# Epicoccarines A, B and epipyridone: tetramic acids and pyridone alkaloids from an *Epicoccum* sp. associated with the tree fungus *Pholiota squarrosa*†

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Investigation of the metabolic profile of a fungus (*Epicoccum* sp.) isolated from the fruiting body of the tree fungus *Pholiota squarrosa* led to the discovery of two novel tetramic acid derivatives, epicoccarine A (**2**) and B (**3**), as well as a new pyridone alkaloid, epipyridone (**1**), with an unusually cyclized side chain. It appears that **1** is biogenetically derived from the ring expansion of **2** followed by a proposed hetero-Diels–Alder reaction. **2** shows selective antibacterial activity against Gram positive bacteria, in particular *Mycobacterium vaccae*.

## Introduction

Natural products containing the tetramic acid (2,4-pyrrolidinedione) ring system represent an important class of medicinally relevant compounds, most of which have been isolated from marine sponges or fungi.[1] Important examples are the aurantosides,[2,3] the militarinones,[4–6] erythroskyrin,[7] the melophlins,[8] α-lipomycin,[9] and epicoccamide.[10] The range of biological activities they exhibit includes antibiotic,[11,12] antiviral,[13] antifungal,[3,8,14,15] phyto-[16] and mycotoxic,[17] and neurotrophic activities.[4,5] Other members of this class inhibit aflatoxin biosynthesis.[18,19] Tetramic acids have raised interest due to their intriguing biosynthetic pathway, which involves the condensation of an amino acid with an activated polyketide moiety.[1] Schmidt and co-workers demonstrated that a rare fungal PKS–NRPS hybrid synthase is involved in the formation of the tetramic acid derivative equisetin.[20] Interestingly, the pyrrolinones play a key role also in the biosynthesis of pyridone alkaloids, such as tenellin[21–24] and antibiotic PF1140.[25] Only recently, two gene clusters have been identified in fungi that encode the biosynthesis of fungal pyridones (tenellin[26] and aspyridone[27]). In both cases, PKS–NRPS hybrid synthases of the equisetin type are involved, as well as putative oxygenases that catalyze the oxidative rearrangement of proposed pyrrolinone intermediates.[26,27]

In the course of our search for bioactive compounds produced by tree fungi,[28,29] we noted that a filamentous fungus resides within the fruiting body of the saprotrophic tree fungus *Pholiota squarrosa*. We have succeeded in cultivating the fungus, which belongs to the genus *Epicoccum* according to morphological characteristics. Here we present three new members of the tetramic acid–pyridone family of natural products produced by the endofungal *Epicoccum* sp.

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## Results and discussion

The mycelium harvested from a 60 l *Epicoccum* sp. culture was first extracted with ethyl acetate and methanol. The MeOH extract was treated with $H_2O$ to eliminate $H_2O$-soluble substances and re-extracted with ethyl acetate. The ethyl acetate-soluble portion of the re-extracted residue (30 g) was subjected to open column chromatography on silica gel. Further purification of selected fractions afforded three novel nitrogen-containing compounds, **1** (5 mg), **2** (6 mg), and **3** (16 mg), see Fig. 1.

Compound **1** was obtained as a red oil. Its molecular formula was determined as $C_{23}H_{29}NO_3$ by HR-EIMS (*m*/*z* 366.2065 [M − H]$^-$) and $^{13}$C NMR data. The $^1$H NMR spectrum of **1** indicated 27 non-exchangeable protons, including 5 olefinic protons and four methyl groups. Moreover, two signals attributable to an AA′BB′ spin coupling system of a *para* disubstituted phenyl moiety were observed. Analyses of the $^{13}$C NMR, DEPT 135 and HMQC spectra of **1** (Table S1) indicated the presence of four methyl carbons, three methylene carbons, nine methine carbons (five of which are sp$^2$ hybridized) and six quaternary carbons (all of which are sp$^2$ hybridized). A carboxyl amide carbon was also detected at $\delta$ 164.5. This observation was strongly supported by the IR spectrum, which showed a strong absorption band at 1638 cm$^{-1}$. $^1$H–$^1$H COSY helped identify the coupling systems H-7–H-8–H-9–H-10–H-11, H-8–H-18, H-10–H-17 and H-13–H-14–H15. By means of C, H long range coupled NMR spectra (HMBC) all connections of protons and carbons were fully assigned. The correlation of the equivalent protons H-2′ and H-6′ ($\delta$ 7.20, d, $J$ = 8.4 Hz) with C-5 ($\delta$ 116.0) helped connect the *para* disubstituted phenyl moiety at C-5. The pyridone ring was established due to the correlations observed between the olefinic proton H-6 ($\delta$ 7.11, s) and the carboxyl amide carbon C-2 ($\delta$ 164.5) and the oxygenated quaternary sp$^2$ carbon C-4 ($\delta$ 162.1). The methyl protons H-18 ($\delta$ 1.14, d, $J$ = 5.7 Hz) were correlated with C-7 ($\delta$ 49.1), C-8 ($\delta$ 27.8) and C-9 ($\delta$ 45.0), indicating that the methyl carbon C-18 ($\delta$ 24.7) is bound to C-8. The correlation of the methyl protons H-17 ($\delta$ 0.86, d, $J$ = 6.5 Hz) with the carbons C-9, C-10 ($\delta$ 26.0) and C-11 ($\delta$ 46.3) and that of the methyl protons H-16 ($\delta$ 0.68) with C-11, C-12 ($\delta$ 37.0) and C-13 ($\delta$ 93.3) were evidence of the connection of the methyl carbons

