DOI: 10.1002/ejoc.200800447

Epicoccalone, a Coumarin-Type Chymotrypsin Inhibitor, and Isobenzofuran Congeners from an *Epicoccum* sp. Associated with a Tree Fungus

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Keywords: Coumarins / Fungi / Natural products / Polyketides / Protease inhibitors

The bioactivity-guided fractionation of metabolites from an *Epicoccum* sp. isolated from the fruiting body of the tree fungus *Pholiota squarrosa* led to the discovery of a new inhibitor of the serine protease α-chymotrypsin. The structure of the active compound, epicoccalone (**1**), was fully elucidated by spectrometric and spectroscopic methods, revealing a coumarin ring system with an unprecedented substitution pattern. Two isobenzofuran-derived co-metabolites were identified that are remarkably similar to the epicoccalone coumarin core, strongly suggesting that these metabolites share the same orsellinic acid like polyketide pathway.

Introduction

Microorganisms that live in association with other organisms are a rich source of biologically active compounds.[1–3] In the kingdom fungi this is well reflected by so-called mycophilic fungi[4–8] and plant endophytes,[9,10] as well as fungicolous or mycoparasitic fungi.[11–13] Another rare example in which the fungus serves as a host are the fungal-bacterial symbioses producing the toxins rhizoxin[14–16] and rhizonin.[17] In the course of our search for bioactive compounds produced by tree fungi,[18–22] we have identified an *Epicoccum* species growing within the fruit body of the tree fungus *Pholiota squarrosa*. We have succeeded in cultivating the fungus and characterized six new polyketide–amino acid hybrid metabolites, including the potent antibacterial tetramic acid epicoccarines A and B, and cytotoxic tetramic acid epicoccamide D.[23,24] Here we report on the isolation, structural elucidation and biological evaluation of a new serine protease (chymotrypsin) inhibitor epicoccalone (**1**) along with one known (**2**) and one new (**3**) benzofuran-derived compound from an *Epicoccum* sp. that was isolated from a saprotrophic tree fungus. The cooccurrence of compounds **1**, **2**, and **3** strongly suggests a common polyketide pathway.

Results and Discussion

The culture broth and biomass obtained from a 30 L *Epicoccum* sp. fermentation was extracted with EtOAc and MeOH, respectively, and the combined EtOAc-soluble extracts were subjected to open column chromatography on silica gel. Further purification of selected bioactive (protease inhibiting) fractions afforded **1** as a yellow oil in 6.2 mg yield. The structure of **1** was fully elucidated by mass spectrometry and IR and NMR spectroscopy (Figure 1, Table 1). Its molecular formula was determined as $C_{16}H_{16}O_7$ based on HR-EIMS and ^{13}C NMR data. The IR spectrum of **1** showed typical absorption bands for hydroxy (3244 cm^{-1}), carbonyl (1699 cm^{-1}) and aryl (1598, 1562 and 1465 cm^{-1}) groups. The ^1H NMR spectrum of **1** showed three signals attributable to methyl groups at δ = 1.23 (d, J = 7.1 Hz, 3 H), 2.22 (s, 3 H) and 2.28 (s, 3 H) ppm, as well as signals for oxymethylene [δ = 4.68 (s, 2 H) ppm], and two methine protons, out of which one is of olefinic character [δ = 4.71 (q, 1 H), 8.89 (s, 1 H) ppm]. The ^{13}C NMR and DEPT 135 spectra of **1** showed the occurrence of five sp^3-carbon signals related to three methyl groups, one oxymethylene and one methine group, and eleven sp^2-carbon signals including one methine sp^2-carbon signal, seven quaternary sp^2-carbon signals (three of which are oxygenated). Furthermore, the ^{13}C NMR spectrum of **1** indicated the occurrence of two carbonyl signals and one carboxy carbon signal (δ = 194.0, 206.2 and 159.2 ppm). HMQC and HMBC experiments revealed all significant connectivities. The correlation of the methyl protons 5′-H_3 (δ = 1.23 ppm) with carbon atoms C-1′ (δ = 194.0 ppm), C-2′ (δ = 57.3 ppm), C-3′ (δ = 206.2 ppm), the correlation of the protons 4′-H_3 (δ = 2.28 ppm) with the carbon atoms C-2′ (δ = 57.3 ppm) and C-3′ (δ = 206.2 ppm) and the correlation of the proton 2′-H (δ = 4.71 ppm) with the carbon atoms C-1′ (δ = 194.0 ppm), C-3′ (δ = 206.2 ppm) and C-5′ (δ = 12.1 ppm) unambiguously established the 2-methyl-1,3-dioxobutyl side chain of **1**. For the identification of the second part of the metabolite, long-range HMBC played also

