Isolation and purification of ent-pimara-8(14),15-diene from engineered Aspergillus nidulans by accelerated solvent extraction combined with HPLC†

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We recently engineered Aspergillus nidulans to produce a pimarane-type diterpene, ent-pimara-8(14),15-diene. Here, we describe methods for its isolation and purification from the engineered A. nidulans production strain and extend these findings with structural confirmation for the diterpene from this fungal source. The extraction protocol was optimized using accelerated solvent extraction (ASE) with three varying parameters: solvent composition, pressure, and temperature. This ASE method using 100% ethyl acetate at 90 °C with 12.07 MPa was more efficient and faster compared to ultrasonic-assisted extraction. Using both analytical and preparative C18 columns at isocratic elution of acetonitrile developed an HPLC separation; and, the diterpene was well separated within 28 min and 35 min, respectively. Also, TLC of the fungal culture extracts was performed with visualization using rhodamine. The reproduction and efficiency of the purification methods were evaluated by GC-MS; and, the structure of the compound was verified by NMR. The method enabled the isolation of this very lipophilic diterpene in pure form, allowing its testing in a number of bioactivity assays. Notably, we demonstrate for the first time the antioxidant activity for ent-pimara-8(14),15-diene using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

Introduction

Aspergillus nidulans is a filamentous fungus widely used as a model organism for studies of cell biology and gene regulation. It is a close relative to other Aspergillus species with industrial and medical significance – e.g., A. niger, A. oryzae, A. flavus, and A. fumigatus. The genus Aspergillus produces a variety of natural products, and offers a model system to study secondary metabolism in eukaryotes.1 Secondary metabolites are microbial organic compounds that are not crucial for the survival of the organism but many times exhibit interesting or useful bioactivities. These are also referred to as natural products. Major groups of secondary metabolites include polyketides, ribosomal and non-ribosomal peptides, and terpenoids. In fungi, the biosynthetic genes of secondary metabolism are organized into clusters.2 The clustering of the genes along with the sequenced genomes of many Aspergillus species has accelerated the discovery of novel natural products.3 Of the numerous secondary metabolites produced by Aspergilli, the most notorious ones include mycotoxins, such as A. flavus aflatoxin4,5 and A. carbonarius ochratoxin.6 On the other hand, many of the Aspergillus natural products also exhibit beneficial properties and include the antibiotic penicillin7 and the cholesterol reducing agent lovastatin.8 Although the functional role of secondary metabolites in the producing organism is often unclear, it has been speculated that secondary metabolites function as chemical signals in communication and defense to enhance the survival of the organism within its ecological environment.9,10

Terpenoids are particularly interesting secondary metabolites because of their many pharmaceutical and toxic bioactivities.11-13 Specifically, oxygenated derivatives of pimaradiene and kaurene have vasorelaxant, anti-inflammatory, anti-microbial and trypanocidal properties.14-19 Biosynthesis of fungal natural products is regulated by environmental as well as genetic factors.8 Recently, we reported that overexpression of a Zn(n)Cys6-type transcription factor in A. nidulans, called pimaradiene biosynthetic cluster regulator (pbrC), led to production of a diterpene hydrocarbon, ent-pimara-8(14),15-diene.20 ent-Pimara-8(14),15-diene has previously been identified as a product of the rice (Oryza sativa subspecies japonica) pimaradiene synthase, OsKSL5.21 Many of the diterpene hydrocarbons in rice, for example, ent-sandaracopimaradiene, ent-cassa-12,15-diene, 9βH-pimara-7,15-diene, and stemar-13-ene groups function as pathway intermediates for diterpene...
Analytical Methods