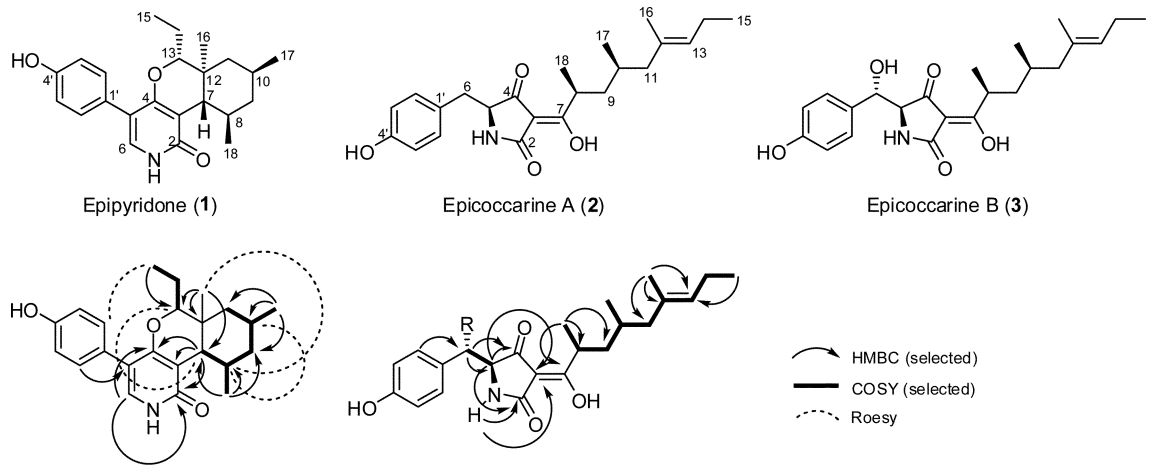
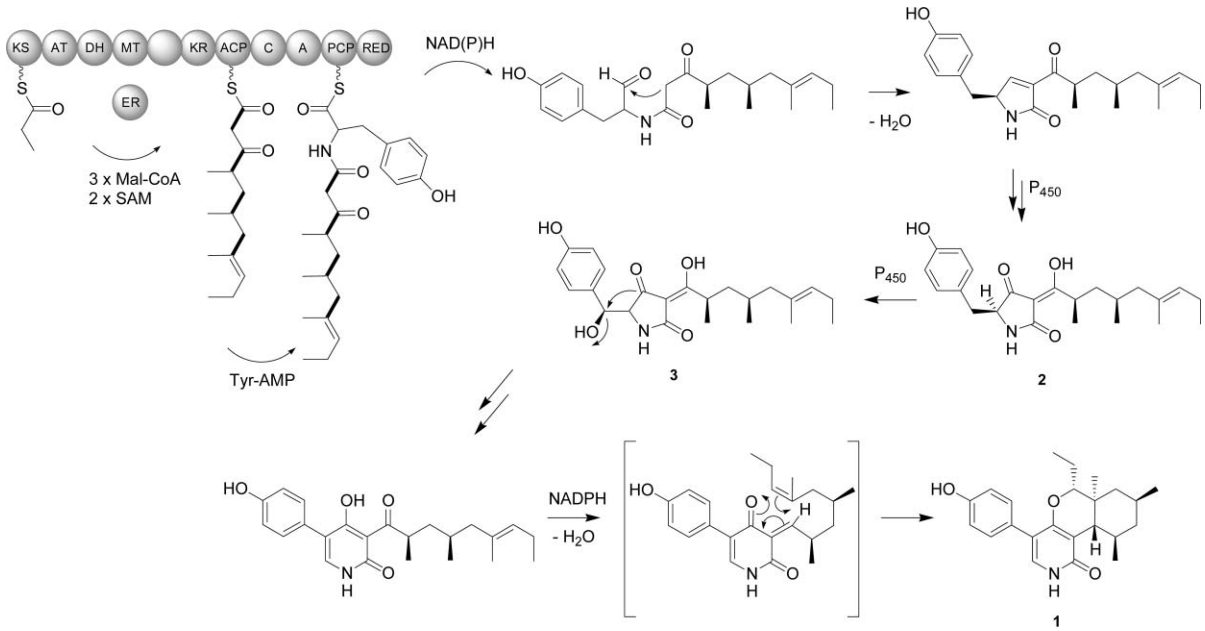



