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# Psychrophilin E, a new cyclotripeptide, from co-fermentation of two marine alga-derived fungi of the genus Aspergillus

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# Psychrophilin E, a new cyclotripeptide, from co-fermentation of two marine alga-derived fungi of the genus *Aspergillus*

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Chemical investigation of the mycelial extract of a mixed culture of two marine alga-derived fungal strains of the genus *Aspergillus* has yielded one new cyclotripeptide, psychrophilin E (**1**), the recently reported oxepin-containing alkaloids, protuboxepin A (**2**) and oxepinamide E (**3**), together with three other polyketide derivatives (**4**–**6**). The chemical structure and relative and absolute configurations of psychrophilin E (**1**) were unambiguously established based on HRMS, 1D, 2D NMR and chiral-phase HPLC analysis of its hydrolysate. All the isolated compounds were assessed for their anti-proliferative activity against four different human cancer cell lines and some of them revealed selective activities.

**Keywords:** *Aspergillus*; co-fermentation; marine-derived fungi; anti-proliferative



## 1. Introduction

Marine-derived fungi continue to be a prolific source providing a plethora of hitherto unprecedented chemical scaffolds and/or new metabolites with potent pharmacologically significant activities (Rateb & Ebel 2011). This notion was proven by the increasing research efforts directed towards marine-derived fungi during the last few decades, which yielded a sixfold increase in the number of identified marine fungal metabolites in 2007 compared with those reported from 1965 to 2005 (Imhoff et al. 2011).

Recently, co-culturing of two or more marine-derived fungal strains in order to imitate their natural environment and/or to induce other biosynthetic interactions has gained a considerable attention aiming at identifying novel chemical skeletons produced through different biosynthetic pathways such as aspergicin, a potent anti-microbial alkaloid recently reported from a mixed culture of two marine-derived mangrove epiphytic fungal strains of the genus *Aspergillus* (Zhu et al. 2011).

Psychrophilins (Psp's) are a class of cyclic nitrotripeptides comprising four metabolites that have been reported from psychrotolerant fungal species of the genus *Penicillium* (Dalsgaard et al. 2004a, 2004b; 2005). The core structural elements of these peptides are one anthranilic acid (2-aminobenzoic acid) and one tryptophan residue together with variable amino acid residues including L-proline (Psp A) (Dalsgaard et al. 2004a), L-valine (Psp B) (Dalsgaard et al. 2004b),

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L-alanine (Psp C) (Dalsgaard et al. 2004b) and L-leucine (Psp D) (Dalsgaard et al. 2005). During our ongoing search for bioactive secondary metabolites from marine-derived fungi through co-cultures, we have studied an EtOAc extract of a mixed culture of two marine alga-derived fungal strains belonging to the genus *Aspergillus* which were derived from the brown algae *Sargassum* sp. collected off the North Sea shores in Germany. The two fungi were cultivated as a co-culture under static conditions at 28°C for four weeks till the dark brown pigment of conidia was noticed and then EtOAc was added to the culture mixture to end the fermentation process and to extract the whole biomass.

## 2. Results and discussion

Chemical investigation of EtOAc extract yielded one new psychrophilin congener (Psp E, **1**), two recently reported oxepin-containing pyrimidine alkaloids, protuboxepin A (**2**) (Lee et al. 2011) and oxepinamide E (**3**) (Lu et al. 2011), together with three mycotoxins, namely sterigmatocystin (**4**) (Seto et al. 1974; Shao et al. 2007), 5-methoxysterigmatocystin (**5**) (Seto et al. 1974; Shao et al. 2007; Cai et al. 2011) and aversin (**6**) (Shao et al. 2007).

