thickness), injection temperature 250 °C, interface temperature 300 °C, carrier gas He, flow rate 1.0 mL/min, constant flow mode, splitless injection, column temperature program 60 °C for 1 min, then raised to 300 °C at a rate of 10 °C min^{-1} and then held at 300 °C for 15 min. The GC-EIMS measurement for **4** was analyzed by a Fisons Instruments GC 8000 series and MD 800 equipped with the same column used to analyze **3a**. Temperature program: starting from 100 to 160 °C at 30 °C/min, then 160 to 200 °C with 1 °C/min, finally 200 to 270 °C at 30 °C/min. Mass selective detector: m/z 202, 240, 248, and 332.

Fungus Material. Fruiting bodies of *Pseudobaeospora pyrifera* were collected in Germany, state of Bavaria, in Lower Franconia near Kitzingen (Klosterforst) at the loco-type (leg. and det. L. Krieglsteiner). A voucher specimen is deposited in REG.

Extraction and Isolation. Dried basidiocarps of *P. pyrifera* (3.08 g) were extracted two times with MeOH (100 mL) in an ultrasonic bath for 45 min. The methanolic extract was concentrated *in vacuo* to obtain a red-brown residue (400 mg), which was subjected to Sephadex LH-20 column chromatography using $CHCl_3$/MeOH (1:1) as eluent. Further purification was carried out by repeated preparative HPLC on an ODS C-18 column, using H_2O (A) and CH_3CN (B) as a solvent system (linear gradient: 0−20 min, 3% to 25% B; 20 to 23 min: 25% to 100% B; isocratic flow of B to 27 min; linear gradient: 27−30 min, 0 to 97% A), flow rate 20 mL/min to afford **1** (2.7 mg, 4.4 min), **2** (1.6 mg, 16.2 min), and **3** (4.8 mg, 22.6 min).

Pyriferine A (1): colorless oil; $[\alpha]_D^{20}$ −8.0 (MeOH, 0.08); UV λ_{max} nm (log ε) 269 (3.7), 234 (3.5); IR (ATR) 3359, 3252, 2925, 2852, 1681, 1615, 1538, 1448, 1258, 1139, 1021, 914 cm^{-1}; ^{1}H NMR (CD_3OD, 600 MHz) δ 1.48 (3H, s, 8-Me), 1.56 (3H, s, 6-Me), 1.68 (1H, m, H-5), 1.90 (1H, m, H-4), 1.92 (1H, m, H-5), 2.05 (2H, m, H-12), 2.13 (1H, m, H-4), 2.45 (2H, dd, J = 7.3, 7.3 Hz, H-11), 3.42 (3H, s, 3-OMe), 3.56 (1H, dd, J = 5.6, 5.6 Hz, H-13), 4.03 (1H, dd, J = 4.4, 4.4 Hz, H-3); ^{13}C NMR (CD_3OD, 151 MHz) δ 22.0 (CH_3, 8-Me),

26.6 (CH_2, C-4), 26.6 (CH_3, 6-Me), 27.4 (CH_2, C-12), 31.9 (CH_2, C-11), 36.4 (CH_2, C-5), 55.5 (CH, C-13), 58.2 (CH_3, 3-OMe), 68.2 (C, C-6), 73.4 (CH, C-3), 91.9 (C, C-8), 117.6 (C, C-1), 173.7 (C, C-14), 174.2 (C, C-10), 183.1 (C, C-2), 201.3 (C, C-7); ^{1}H NMR (DMSO-d_6) δ 1.32 (3H, s, 8-Me), 1.37 (3H, s, 6-Me), 1.52 (1H, m, H-5), 1.70 (1H, m, H-5), 1.75 (1H, m, H-4), 1.77 (2H, m, H-12), 2.01 (1H, m, H-4), 2.77 (2H, t, J = 7.6 Hz, H-11), 3.12 (1H, t, J = 6.2 Hz, H-13), 3.27 (3H, s, 3-OMe), 3.93 (1H, t, J = 4.1 Hz, H-3), 9.22 (1H, s, H-9); ^{13}C NMR (DMSO-d_6) δ 21.9 (8-Me), 24.8 (C-4), 26.4 (6-Me), 26.7 (C-12), 30.8 (C-11), 35.4 (C-5), 53.3 (C-13), 56.8 (3-OMe), 65.7 (C-6), 71.3 (C-3), 90.7 (C-8), 116.4 (C-1), 169.5 (C-14), 171.3 (C-10), 179.3 (C-2), 198.2 (C-7); positive ion ESIMS 379 $[M + Na]^+$; negative ion ESIMS 355 $[M - H]^-$; LC-ESIMS/MS (positive) 357 (18) $[M + H]^+$, 339 (100), 210 (82); LC-MS/MS (negative) 355 (10) $[M - H]^-$, 337 (25), 293 (12), 276 (22), 226 (100), 208 (25), 145 (20); ESI-FTICR-MS $[M + Na]^+$, *m/z* 379.1476, calcd for $C_{16}H_{24}O_7N_2Na^+$, 379.1481.