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a significant role. The correlation of the proton of the oxymethylene carbon atom 9-H_2 (δ = 4.68 ppm) with C-4a (δ = 109.9 ppm), C-5 (δ = 131.6 ppm) and C-6 (δ = 122.4 ppm) and the correlation of the methyl protons 10-H_3 (δ = 2.22 ppm) with C-5 (δ = 131.6 ppm), C-6 (δ = 122.4 ppm) and C-7 (δ = 151.6 ppm) suggested the connection of the oxymethylene carbon atom C-9 (δ = 55.6 ppm) and the methyl carbon atom C-10 (δ = 11.3 ppm) at positions 5 and 6, respectively. The most important correlation was that of the olefinic proton 4-H (δ = 8.89 ppm) with C-2 (δ = 159.2 ppm), C-3 (δ = 116.5 ppm), C-5 (δ = 131.6 ppm) and C-8 (δ = 130.2 ppm). This information denoted the second part of the molecule as 7,8-dihydroxy-5-(hydroxymethylene)-6-methylcoumarin. Due to the strong correlation between the olefinic proton 4-H (δ = 8.89 ppm) and the carbonyl carbon atom C-1′ (δ = 194.0 ppm) it is obvious that the two parts are connected at the position 3. Thus, the structure of **1** was established as 7,8-dihydroxy-5-(hydroxymethylene)-3-(2-methyl-1,3-dioxobutyl)-6-methylcoumarin, named epicoccalone.

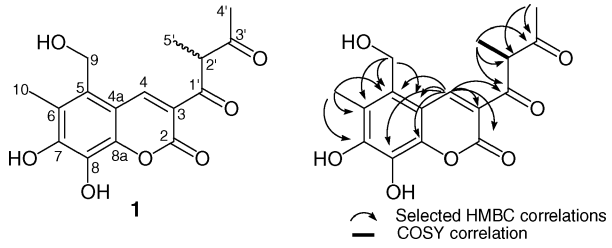


Figure 1. Structure of epicoccalone (**1**) with selected connectivities.

Table 1. NMR spectroscopic data of **1** and **3** (in [D_6]DMSO).

Pos.	**1**		**3**	
	δ_H	δ_C, mult.	δ_H	δ_C, mult
1				169.8, qC
2		159.2, qC		
3		116.5, qC	5.09 (s)	67.6, CH_2
4	8.89 (s)	147.0, CH		109.1, qC
4a		109.9, qC		138.0, qC
5		131.6, qC		151.1, qC
6		122.4, qC		132.8, qC
7		151.6, qC		143.0, qC
7a				102.7, qC
8		130.2, qC	1.96 (s)	10.7, CH_3
8a		144.0, qC		
9	4.68 (s)	55.6, CH_2		
10	2.22 (s)	11.3, CH_3		
1′		194.0, qC		
2′	4.71 (q)	57.3, CH		
3′		206.2, qC		
4′	2.28 (s)	28.7, CH_3		
5′	1.23 (d, 7.1 Hz)	12.1, CH_3		

The proposed structure is fully supported by the observed MS/MS spectrum (negative mode) showing daughter ion fragments at *m*/*z* = 221, 203, and 175. These fragments result from the cleavage of the side-chain moiety (*m*/*z* = 221) followed by the elimination of H_2O (*m*/*z* = 203), and the loss of the carbonyl group of the carboxy moiety (*m*/*z* = 175). Epicoccalone has a chiral center at C-2′. However, this position is prone to enolization resulting in a racemate. While numerous coumarins are known, the substituted alkyl side chain at position 3 makes the structure of epicoccalone unique. The only coumarin sharing a similar feature is armillarisin A from *Armillariella tabescens*, clinically used as a choleretic to improve bile secretion and regulate the pressure of the bile duct to ease inflammation and adjust liver function.[25]