Psychrophilin E (**1**), $[\alpha]_D^{25}$ + 32.0 (*c* 0.2, $CHCl_3$), IR (film): 3296, 2926, 2851, 1693, 1623, 1603, 1524, 1453, 1363, 754 $cm^{-1}$, UV (MeOH) $\lambda_{max}$ = 222, 246, 306 nm, was isolated as a white amorphous solid featuring in $^{13}C$ NMR spectrum and DEPT experiment the existence of 25 carbon signals differentiated into nine quaternary carbons including four carbonyl $sp^2$ carbons at $\delta_C$ 166.2, 167.4, 170.1 and 172.4, eleven tertiary carbons including two $sp^3$ at $\delta_C$ 51.0 and 59.8, four secondary carbons, and one primary methyl carbon ($\delta_C$ 23.0). The molecular formula of **1** was established to be $C_{25}H_{24}N_4O_4$ on the basis of HR-ESI-MS revealing ion peak at *m/z* 445.1870 $[M + H]^+$ (calcd for $C_{25}H_{25}N_4O_4$ 445.1876) implying the presence of 16 degrees of unsaturation in the compound. In addition, UV spectrum of **1** in MeOH indicated the presence of aromatic and indole moieties in the compound as revealed by psychrophilins A–D (Dalsgaard et al. 2004a, 2004b; 2005). Based on the literature search, a match was found in psychrophilins, a class of cyclic nitrotripeptides. Further investigation was conducted through IR spectrum, showing an intense stretching vibration at 1693 $cm^{-1}$ indicating the presence of amide carbonyl groups and missing those characteristic stretching vibrations of the nitro functionality at 1553 and 1360 $cm^{-1}$ as for psychrophilins A–D (Dalsgaard et al. 2004a, 2004b; 2005). These findings suggested that **1** is a cyclic tripeptide resembling the psychrophilins except in the absence of the nitro group (Figure 1).

Structural elucidation was continued through extensive 2D NMR spectral analyses including $^1H$–$^1H$ COSY and HSQC (see Supplementary materials, Figures S9 and S10) which confirmed the existence of an *ortho*-substituted benzene and indole ring systems similar to those in psychrophilins A–D representing an anthranilic acid (Abz) and tryptophan (Try) residues in addition to two aliphatic spin systems of the types X–CH(X′)–$CH_2$–$CH_2$–$CH_2$–X″ and –XNH–CH(X′)–$CH_2$–X″ which were attributed to proline and tryptophan residues, respectively. The arrangement of amino acid residues was confirmed through the HMBC spectrum (see Supplementary materials, Figure S11) which revealed correlations between H-11 and C-12 ($\delta_C$ 166.2), 18-N*H* to C-19 ($\delta_C$ 167.4) and H-20 to C-1 ($\delta_C$ 172.4) together with the correlations of 2-N*H*, H-2 and a singlet methyl resonance at $\delta_H$ 2.04 (s, 3H) to C-24 ($\delta_C$ 170.1) which established the amino acid sequence of psychrophilin E (**1**) as *cyclo*-(*N*-acetyltryptophan-prolyl-*O*-aminobenzoyl).

The absolute configuration of **1** was determined through implementing chiral-phase gas chromatographic (GC) analysis of methyl *N*-(trifluoroacetyl)proline derivative obtained from hydrolysis, using methanolic HCl followed by acetylation using trifluoroacetic acid anhydride (TFAA) compared with those of D- and L-proline standards (Stenerson & Lee 2011). Comparison of GC retention times of the derivatised proline with D- and L-proline standards

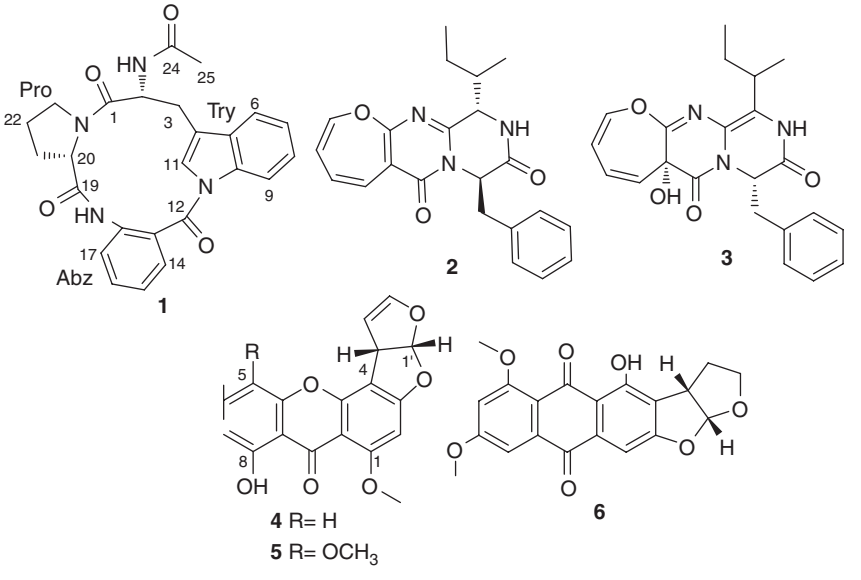


Figure 1. Structures of compounds **1**–**6**.

unambiguously proved proline to possess the L-configuration assigning *S* configuration to C-20. The absolute configuration of C-2 cannot be directly established due to the destruction of *N*-acetyltryptophan during acid hydrolysis.