**Phytoalexins**, which have been suggested to act as antibiotics. Also, ent-kaur-16-ene is a precursor of a diterpene phytosterol content. giibberellin that regulates many aspects of development in higher plants. However, the biological activity for ent-pimara-8(14),15-diene in rice is still unknown, since OsKSL5 is not involved in the biosynthesis of diterpene phytoalexins or gibberellins. In addition to higher plants, diterpene hydrocarbons have also been found in fungi. For example, sandaracopimaraadiene, isopimaradiene, and pimaraadiene have previously been identified among other terpene hydrocarbons in crude extracts of the fungus *Phomopsis amygdali*. Also, many pimarane-type diterpene hydrocarbons are found in the fungus *Phoma betae*, where 8β-pimaradiene-9(11),15-diene has been proposed to be an intermediate in a biosynthetic pathway of aphidicolin, a specific inhibitor of DNA polymerase α. The identification of different diterpene hydrocarbons is many times based on the analysis of terpene synthase products of *in vitro* enzyme assays and, the few reports on the identification of fungal diterpenes do not report bioactivity assay data for these compounds. Several techniques are used for isolation of oxygenated terpenoids from crude plant and fungal extracts, such as silica gel chromatography, reverse phase chromatography, vacuum liquid chromatography, flash chromatography, preparative thin-layer chromatography, capillary electrophoresis, and recrystallization with methanol. These methods are most suitable for terpenoids with functional groups, which add hydrophilic properties to the compound and provide UV-absorbing groups. ent-Pimara-8(14),15-diene is a very lipophilic compound, and it does not have any functional groups or bonds that have UV absorbance. The compound is not detectable directly by UV fluorescence. In this report we describe the methods for isolation and purification of ent-pimara-8(14),15-diene from *A. nidulans* by using ASE combined with reverse phase HPLC. Compared to ultrasonic extraction, ASE was more efficient and was further optimized for *A. nidulans* mycelia. GC-MS was used to analyze the efficiency of the extraction methods and the HPLC separation. We also describe a TLC separation and rhodamine staining detection method, as well as structural confirmation for *A. nidulans* ent-pimara-8(14),15-diene by NMR.

The purification methods enabled us to study the activity of pure ent-pimara-8(14),15-diene in a number of bioactivity assays. In DPPH radical scavenging assays, purified ent-pimara-8(14),15-diene had higher activity than the known tetraterpene antioxidant, beta-carotene, demonstrating for the first time the antioxidant activity of ent-pimara-8(14),15-diene. With our production strain and purification method larger scale production of ent-pimara-8(14),15-diene is possible.

**Experimental**

**Aspergillus nidulans** strains and culture media

*A. nidulans* FGSC A4 (Glasgow wild type, veA+) was obtained from the Fungal Genetics Stock Center (Kansas City, Missouri, USA) and used as a wild type control strain. ent-Pimara-8(14),15-diene producing strain, oe-PbcR, has been described earlier. Briefly, the genome sequence of the Zn(II)Cys6-type transcription factor PbcR (AN1599.4, GenBank:BN001307) was cloned into a fungal expression vector pKB1, where the expression of pbcR is under the *A. nidulans* gpdA promoter. 20 mg of linearized expression plasmid DNA was transformed into *A. nidulans* FGSC A4 by protoplasting, and the positive colonies were selected on minimal medium agar plates with 200 mg mL⁻¹ of glufosinate ammonium. Strains were grown in liquid YES-media (2% yeast extract, 4% sucrose) supplemented with 3% gelatin at 30 °C with 250 rpm.

**Extraction**

Extraction conditions of the sonication method have been described earlier. Filtered and freeze-dried *A. nidulans* mycelia were homogenized with a Retsch MM301 Ball Mill at 29 Hz for 30–60 s. The homogenized powder was mixed with diatomaceous earth in ASE sample cells (Dionex) and extracted with ASE 200 (Dionex) using hexane:ethyl acetate (Hex: EtOAc) (1:1), EtOAc, or acetonitrile (ACN). Extraction conditions were as follows: pressure, 12.07 MPa or 15.51 MPa; temperature, 70 °C, 80 °C, or 90 °C; preheat time, 1 min; heat time, 5 min; static time, 10 min; flush volume, 30%; and purge time, 90 seconds. When assessing the efficiency of the sonication and ASE methods, one batch of mycelia was extracted three times with EtOAc.

**Thin layer chromatography**

An aliquot (200 μL) of the Hex: EtOAc (1:1) crude extract of FGSC A4 (wild type) and oe-PbcR (pimaradiene producing strain) was applied to silica plates (Silica Gel 60, Pre-coated TLC Plates, Merck, Germany) and purified by thin-layer chromatography. The separation was done with EtOAc : heptane : acetic acid (9:90:1), and the different compound groups were visualized under UV light after spraying the silica plates with 0.01% rhodamine 6B (Merck, Germany). The areas with different compounds were marked with a pencil, and silica was scraped and extracted with Hex: EtOAc (1:1). The purity of obtained fractions was analyzed by GC-MS.

