Phytochemistry 117 (2015) 10–16

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Entonalactams A–C: Isoindolinone derivatives from an Australian rainforest fungus belonging to the genus *Entonaema*

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ARTICLE INFO

Article history:
Received 19 February 2015
Received in revised form 25 May 2015
Accepted 26 May 2015

Keywords:
Entonaema
Isoindolinone
Lactam
Entonalactam
Rainforest
Fungi
Natural product
Malaria

ABSTRACT

Bioassay-guided fractionation of an antimalarial DCM/MeOH extract derived from the Australian rainforest fungus *Entonaema* sp. resulted in the isolation of three new isoindolinone derivatives, entonalactams A–C (**1**–**3**), along with the known natural products 3-methoxy-5-methylbenzene-1,2-diol (**4**), daldinal B (**5**), and ergosta-4,6,8(14),22-tetraen-3-one (**6**). The chemical structures of the new secondary metabolites were determined following extensive 1D/2D NMR and MS data analysis. A single crystal X-ray structure for entonalactam A (**1**) confirmed the NMR-based structure assignment. Entonalactams A–C (**1**–**3**) were all determined to be racemic based on chiro-optical data. All secondary metabolites were tested *in vitro* against *Plasmodium falciparum* malaria parasites, and ergosta-4,6,8(14),22-tetraen-3-one (**6**) was identified as the most active compound with 66% inhibition at 50 μM.

1. Introduction

Entonaema is a genus of ascomycetous fungi belonging to the family Xylariaceae, which is considered one of the most diverse fungal families (Stadler et al., 2013; Tang et al., 2009). A total of at least 75 genera and more than 1300 species are described worldwide for this particular family (Stadler et al., 2013; Tang et al., 2009). It has been reported that Xylariaceous species are typically saprobes, but they are also commonly isolated as endophytes and some species are pathogenic (Kodsueb et al., 2008; Pinruan et al., 2007; Sánchez Márquez et al., 2008). The genus *Entonaema* has characteristic hollow, gelatinous stromata that accumulate liquid and are morphologically similar to *Sarcoxylon* spp. (Stadler et al., 2008; Rogers, 1981). *Entonaema*, together with *Phylacia*, *Pulveria*, *Rhopalostroma*, *Sarcoxylon*, and *Thamnomyces* are distinguished from other genera within Xylariaceae due to their atypical stromatal anatomy, morphology, and/or the development of ascal structures (Stadler et al., 2004).

Chemical studies of Xylariaceous fungi have shown that this family is a rich source of structurally novel and pharmacologically active secondary metabolites (Dictionary of Natural Products, 2014; Davis, 2005; Davis et al., 2005; Healy et al., 2004). Examples include the cytotoxic metabolite 13-hydroxy-3-,7(11)-eudesmadien-12,8-olide (Pittayakhajonwut et al., 2005), the HIV-1 integrase inhibitor integric acid (Singh et al., 1999) and xylarinic acids A and B, which are potent antifungal natural products (Jang et al., 2007). The chemotaxonomic alignment of *Entonaema* to the mainstream and/or atypical members of Xylariaceae is not well understood due to the very limited number of documented species and sporadic chemotaxonomic studies (Stadler et al., 2004). Although their distribution appears to be widespread, to date, only ten species of *Entonaema* have been described in the world (Mycobank, 2014) and with the aid of chemotaxonomy, taxonomic revisions of this genus are ongoing (Stadler et al., 2004, 2008).

The *Entonaema* species associated with this study was chosen on the basis of the moderate antimalarial activity that was identified during the screening of fractions derived from this fungus, in conjunction with the analytical HPLC and MS data profiling of the DCM/MeOH extract. The details of the antimalarial screening of the pre-fractionated fungal-derived library have been reported previously (Choomuenwai et al., 2012, 2013).

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http://dx.doi.org/10.1016/j.phytochem.2015.05.018
0031-9422/

This paper describes the bioassay-guided fractionation and structure elucidation of three new isoindolinones, which we have named entonalactams A–C (**1**–**3**), along with the known natural products 3-methoxy-5-methylbenzene-1,2-diol (**4**), daldinal B (**5**), and ergosta-4,6,8(14),22-tetraen-3-one (**6**) (Fig. 1) from the rainforest fungus *Entonaema* sp. Furthermore, the *in vitro* antimalarial evaluation of all compounds towards a chloroquine-sensitive line of *Plasmodium falciparum* (3D7) is reported.