**Fig. 1** Structures of novel *Epicoccum* metabolites and key HMBC, COSY and ROESY correlations.

C-17 ($\delta$ 22.7) and C-16 ($\delta$ 15.4) at C-10 and C-12 respectively. As far as the ethyl group is concerned, its linkage to C-13 ($\delta$ 93.3) was suggested by the correlation of the methyl protons H-15 ($\delta$ 0.89) with C-13. According to all of these data, the structure was assigned as a novel pyridone derivative named epipyridone (**1**). **1** has five stereocenters. The relative stereochemistry of **1** was determined based on the ROESY spectrum, which showed correlations between the protons H-8 ($\delta$ 2.63), H-10 ($\delta$ 1.60) and H-16 ($\delta$ 0.68). A strong correlation between the protons H-7 ($\delta$ 1.98) and H-13 ($\delta$ 3.84) was also observed. Epipyridone shows structural similarities with fusaricide isolated from *Fusarium* sp.[30] and leporine A isolated from *Aspergillus leporus*.[31,32]

The molecular formula of compound **2** was established as $C_{23}H_{31}NO_4$ by HR-EIMS. The $^1$H NMR spectrum in $CDCl_3$ showed signals for one exchangeable and 28 non-exchangeable protons. Comparison of $^{13}$C NMR, DEPT, and HSQC spectra revealed the presence of 21 carbon resonances, which were attributable to four methyl groups, four methylene, six methine (three of which are sp$^2$ hybridized), and seven quaternary carbons, including two carbonyl carbons and one carboxyl amide carbon. A noticeable feature attributable to a *para* disubstituted phenyl moiety was the appearance of four sp$^2$ methine carbons as two chemically equivalent pairs. Evident in the $^{13}$C NMR spectrum of epicoccarine A (**2**) were four broad resonances at $\delta$ 193.9 (C-7, s), 193.7 (C-4, s), 175.3 (C-2, s), 100.4 (C-3, s) and 63.5 (C-5, d), which were best assigned to a tetramic acid moiety. From all of these observations it became obvious that **2** was composed of three distinct fragments, a *para* disubstituted phenyl moiety, an unsaturated aliphatic side chain and a tetramic acid moiety. The presence of a *p*-hydroxyphenyl ring was corroborated by COSY and HMBC correlations and by comparison with data for **1**. The structure of the aliphatic side chain was established by combined analysis of COSY, HSQC, and HMBC spectra. The coupling systems H-8–H-9–H-10–H-11 and H-13–H-14–H-15 were identified based on COSY data. The methyl protons H-16 ($\delta$ 1.49) correlated with C-11 ($\delta$ 47.9), C-12 ($\delta$ 132.6) and C-13 ($\delta$ 128.3), indicating that the methyl group $CH_3$-16 is bound to the quaternary sp$^2$ carbon C-12. The correlations of the methyl protons H-17 ($\delta$ 0.82, d, $J$ = 6.5 Hz), with C-9 ($\delta$ 40.4), C-10 ($\delta$ 28.9), and C-11 as well as those of H-18 ($\delta$ 1.15, d, $J$ = 6.8), with C-7, C-8 and C-9 were also observed. These helped elucidating the structure of the aliphatic side chain as 4,6,8-trimethyloctan-3-enyl, which was linked to C-7 with the aid of diagnostic HMBC correlations, in particular *via* three-bond connectivity between H-18 and C-7 as well as connectivity between H-8 and C-7. Further analyses of the HMBC spectrum [three-bond correlation of H-5 ($\delta$ 3.97, dd, $J$ = 3.6, 9.4 Hz) with C-1′ ($\delta$ 127.1) and correlation of H-6 ($\delta$ 2.64–3.18, m) with C-5 and C-1′] revealed that the *p*-hydroxyphenyl moiety is connected to C-5 through the methylene bridge. Thus, the structure of **2** was elucidated as 5-(4-hydroxybenzyl)-3-(1-hydroxy-2,4,6-trimethylocta-6-ene)-pyrrolidine-2,4-dione. This compound represents a novel tetramic derivative named epicoccarine A.