**Gas chromatography-mass spectrometry**

GC-MS analysis has been previously described by Bromann et al. (2012). The separations were performed using an Agilent 6890 Series GC combined with a 5973 Network MS detector (Agilent, USA) and a Compibay injector (Varian Inc., USA). Samples were dissolved in EtOAc, injected in split mode (10:1) and separated on a 25 m × 0.2 mm × 0.33 μm HP-1 capillary column (Agilent, USA) with a helium flow rate of 1.3 mL min⁻¹. The ion source temperature was 230 °C, the interface was 280 °C, and the oven temperature was equilibrated at 100 °C for 30 seconds and then increased to 320 °C at a rate of 10 °C min⁻¹ and held at that temperature for 25 minutes. The MS detector was operated in EI mode at 70 eV with a mass range of 40–550. Compounds were identified with the Palisade Complete 600K Mass spectral library (Palisade Mass Spectrometry, USA).

**High-performance liquid chromatography**

*A. nidulans* oe-PbcR cell extracts were evaporated to dryness and resuspended in HPLC grade ACN. Analytical HPLC was...
performed using an XTerra MS C18 column (250 × 4.6 mm; 3.5 μm; Waters) with a Waters 616 pump with a Waters 600 S system controller equipped with a Waters in-line degasser AF, a Waters 717plus autosampler, a Waters 2996 photodiode array detector (200–600 nm), and Waters Empower Pro chromatography software for data processing (Milford, MA). Samples were eluted with a flow rate of 0.5 mL min⁻¹. The injected volume of the crude extract per run was 150 μL and the total analysis time was set to 60 min. Preparative scale purification was done using a Waters XTerra Prep MS C18 column (300 × 10 mm; 10 μm; Waters) with a 2 mL sample loop and a 2.5 mL syringe (Waters). The injected volume was 2 mL of the crude extract and the flow rate was set to 2.5 mL min⁻¹. The total analysis time was set to 60 min. The elution of the different fractions was monitored at 225 nm, and the collected fractions were evaporated to dryness under N₂ flow at 30 °C. Fractions were dissolved in a small volume of EtOAc and the purity was analyzed by GC-MS.

### Results and discussion

Previously, we reported the identification of the transcription factor, PbcR, in *A. nidulans*. In the strain overexpressing *pbcR* (oe:*PbcR*), transcription of seven genes within a cryptic secondary metabolite gene cluster is activated; and, this results in the production of a pimarane-type diterpene that we identified as *ent*-pimar-8(14),15-diene.²⁰

#### Comparison of extraction methods

In the current study, oe:*PbcR* mycelia were collected at the exponential growth phase and then freeze-dried and homogenized. To compare the previously reported sonication extraction with ASE, we performed three serial extractions of the same batch of mycelia using two methods. In GC-MS analysis of three serial sonication extractions of manually ground mycelia with Hex:EtOAc (1 : 1), 48% of *ent*-pimar-8(14),15-diene was recovered in the first extraction, 19% in the second extraction, and 33% in the third extraction (Fig. 1A). In contrast, ASE efficiently recovered 100% of *ent*-pimar-8(14),15-diene using one extraction (Fig. 1B). Thus, automated homogenization of freeze-dried mycelia and subsequent ASE proved to be superior for the recovery of *ent*-pimar-8(14),15-diene. An additional benefit in

#### DPPH radical scavenging assay

DPPH radical scavenging assay was performed according to Kähkönen et al. with slight modifications. Methanolic 0.1 mM DPPH radical solution was mixed with *ent*-pimar-8(14),15-diene or beta-carotene solution. The concentrations of pure compounds were either 1 mM, 2.5 mM or 5 mM. The absorption at 517 nm was monitored up to 120 min using a UV-1800 UV-Vis spectrophotometer (Shimadzu). The results were calculated as the percentage of radicals scavenged at four different time-points: 20, 60, 90, and 120 min.

#### IR, NMR, HRMS experimental

The IR spectrum was obtained using a Vertex 70 (Bruker Optics Inc., MA, USA) FTIR instrument. The FTIR measurements were made directly in solids with a horizontal Attenuated Total Reflectance (ATR) accessory (MIRacle, Pike Technology, Inc, WI, USA). The transmittance spectrum was recorded at a 4 cm⁻¹ resolution between 4000 and 600 cm⁻¹ using the OPUS 5.5 software (Bruker Optics Inc., MA, USA). ESI-MS was performed by direct injection using a Synapt G2 HDMS (Waters, MA, USA) instrument. NMR spectra were obtained using a Varian Mercury Plus 300 spectrometer, in CDCl₃ with tetramethylsilane (TMS) as the internal standard. The chemical shifts were reported in parts per million (ppm) and on the δ scale from TMS as an internal standard. The coupling constants *J* are quoted in hertz (Hz).