However, the ROESY spectrum (see Supplementary materials, Figures S14 and S15) of psychrophilin E (**1**) revealed key correlations which were used to determine the most likely configuration. Using the 3D models of the minimal energy conformation of (2*R*, 20*S*) and (2*S*, 20*S*) psychrophilin E (**1**) together with the ROESY correlations (see Supplementary material, Figure S15) between the α-proton in *N*-acetyltryptophan (H-2) and H-3b; H-2 and H-23a/b; and the correlations between H-3b and H-6; H-3a and H-11, the absolute stereochemistry of C-2 was assigned as *R* configuration and hence (2*R*, 20*S*) for psychrophilin E (**1**) unlike the absolute configuration reported for psychrophilins A–D (Dalsgaard et al. 2004a, 2004b; 2005).

All the isolated compounds were assessed for their *in vitro* anti-proliferative activities using a microplate (MTT) assay (Müller et al. 2004) against four different human tumour cell lines, namely K562 (leukaemia), HCT116 (colon), A2780 (ovary) and A2780CisR (cisplatin-resistant mutant). Among the tested compounds (Table 1), psychrophilin E (**1**), sterigmatocystin (**4**) and 5-methoxysterigmatocystin (**5**) exhibited selective anti-proliferative activities in particular towards HCT116 (colon) cell line with $IC_{50}$ values of 28.5, 10.3 and 4.4 μM, respectively, compared with cisplatin as a positive control ($IC_{50} = 33.4$ μM). In addition, 5-methoxyster-

Table 1. $IC_{50}$ values of selected compounds against four different human tumour cell lines.

| Compound | $IC_{50}$ (μM) | | | |
|---|---|---|---|---|
| | K562 | HCT116 | A2780 | A2780CisR |
| Psychrophilin E (**1**) | 67.8 | **28.5** | 27.3 | 49.4 |
| Sterigmatocystin (**4**) | 57.0 | **10.3** | 30.6 | 95.5 |
| 5-Methoxysterigmatocystin (**5**) | **13.4** | **4.4** | 51.0 | >140 |
| Cisplatin | 7.8 | 33.4 | 0.8 | 8.4 |

igmatocystin (**5**) showed significant anti-proliferative activity against K562 (leukaemia) cell line with $IC_{50}$ of 13.4 μM compared with cisplatin ($IC_{50} = 7.8$ μM).

## 3. Experimental

### 3.1. *General experimental procedures*

Optical rotation was recorded using a Perkin-Elmer-341 MC polarimeter (Perkin-Elmer®, Waltham, MA, USA) at 589 nm using a cylinder with 10 cm length at 20°C. IR spectrum was recorded using Perkin-Elmer Spectrum One IR-Spectrometer (Perkin-Elmer®, Waltham, MA, USA). 1D and 2D NMR spectra were measured using Bruker ARX 500 and AVANCE DRX 600 NMR spectrometers (Bruker®, Billerica, MA, USA). MS (ESI) and HRMS (ESI) were determined with a ThermoFinnigan LCQ DECA (Thermo®, Waltham, MA, USA) and Micromass QTOF 2 (Waters®, Milford, MA, USA) mass spectrometers, respectively. Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements.