The structure of **1** is suggestive for a polyketide pathway leading to the unusual coumarin. Unfortunately though, ^{13}C isotope labeling studies were hampered by the low production of **1** (0.2 mg L^{-1}). However, we identified two co-metabolites (**2** and **3**) that could shed light on the biogenesis of **1**. Compounds **2** and **3** were obtained by preparative HPLC in 2.5 mg and 1.3 mg yield, respectively. All physical and spectroscopic data of **2** were identical to those reported for an *Aspergillus terreus* metabolite, 1,3-dihydro-4,5,6-trihydroxy-7-methylisobenzofuran that functions as a lipoxygenase inhibitor.[26] More recently, **2** has also been described as a metabolite of *Epicoccum purpurascens*.[27] Compound **3** appeared to be a related substance with a molecular formula of $C_9H_8O_5$ according to HR-EIMS and ^{13}C NMR data. The IR spectrum of the metabolite showed absorption bands for hydroxy groups at 3420 and 3134 cm^{-1}, for a carboxy group at 1725 cm^{-1}, and an aromatic ring system and 1628, 1515, and 1481 cm^{-1}. The ^1H NMR spectrum of **3** showed two signals attributable to three methyl [δ = 1.96 (s, 1 H) ppm] and two methylene protons [δ = 5.09 (s, 2 H) ppm]. As in the ^1H NMR spectrum of **2**, the absence of aromatic proton signals in the ^1H NMR spectrum of **3** suggested that the aromatic part of the metabolite was fully substituted. The ^{13}C NMR and DEPT 135 spectra of **3** showed the occurrence of two sp^3-carbon signals including one methyl carbon signal at δ = 10.7 ppm and one oxymethylene carbon signal at δ = 67.6 ppm, seven quaternary sp^2-carbon atoms including a carboxy carbon atom with signal at δ = 169.8 ppm. The protonated carbon atoms and their corresponding protons and the full connection of compound **3** were established by using HMQC and HMBC experiments, respectively. The HMBC data showed the correlations between the methyl protons 8-H_3 (δ = 1.96 ppm) and C-4 (δ = 109.1 ppm), C-4a (δ = 138.0 ppm) and C-5 (δ = 151.1 ppm). The correlation between the oxymethylene protons 3-H_2 [δ = 5.09 (s, 1 H) ppm] and C-1 (δ = 169.8 ppm), C-4 (δ = 109.1 ppm), C-4a (δ = 138.0 ppm), C-5 (δ = 151.1 ppm), C-7 (δ = 143.0 ppm) and C-7a (δ = 102.7 ppm) were also visible. Thus, the structure of **3** was elucidated as 5,6,7-trihydroxy-4-methyl-1(3*H*)-isobenzofuranone (epicoccone B). Notably, **3** is an isomer of epicoccone (**4**) from a marine-derived fungus of the same genus,[28] and was rediscovered in *Epicoccum purpurascens*.[27] The identity of the isomer was further confirmed by NOESY experiments, which showed a strong correlation between the methyl protons (8-H_3: δ = 1.96 ppm) and the oxymethylene protons (3-H_2: δ = 5.09 ppm) (Figure 2).

A remarkable fact is that both co-metabolites of **1** represent a part of its coumarin substructure. On the basis of the metabolite structures, the most plausible deduction is that

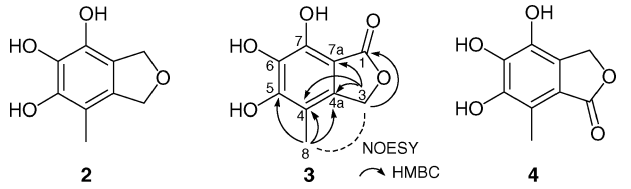
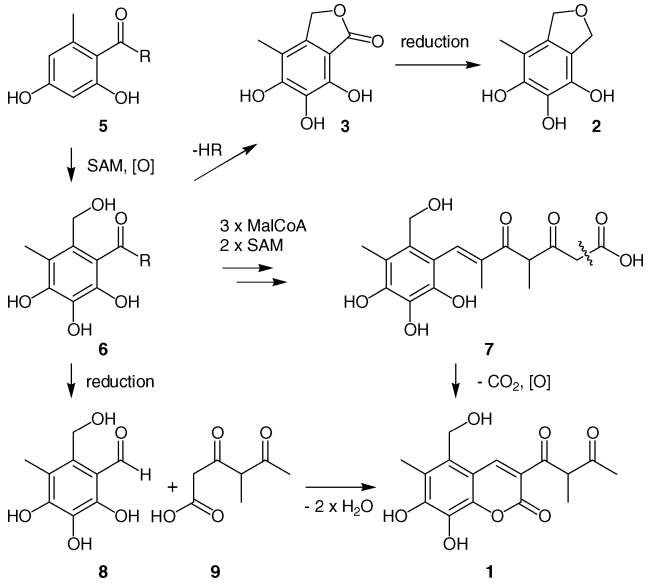