Pyriferine B (2): colorless oil; $[\alpha]_D^{20}$ +34.3 (MeOH, 0.06); UV λ_{max} nm (log ε) 297 (3.3), 219 (3.9), 204 (3.9); IR (ATR) 3209, 3041, 2927, 2851, 1673, 1641, 1590, 1539, 1410, 1352, 1242, 1141, 1086, 874 cm^{-1}; ^{1}H NMR (CD_3OD, 600 MHz) δ 1.49 (3H, s, 8-Me), 1.96 (2H, m, H-4), 2.04 (2H, m, H-12), 2.35 (1H, m, H-5), 2.47 (2H, dd, J = 7.3, 7.3 Hz, H-11), 2.56 (1H, m, H-5), 3.45 (3H, s, 3-OMe), 3.55 (1H, dd, J = 5.9, 5.9 Hz, H-13), 4.21 (1H, dd, J =, 5.4, 5.4 Hz, H-3), 4.89 (1H, s, 6-CH_2), 5.62 (1H, s, 6-CH_2); ^{13}C NMR (CD_3OD, 151 MHz) δ 22.2 (8-Me), 27.3 (C-12), 28.9 (C-5), 29.7 (C-4), 31.9 (C-11), 55.5 (C-13), 58.2 (3-OMe), 74.1 (C-3), 92.3 (C-8), 108.7 (6-CH_2), 114.5 (C-1), 135.9 (C-6), 173.7 (C-14), 174.3 (C-10), 183.5 (C-2), 200.2 (C-7); positive ion ESIMS 361 $[M + Na]^+$, negative ion ESIMS 337 $[M - H]^-$; LC-ESIMS/MS (positive) 339 (10) $[M + H]^+$, 210 (100), 192 (72); LC-ESIMS/MS (negative) 337 (35) $[M - H]^-$, 305 (15), 243 (15), 208 (70), 145 (100); ESI-FTICR-MS $[M + Na]^+$, *m/z* 361.1370, calcd for $C_{16}H_{22}O_6N_2Na^+$, 361.1376.

Pyriferine C (3): oil; $[\alpha]_D^{20}$ +12.4 (MeOH, 0.1); UV λ_{max} nm (log ε) 346 (3.1), 267 (3.7), 205 (4.1); IR (ATR) 3254, 3040, 2936, 2835, 1720, 1673, 1621, 1596, 1518, 1267, 1232, 1071, 1022, 816 cm^{-1}; ^{1}H NMR (CD_3OD, 600 MHz) δ 1.51 (3H, s, 8-Me), 2.02 (2H, m, H-12), 2.47 (3H, s, 6-Me), 2.48 (2H, dd, J = 7.3, 7.3 Hz, H-11), 3.53 (1H, dd, J = 5.6, 5.6 Hz, H-13), 3.86 (3H, s, 3-OMe), 6.78 (1H, d, J = 7.8 Hz, H-5), 7.13 (1H, d, J = 7.8 Hz, H-4); ^{13}C NMR (CD_3OD, 151 MHz) δ 17.0 (6-Me), 22.0 (8-Me), 27.5 (C-12), 31.9 (C-11), 55.5 (C-13), 56.8 (3-OMe), 91.6 (C-8), 120.1 (C-1), 120.5 (C-4), 123.9 (C-5), 131.2 (C-6), 145.3 (C-3), 160.9 (C-2), 174.6 (C-10, C-14), 200.7 (C-7); positive ion ESIMS 359 $[M + Na]^+$, negative ion ESIMS 335 $[M - H]^-$; LC-ESIMS/MS (positive) 337 (10) $[M + H]^+$, 208 (40), 191 (100); LC-ESIMS/MS (negative) 335 (13) $[M - H]^-$, 273 (32), 206 (100), 191 (10), 128 (26); ESI-FTICR-MS $[M + Na]^+$, *m/z* 359.1214, calcd for $C_{16}H_{20}O_6N_2Na^+$, 359.1219.

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Supporting Information Available: Silylation of pyriferine C (**3**), determination of absolute configuration of compound **3** by GC-EIMS, fragmentation scheme of compound **1** in the LC-ESIMS/MS, and 1D and 2D NMR spectra of pyriferines A−C (**1**−**3**). This material is available free of charge via the Internet at http://pubs.acs.org.

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