*ent*-Pimar-8(14),15-diene (1). IR (ATR) 3085, 2925, 2895, 1456, 1365, 912, 856 cm⁻¹. ¹H-NMR (300 MHz, CDCl₃) δ 0.80 (s, 3H, 20-CH₃), 0.85 (s, 3H), 0.88 (s, 3H), 1.01 (m, 1H, 5-H); 1.04 (s, 3H, 17-CH₃), 1.70 (m, 2H), 2.05 (m, 1H, 7β-H), 2.26 (m, 1H, 7α-H), 4.88 (dd, 1H, J₁=1.5 Hz and J₂=10.6 Hz, 16-Ha), 4.91 (dd, 1H, J₁=1.5 Hz and J₂=17.5 Hz, 16-Ha), 5.21 (s, 1H, 14-H), 5.78 (dd, 1H, J₁=10.6 Hz and J₂=17.5 Hz, 15-H). ¹³C-NMR (75 MHz, CDCl₃) δ 14.9 (C20), 18.7, 19, 22 (C19), 22.5, 25.9 (C17), 33.2 (C4), 33.7 (C18), 34.6, 36 (C7), 37.3 (C13), 38.2 (C10), 39.3, 42.1, 50.6 (C9), 54.8 (C5), 109.8 (C16), 128.4 (C14), 137.2 (C8), 149.1 (C15). HMRS calc. for C₂₀H₃₂ 273.2582 [M + 1][⁺], found 273.2578.

Fig. 1 Comparison of relative yields of *ent*-pimar-8(14),15-diene from two different extraction techniques. The sonication extraction (A) recovers only 48.5% of *ent*-pimar-8(14),15-diene in the first extraction, whereas ASE (B) efficiently recovers 100% of the *ent*-pimar-8(14),15-diene from *A. nidulans* mycelia in the first extraction.
using ASE is that weighing of the freeze-dried sample also makes it possible to quantitate the extraction yield and method efficiency.

The ASE method was optimized for the powdered sample by using different solvent compositions, temperatures, and pressures. The recovery of ent-pimara-8(14),15-diene was monitored by analyzing GC-MS peak areas. We tested three different solvents: Hex : EtOAc (1 : 1), 100% EtOAc, and ACN. The highest relative recovery was marked as 100% extraction efficiency, and other peak areas were calculated as a percentage of this value. The recovery of ent-pimara-8(14),15-diene from the fungal mycelia was highest by using 100% EtOAc (Fig. 2A). Hex : EtOAc was nearly as efficient (92%) in extracting ent-pimara-8(14),15-diene as EtOAc, whereas ACN could extract only 52% of the ent-pimara-8(14),15-diene compared to EtOAc (Fig. 2A).

The extraction method was further optimized with EtOAc by testing at two different pressures: 12.07 MPa and 15.51 MPa. Recovery of ent-pimara-8(14),15-diene was efficient at 12.07 MPa, and increasing pressure slightly lowered the yield (data not shown), suggesting that pressure did not exert a great influence on extraction efficiency. Extraction with EtOAc at three different temperatures (70 °C, 80 °C, and 90 °C), with 12.07 MPa pressure, demonstrated that the highest recovery was achieved at 90 °C (Fig. 2B). In a previous study, Kawamura et al. reported that increasing temperature resulted in higher recovery of paclitaxel from bark, although pressure was less important for recovery. In their study, the temperature range used for paclitaxel was 100–150 °C, so it is possible that we could have achieved higher recovery of ent-pimara-8(14),15-diene using temperatures over 100 °C. However, to prevent the possible degradation of the compound, we did not test the extraction at temperatures above 90 °C. Taken together, our data suggest optimal extraction conditions for ent-pimara-8(14),15-diene from mycelia of oe:PbcR.