The NMR spectra of **3** indicated four methyl, three methylene, nine methine, and seven quaternary carbons. The analyses of 1 and 2-D NMR spectra of **3** revealed that major portions of the molecule are identical to that of epicoccarine A (**2**), such as the *p*-hydroxyphenyl moiety, the aliphatic side chain linked to C-7 ($\delta$ 193.9), and the tetramic acid moiety. A notable feature for **3** is the presence of a hydroxymethine group at C-6 ($\delta$ 74.1) bearing two chemically equivalent protons. This information was strongly supported by the COSY spectrum, which showed correlation between H-5 ($\delta$ 3.96) and H-6 ($\delta$ 4.74) as well as between H-6 and H-2′ ($\delta$ 7.19). Thus the structure of epicoccarine B (**3**) was elucidated as 5-[hydroxy(4-hydroxyphenyl)methyl]-3-(1-hydroxy-2,4,6-trimethylocta-6-ene)pyrrolidine-2,4-dione, obviously the 6-hydroxy derivative of **2**. It should be highlighted that epicoccarines A (**2**) and B (**3**) feature an unprecedented side chain not previously encountered in microbial metabolites. These structures were also supported by MS–MS data, which showed the ion fragments $m/z$ = 278, 194, and 99. These fragments correspond to the cleavage of the *p*-hydroxybenzyl (epicoccarine A) or the hydroxy-*p*-hydroxybenzyl (epicoccarine B) moiety followed by the α-cleavage of the tetramic acid moiety and the degradation of the aliphatic side chain (Fig. S1). Moreover, epicoccarines have three to four stereocenters in two distant portions of the molecule. The *trans* configuration of the protons H-5 and H-6 in **3** was established based on the diagnostic coupling constant ($J^{\text{H-5,H-6}}$ = 7.5 Hz). The absolute configuration of the amino acid moiety (tyrosine) of the tetramic acid derivatives could not yet be elucidated due to the




**Scheme 1** Model for the biosynthesis of the tetramic acids, epicoccarines A and B (**2**, **3**), according to ref. 20,26,27 and formation of epipyridone (**1**) involving a hetero-Diels–Alder reaction.

limited quantity of material. As far as the relative configurations at C-8 and C-10 are concerned, we concluded that they were the same as that of epipyridone A at C-8 and C-10 respectively, since the three compounds likely share the same biosynthetic pathway (Scheme 1).

The cooccurrences of compounds **1–3** in the same organism strongly suggest a biogenetic relationship. According to the molecular studies on the equisetin,[20] tenellin,[26] and aspyridone[26] pathways, the biosynthesis of tetramic acid derivatives and derived natural products involves the condensation of amino acids and activated polyketide moieties catalyzed by rare polyketide synthase–non-ribosomal peptide synthetase (PKS–NRPS) hybrids. According to the currently accepted model reductive cleavage of the thioester with subsequent ring closure and oxygenation gives rise to the corresponding pyrrolidinones, *e.g.* **2**. The tetramic derivative undergoes a rearrangement, leading to pyridone-like alkaloids like **1**. This step most likely involves a hydroxy intermediate, such as **3**. The side chain of **1** is particularly intriguing. Since **2** and **3** exhibit the same carbon backbone, it is likely that the formation of **1** takes place by an intramolecular cyclisation in a hetero-Diels–Alder fashion (Scheme 1). This proposed model could be generally applicable to the biosynthesis of similar compounds, such as PF1140,[33] fusariside[30] and leporin[31] from *Eupenicillium*, *Fusarium*, and *Aspergillus* sp., respectively, and likely represents a new type of Diels–Alder reaction in nature.[34,35]

As mentioned above, the polyketide–amino acid hybrid metabolites of the tetramic acid and pyridone families of secondary metabolites exhibit a wide spectrum of biological activities. It is striking that most of the compounds are produced by pathogenic organisms or in symbioses. Interestingly, a tetramic acid derivative has been isolated by König and coworkers from an *Epicoccum* sp. isolated from jellyfish,[10] while the *Epicoccum* isolate investigated in the present study originates from a tree fungus. In a panel of biological assays we found that epipyridone and epicoccarine B had only moderate activity against Gram positive bacteria, but epicoccarine A exhibited a very selective activity against *Mycobacterium vaccae* with a MIC value of 6.25 μg ml$^{-1}$ (Table S2).

## Conclusions

In summary, we have isolated a filamentous fungus of the genus *Epicoccum* from the tree fungus *Pholiota squarrosa* and succeeded in the isolation and characterization of a new α-pyridone alkaloid, epipyridone (**1**), and two new tetramic acid derivatives, epicoccarine A (**2**) and B (**3**). The three metabolites obviously represent polyketide–amino acid hybrids, which fit well into the biosynthetic model for pyridone formation. While all side chains are new, the structure of the tricyclic pyridone is most intriguing from a mechanistic point of view. A model for its formation is suggested, which may involve a hetero-Diels–Alder reaction. While **1** and **3** exhibited only weak to moderate activity against Gram positive bacteria, **2** proved to be a potent antibacterial agent with a high selectivity to *Mycobacterium vaccae*. However, it is very likely that this activity does not reflect the original function of these metabolites in the unusual fungal association.

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