2. Results and discussion

The air-dried and ground sample of *Entonaema* sp. was exhaustively extracted with DCM followed by MeOH. Both extracts were combined to afford a dark brown extract, and a portion of this was subjected to bioassay-guided fractionation using semi-preparative C_{18} HPLC and a MeOH-H_2O-0.1% TFA gradient and the previously described *in vitro* antimalarial assay (Andrews et al., 2000). This process readily identified antimalarial HPLC-generated fractions which, following lyophilisation and NMR/MS data analysis, was shown to contain three new isoindolinone derivatives, entonalactam A (**1**, 8.0 mg, 0.34% dry wt), entonalactam B (**2**, 3.0 mg, 0.13% dry wt), entonalactam C (**3**, 1.0 mg, 0.04% dry wt), together with three known fungal metabolites that included 3-methoxy-5-methylbenzene-1,2-diol (**4**, 2.0 mg, 0.08% dry wt), daldinal B[1] (**5**, 1.0 mg, 0.04% dry wt), and ergosta-4,6,8(14),22-tetraen-3-one (**6**, 1.6 mg, 0.07% dry wt; Fig. 1).

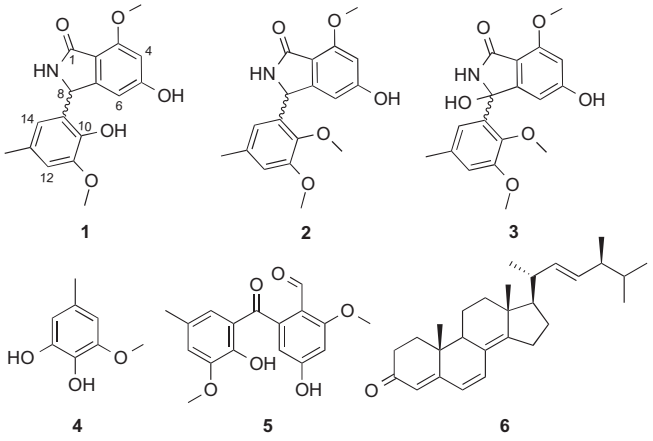

Fig. 1. The chemical structures of the secondary metabolites isolated from *Entonaema* sp.; entonalactam A (**1**), entonalactam B (**2**), entonalactam C (**3**), 3-methoxy-5-methylbenzene-1,2-diol (**4**), daldinal B (**5**), ergosta-4,6,8(14),22-tetraen-3-one (**6**).