**Purification of ent-pimara-8(14),15-diene with TLC and HPLC**

In order to isolate ent-pimara-8(14),15-diene from crude extracts of oe:PbcR, a TLC method was developed. Mycelia from wild-type *A. nidulans* (FGSC A4) and oe:PbcR were extracted, and the extracts were separated on silica plates. *ent-Pimara-8(14),15-diene* was visualized by rhodamine staining of the developed silica plates (Fig. 3A), since the compound was not detectable directly by UV fluorescence. *ent-Pimara-8(14),15-diene* was separated by using EtOAc : heptane : acetic acid (9 : 90 : 1). Using this eluent composition, the compound moved along the solvent front (Fig. 3A, lane 1). *ent-Pimara-8(14),15-diene* was then extracted from the silica, and the purity of the compound was analyzed by GC-MS (Fig. 3B and C). Many fatty acids and other unknown interfering compounds that were present in the crude extract (Fig. 3B) were not detected in the TLC purified fractions (Fig. 3C). However, the TLC-purified compound still contained some non-polar alkanes that interfered with identification by NMR analysis (Fig. 3C).

To further purify *ent-pimara-8(14),15-diene*, we developed an analytical scale HPLC method. In HPLC, both the interactions between the purified compound and the stationary phase as well as the solvent play a role in the separation process. Specifically, *ent-pimara-8(14),15-diene* was purified from crude extracts of oe:PbcR by reverse phase HPLC. The purification method was tested by using three isocratic eluent compositions of 85%, 90%, and 100% ACN; and efficient separation was achieved with 100% ACN in one hour. Low wavelengths (190 nm, 210 nm, 225 nm, and 250 nm) were used to monitor fractionation, because this diterpene hydrocarbon does not have any functional groups or bonds that have UV absorbance; and, the peak corresponding to *ent-pimara-8(14),15-diene* was not detectable at wavelengths 250 nm and greater. To identify the compound peak, eluent fractions were collected in six 10 minute intervals. *ent-Pimara-8(14),15-diene* was detected in the third interval (20–30 minutes), and the individual peaks were then collected and analyzed by GC-MS. The UV absorption maximum for the peak corresponding to *ent-pimara-8(14),15-diene* was 222.1 nm (Fig. 4A), and monitoring of the following purifications was done at 225 nm.

In the analytical scale HPLC, *ent-pimara-8(14),15-diene* was eluted at 25 to 28 minutes after the sample injection (Fig. 4B), and the total analysis time was kept at 60 minutes. In the preparative scale HPLC, the *ent-pimara-8(14),15-diene* peak

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**Fig. 2** Influence of different solvents (A) and temperatures (B) on recovery of *ent-pimara-8(14),15-diene* from oe:PbcR mycelia. The error bars represent the standard error of the mean (SEM, n = 3).
was detected at 30 to 35 minutes after injection (Fig. 4B, lower panel). The shoulder seen at the preparative scale HPLC peak was not collected. Isolated ent-pimara-8(14),15-diene fractions were combined, evaporated to dryness, and dissolved in EtOAc prior to GC-MS analysis. Comparison of the GC-MS peak areas for the crude extract and for the HPLC purified ent-pimara-8(14),15-diene (Fig. 4C) indicated that there were no impurities in the collected fraction; and, the recovery of the diterpene was consistently over 90% (Fig. 4C, small panel). After multiple rounds of preparative scale HPLC, 25 mg of pure compound was collected.

Bioactivity testing of ent-pimara-8(14),15-diene

With its isolation and purification, we could now test ent-pimara-8(14),15-diene in a number of bioactivity assays. In Aspergilli, the production of secondary metabolites has been linked to oxidative stress.\textsuperscript{36,37} For example, lovastatin biosynthesis in \textit{A. terreus}, and aflatoxin biosyntheses in \textit{A. parasiticus} and \textit{A. flavus} respond to accumulation of reactive oxygen species (ROS) in these fungi.\textsuperscript{38,39} Because oxidative stress has also been shown to activate secondary metabolite biosynthesis in \textit{A. nidulans}, we tested the anti-oxidant activity of pure ent-pimara-8(14),15-diene hydrocarbon in a DPPH radical scavenging assay with three different concentrations and four time-points. We compared its activity to a known tetraterpene anti-oxidant, beta-carotene. Pure ent-pimara-8(14),15-diene had significantly higher DPPH radical scavenging activity than beta-carotene in all test conditions (Fig. 5).