Figure 2. Structures of isobenzofuran-derived metabolites **2**–**4** from *Epicoccum* spp. with selected connectivities for **3**.

they derive from an orsellinic acid (**5**, Scheme 1) derived pathway involving methylation and oxidoreductive tailoring steps. In this context it is also worthwhile mentioning that **3** is reminiscent of mycophenolic acid, which results from aryl prenylation in lieu of hydroxylation.[29] For the biosynthesis of **1**, two general pathways are now conceivable. A common precursor of **1**–**3** could be **6** that might be further elongated by three repetitive Claisen condensations. Subsequent or concomitant methylation would yield **7**. The latter compound would then undergo decarboxylation, oxygenation and cyclization to yield the coumarin structure (Scheme 1). An alternative pathway would involve the condensation of aldehyde **8** with a triketide chain (**9**) in a Knoevenagel-type reaction. In fact, an aldehyde might be formed by reductive downloading from the polyketide synthase;[30,31] but considering the short lifetime of aldehydes and competing reactions, this scenario is slightly less likely.



Scheme 1. Model for the biogenetic relationship of **1**–**3** (R = OH or SEnz; SAM: *S*-adenosylmethionine).

Pure **1** was evaluated for its inhibitory activity against the serine protease α-chymotrypsin according to an established enzyme assay.[32] We found that **1** inhibits α-chymotrypsin at an IC_{50} value of 84 μM. The control of proteases by inhibition is an important approach in therapy.[33] Apart from medical implications, natural protease inhibitors play crucial roles in organismic interactions. Many microorganisms produce compounds that specifically inhibit various types of proteases[34–36] that may interfere with other organisms. Microviridins from bloom-forming cyanobacteria (e.g. *Microcystis* spp.), for example, affect the molting process of *Daphnia*.[37] Compared to various other chymotrypsin inhibitors that are active in the nanomolar range, **1** can be considered as moderately or weakly active. Nonetheless, it is well conceivable that a fungus growing within the tissue of a saprotrophic tree fungus benefits from excreting a protease inhibitor. One might imagine that such an inhibitor could facilitate colonizing the fungus or even affect the host tree, which cannot be experimentally tested at this stage. We have observed earlier that tree fungi produce anti-inflammatory agents that likely interfere with hypersensitive response of plants during infection.[18] Interestingly, **2** has been reported as a potent antioxidant, and the structures of **1**–**3** suggest that they are capable of scavenging radicals or neutralizing reactive oxygen species.[26] The metabolic potential of the fungus growing within the saprotrophic fungus might thus well support the lifestyle of its host.

Conclusions

We have discovered and characterized epicoccalone, a novel chymotrypsin inhibitor from a fungus associated with the tree fungus *Pholiota squarrosa*. Its structure was fully elucidated by 2D NMR spectroscopy, revealing a coumarin ring system with an unprecedented substitution pattern. The isolation and structural elucidation of two co-metabolites, the new compound **3** and the known compound **2**, lend support for a shared orsellinic acid like biosynthetic pathway. Epicoccalone is a new addition to the growing body of knowledge on diverse metabolomes of microorganisms associated with higher organisms – an emerging source for bioactive compounds.