Entonalactam A (**1**) was isolated as colourless crystalline needles, which showed pseudomolecular ions at *m*/*z* 316 $[M+H]^+$ and 314 $[M-H]^-$ in the LRESIMS that indicated the presence of an odd number of nitrogen atoms. The molecular formula for **1** was established as $C_{17}H_{17}NO_5$ (10 degrees of unsaturation) based on HRESIMS (*m*/*z* 338.1001 $[M+Na]^+$) and 1D NMR data (Table 1). The ^1H and HSQC NMR spectrum of **1** showed the presence of one *C*-methyl group (δ_H 2.12); two methoxy groups (δ_H 3.76, 3.80); five methine protons (δ_H 5.78, 6.29, 6.30, 6.32, 6.68), and three exchangeable protons (δ_H 8.08, 8.73, 9.97). The ^{13}C NMR spectrum showed two downfield signals at δ_C 168.9 and 162.4, one *C*-methyl group resonating at δ_C 20.7; one heteroatom substituted carbon at δ_C 52.7; two methoxy signals (δ_C 55.2 and 55.8), and another eleven aromatic carbons (δ_C 98.5, 101.9, 109.8, 111.5, 117.6, 126.6, 127.7, 141.4, 147.5, 153.4 and 157.8). Furthermore, the presence of phenol group(s) was supported by the UV spectrum of **1**, which underwent a bathochromic shift on addition of base. These data accounted for all ^1H and ^{13}C NMR resonances and suggested the presence of at least two highly-substituted aromatic ring systems. One aryl moiety was elucidated as a 2-hydroxy-3-methoxy-5-methylphenyl system based on COSY and HMBC data analysis (Fig. 2). Analysis of the ^1H–^1H coupling constants and COSY data for the methine doublets at δ_H 6.68 (J = 1.8 Hz) and 6.29 (J = 1.8 Hz), indicated a *meta* orientation for these two protons. Strong HMBC correlations from the *C*-methyl resonating at δ_H 2.12 to both aromatic methine carbons (δ_C 111.5 and 117.6) to which the *meta*-coupled protons were directly attached, enabled the methyl to be positioned between these two protons. HMBC correlations from the methoxy at δ_H 3.80 to the downfield carbon at δ_C 147.5, and the phenolic at δ_H 8.73 to the carbons at δ_C 126.6, 141.4 and 147.5 established that these oxygenated moieties were *ortho* to each other. A strong ROESY correlation between the methoxy at δ_H 3.80 to the aromatic methine at δ_H 6.29 completed the assignment of the 2-hydroxy-3-methoxy-5-methylphenyl system. In a similar manner, the remaining upfield aromatic methine protons resonating at δ_H 6.32 and 6.30 were positioned in a *meta* orientation following interpretation of the HSQC and HMBC data. An hydroxyl group was positioned between these two protons on account of strong HMBC correlations from the phenolic proton at δ_H 9.97 to the methine carbons at δ_C 98.5 and 101.9, along with the oxygenated aryl carbons at δ_C 162.4 and 157.8. The remaining methoxy signal of **1** resonated at δ_H 3.76 and was positioned *ortho* to the aromatic methine proton at δ_H 6.30 on account of a strong HMBC correlation to the downfield carbon at δ_C 157.8 and a shared ROESY cross peak between these protons, thus a 1,2-disubstituted-3-methoxy-5-hydroxy benzenoid system was elucidated. The remaining unassigned atoms of **1** consisted of a C_2H_2N unit. COSY data analysis established the presence of a –NH-CH– moiety, while the final unassigned carbon at δ_C 168.9 was indicative of a carbonyl group. With all atoms and nine of the 10 degrees of unsaturation accounted for in **1**, the remaining partial structure for **1** had to incorporate a third ring, and the only possibility was a five-membered lactam system. The linkage of the lactam to both of the elucidated aryl rings was accomplished via HMBC data analysis. Notably, the shared three-bond correlation from the NH proton at δ_H 8.08 and the methine at δ_H 6.30 to the carbon at δ_C 109.8, and the strong $^3J_{CH}$ correlations from the methine proton at δ_H 5.78 to the phenyl system carbons at δ_C 141.4 and 117.6. Thus the planar chemical structure of **1** was assigned as 5-hydroxy-3-(2-hydroxy-3-methoxy-5-methylphenyl)-7-methoxyisoindolin-1-one to which the trivial name, entonalactam A, has been assigned.

The structure of entonalactam A (**1**) was unequivocally determined by single crystal X-ray crystallographic analysis, and a perspective ORTEP of **1** is shown in Fig. 3. The X-ray data indicated that entonalactam A (**1**) was purified as a racemic mixture of 8*S* and 8*R* enantiomers, which was further supported by specific rotation and CD data. Of note, other structurally related isoindolinone alkaloids have also been isolated as racemic mixtures, such as pestalachloride A (Li et al., 2008).

Entonalactam B (**2**) was isolated as a white stable solid. The molecular formula $C_{18}H_{19}NO_5$ was determined by interpretation of the $[M+Na]^+$ ion at *m*/*z* 352.1152 in the (+)-HRESIMS. Comparison of the ^1H and ^{13}C NMR data for compounds **1** and **2** clearly identified that **2** was a mono-methyl ether analogue of **1**. The additional methoxy group in **2** (δ_H 3.76/δ_C 60.6) was positioned

[1] The chemical structures of daldinals A and B have been incorrectly drawn in a number of publications. Both SciFinder and the Dictionary of Natural Products database list the IUPAC names for daldinal A (CAS 160889-21-4) and daldinal B (CAS 160889-33-8) as 2-(2,3-dimethoxy-5-methylbenzoyl)-4-hydroxy-6-methoxybenzaldehyde and 4-hydroxy-2-(2-hydroxy-3-methoxy-5-methylbenzoyl)-6-methoxybenzaldehyde, respectively.