Although the function of this compound in \textit{A. nidulans} is not known, the data suggest that one potential role of ent-pimara-8(14),15-diene may be to function as an antioxidant. The antioxidant activity of ent-pimara-8(14),15-diene could be one way for \textit{A. nidulans} to protect itself against stressful situations where accumulation of free radicals inside the cells occurs. We also tested purified ent-pimara-8(14),15-diene for antimicrobial activity against \textit{Staphylococcus aureus}, and for inhibition of axenic amastigotes of \textit{Leishmania donovani}. However, even relatively high concentrations of the compound (50 \textmu M) were ineffective in both of these tests (data not shown). Additional bioactivity and antioxidant assays will give a more complete picture of the potential bioactivity of the compound, and this will be addressed in future studies.

Structural analysis

To verify the structure of HPLC-purified ent-pimara-8(14),15-diene, infra-red (IR), MS, and 1D and 2D NMR experiments were performed (Table 1). Inspection of the \textsuperscript{1}H NMR spectrum...
showed the four methyl group signals as singlets at 0.80, 0.85, 0.88, and 1.04 ppm. The downfield double doublet at 5.78 ppm was assigned to 15-H whereas the double doublets at 4.88 and 4.91 ppm were assigned to 16-Hb and 16-Ha, respectively. Thus, the signal at 4.88 ppm displayed the cis vicinal coupling constant of 10.6 Hz between 16-Hb and 15-H and the geminal
The coupling constant of 1.5 Hz. The *trans* vicinal coupling constant for 16-H$_a$ and 15-H was found to be 17.5 Hz.

The COSY spectrum displayed correlations between the neighboring 15-H, 16-H$_a$, 16-H$_b$ proton signals. The assignment of C15 (149.1), C8 (137.2), C14 (128.4), and C16 (109.8) was made by observation of their respective correlations on the HSQC experiment. The signal at 1.70 ppm displayed correlation to the signal at 50.6 ppm, belonging to a CH carbon, and was assigned to 9-H. The remaining CH/CH$_3$ signals to assign correlated to the methyl group protons and to 5-H (C5 at 54.8 ppm). Assignment of the 17-methyl group to the signal at 1.04 ppm was based on the HMBC correlations with the signals of C15, C14, and the quaternary carbon C13 at 37 ppm. The remaining methyl group signals all displayed HMBC correlations to C5. The signal at 0.80 ppm was assigned to the 20-methyl protons based on the HMBC correlations to C9 and quaternary C10 (38.2 ppm). The signals at 0.85 and 0.88 ppm, corresponding to the 19 and 18-methyl protons, both displayed HMBC correlations to quaternary C4 (33.2 ppm). The stereochemistry of *ent*-pimara-8(14),15-diene was verified by the NOESY experiment. Selected NOESY correlations and a 3-D presentation of *ent*-pimara-8(14),15-diene are depicted in Fig. 6A and B, respectively. The axial position of the 13-methyl group and $\beta$-orientation for 5-H and 9-H was confirmed by the observed NOESY cross peaks of 14-H/17-CH$_3$, 14-H/7a-H, and 16a-H/17-CH$_3$, and is consistent with those found in the literature. Cross-peaks were also observed for the signals of 9-H and 5-H. The assignment of C17 to the signal at 25.9 ppm is also consistent with the axial position of this methyl group, as opposed to the reported values of about 30 ppm for equatorial orientation. These data confirm the structure and stereochemistry of *ent*-pimara-8(14),15-diene isolated from *A. nidulans* strain oe:PbcR$_R$, and are consistent with our earlier report. The structure and the mass spectrum of pure *ent*-pimara-8(14),15-diene are shown in Fig. 6C. NMR spectra are presented in the ESI (Fig. 1S–6S).

**Conclusions**

We developed isolation and purification methods for a lipophilic tricyclic diterpene, *ent*-pimara-8(14),15-diene, produced in fungi. The optimized ASE and HPLC methods developed here were very reproducible and could be scaled up to accommodate larger sample volumes. Biotechnological production of this particular diterpene in *A. nidulans* and the purification methods described here should enable production of large quantities of pure *ent*-pimara-8(14),15-diene. This is necessary for more systematic bioactivity screening of this compound. Finally, the
methods described are relevant for purification of other diterpenes with similar properties.

Conflict of interest
The authors declare no conflict of interest.

Acknowledgements

We thank Andreas Helfenstein and Päivi Tammela for antimicrobial testing, Charles Jaffe for anti-leishmanial testing, and Paul A. Bromann for his critical reading of the manuscript. This work was supported by Fibic Oy, Future Biorefinery Joint Research 2 program.

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