Experimental Section

General Experimental Procedures: IR spectra (film) were recorded with a JASCO FT/IR-4100 Fourier-transform IR spectrometer. High-resolution electron impact mass spectra (EI-MS) were recorded with an AMD 402 double-focusing mass spectrometer with BE geometry (AMD, Intestra, Harpstedt, Germany). NMR spectra were recorded with a Bruker Avance 500 DRX spectrometer (Bruker, Karlsruhe, Germany) at 300 MHz for ^1H and 75 MHz for ^{13}C in $CDCl_3$, chemical shifts are given in ppm relative to TMS as internal standard. Open column chromatography was performed on silica gel (60, 0.063–0.2 μm; Merck) and sephadex LH-20. HPLC was performed by using a Gilson binary gradient HPLC system equipped with a UV detector (UV/Vis-151) monitoring at 300 nm. The preparative column was packed with nucleosil 100-7 C_{18}. TLC was carried out with silica gel 60 F_{254} plates. Spots were visualized by spraying with vanilline/H_2SO_4, followed by heating. All solvents used were spectral-grade or distilled prior to use.

Fermentation, Extraction and Isolation: The fungus was cultivated under the condition of surface fermentation at 25 °C in 500 mL Erlenmeyer flasks containing 100 mL of medium composed of malt extract (30 g L^{-1}), glucose (10 g L^{-1}), yeast extract (1 g L^{-1}) and $(NH_4)_2SO_4$ (5 g L^{-1}) at pH = 6.0. After cultivation at 25 °C for 28 d, the mycelium cake from the culture medium (30 L) was extracted twice with ethyl acetate and methanol (each 10 L). The cul-

ture broth was thoroughly extracted with ethyl acetate. The organic layers were combined and concentrated under reduced pressure. The resulting dark brown oil was washed with *n*-hexane to remove fatty acids, and the residue was dissolved in a small volume of methanol and subjected to column chromatography (Merck silica gel 60, 0.063–0.1 mm; column 4 × 60 cm), by using stepwise $CHCl_3$ and $CHCl_3$/MeOH (9:1, 1:1, v/v) as eluents. Isolation of active components was guided by bioassay (see below). Final purification was achieved by preparative HPLC using a Spherisorb ODS-2 RP_{18} column (250 × 25 mm, 5 μm, Promochem), and acetonitrile/H_2O (83:17, v/v) as eluent (flow rate 10 mL min^{-1}, UV detection at 350 nm) to yield **1** (6.2 mg), **2** (2.5 mg), and **3** (1.3 mg).

Epicoccalone (1): Yellow oil. HR-EIMS: *m/z* = 319.0814 $[M - H]^-$ (calcd. for $C_{16}H_{16}O_7$ 319.0818). MS/MS: *m/z* = 221, 203, 175. UV (MeOH): λ_{max} = 269, 390, 463 nm. IR (film): $\tilde{\nu}$ = 3244, 2925, 1699, 1598, 1562, 1465, 1268, 1197, 1107, 895 cm^{-1}. ^1H NMR ([D_6]DMSO, 300 MHz), ^{13}C NMR ([D_6]DMSO, 75 MHz): See Table 1.

Epicoccone B (3): Brownish oil. HR-EIMS: *m/z* = 196.0376 (calcd. for $C_9H_8O_5$ 196.0372). IR (film): $\tilde{\nu}$ = 3420, 3134, 1725, 1628, 1514, 1249, 1104, 879 cm^{-1}. ^1H NMR ([D_6]DMSO, 300 MHz), ^{13}C NMR ([D_6]DMSO, 75 MHz): See Table 1.

Chymotrypsin Inhibitory Assay: Chymotrypsin inhibitory activity was determined by the method of Cannell et al.[32] Each assay mixture containing 30 μL of 50 mM Tris-HCl buffer (pH = 7.6), 50 μL of chymotrypsin solution (150 U mL^{-1} in 50 mM Tris-HCl buffer adjusted to pH = 7.6) and 20 μL of the test solution were added to each cuvette and pre-incubated at 37 °C for 5 min. Each reaction was started with the addition of 100 μL of substrate solution [*N*-succinylphenylalanine-*p*-nitroanilide in Tris-HCl (pH = 7.6, 1 mg mL^{-1})]. The absorbance of the reaction mixture was immediately measured at 405 nm. The developed color was measured after incubation at 37 °C for 30 min. Compound **1** inhibits α-chymotrypsin at an IC_{50} value of 84 μM.

Supporting Information (see footnote on the first page of this article): ^1H, ^{13}C NMR and HMBC spectra of epicoccalone.

Acknowledgments

This project has been financially supported by the European Community (EUKETIDES). We thank H. Röhrig and M.-G. Schwinger for their assistance in natural product isolation and biological assays.

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Received: May 6, 2008
Published Online: June 24, 2008