For HPLC analysis, a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340U) was used. Routine detection was conducted at 225, 250, 275 and 350 nm. The separation column (125 × 4 mm ID) was pre-filled with C-18 Eurosphere, 5 μM (Knauer, Berlin, Germany). Separation was performed implementing a linear gradient from 10% MeOH to 100% MeOH over 40 min. For preparative HPLC separations, a separation column (250 × 8 mm ID) pre-filled with C-18 Eurosphere, flow rate 3.0 mL/min and UV detection at 240 and 350 nm; the elution system comprised a linear gradient of $CH_3OH$ and nanopure $H_2O$. TLC was performed using Polygram®, plastic sheets pre-coated with silica gel 60 $F_{254}$ (Macherey-Nagel, Düren, Germany). Chiral GC analysis was carried out on Shimadzu® GC-2010 Plus equipped with capillary CP-chirasil Dex CB column using helium as carrier gas and an isothermal program at 120°C for 15 min. Minimal energy conformations of psychrophilin E (**1**) was calculated using ChemDraw® Ultra 12.0 based on 3D models of the two conformers (2*R*, 20*S*) and (2*S*, 20*S*).

### 3.2. *Isolation and cultivation of the microbial material*

Two fungal strains of the genus *Aspergillus* (BM-05 and BM-05ML) were isolated from a brown algal species belonging to the genus *Sargassum* collected off Helgoland, North Sea, Germany in 2001. The two fungi were cultivated together as a co-culture under static conditions at 28°C for four weeks in ten 1-L Erlenmeyer flasks containing the culture medium (500 mL/flask) composed of peptone from soya (4 g/L), maize starch (10 g/L), $MgSO_4$ (3.6 g/L), NaCl (20 g/L), yeast extract (2 g/L) and $CaCO_3$ (1.8 g/L) using demineralised water. The culture medium was sterilised in 1-L Erlenmeyer flasks at 121°C for 20 min. Agar plugs from plated culture were seeded into production medium (10 × 500 mL). The fermentation time was reached when dark brown pigment of conidia was noticed.

### 3.3. *Extraction and isolation*

At the end of the incubation period, the mycelium and medium were extracted four times with ethyl acetate. The obtained extract was concentrated under vacuum to yield residual gum in 6.5 g. This residue was subjected to vacuum liquid chromatography on a silica gel column implementing a step gradient of *n*-hexane–EtOAc and then $CH_2Cl_2$–$CH_3OH$ yielding nine fractions (V1–V9) each of 500 mL. These fractions were dried under reduced pressure and examined using analytical HPLC system and TLC on pre-made silica gel plates using a $CH_2Cl_2$–$CH_3OH$-based solvent system. Further purification of fractions V2 (240 mg), V3 (210 mg) and V8 (160 mg) was obtained separately through column chromatography using Sephadex LH20 as stationary phase and eluted with $CH_2Cl_2$:$CH_3OH$ (2:8). Final purification steps were achieved

through preparative reversed-phase HPLC using a C18 column (MeOH–$H_2O$, linear gradient, 10% MeOH → 100% MeOH over 40 min) to yield **1** (14.0 mg), **2** (3.0 mg), **3** (4.0 mg), **4** (10.0 mg), **5** (2.0 mg) and **6** (1.0 mg). Chemical structures of the isolated known compounds were characterised based on spectrometric and spectroscopic analyses, including mass spectrometry, HRMS, 1D and 2D NMR spectroscopy, and by comparing their NMR spectroscopic data with the literature values (Shao et al. 2007; Cai et al. 2011; Lee et al. 2011; Lu et al. 2011).

#### 3.3.1. *Psychrophilin E (**1**)*

White amorphous solid; $[\alpha]_D^{25}$ + 32.0 (*c* 0.2, $CHCl_3$). IR (film): 3296, 2926, 2851, 1693, 1623, 1603, 1524, 1453, 1363, 754 $cm^{-1}$, UV (MeOH) $\lambda_{max}$ = 222, 246, 306 nm. $^1H$ and $^{13}C$ NMR (500 MHz, $CDCl_3$): 1.23 (m, 1H, H-22b), 1.52 (m, 1H, H-22a), 1.69 (m, 1H, H-21b), 2.02 (m, 1H, H-21a), 2.04 (s, 3H, H-25), 2.14 (m, 1H, H-23b), 2.89 (t, *J* = 12.2 Hz, 1H, H-3a), 3.27 (dd, *J* = 16.7, 9.2 Hz, 1H, H-23a), 3.35 (dd, *J* = 12.2, 5.4 Hz, 1H, H-3b), 4.55 (dd, *J* = 8.3, 2.7 Hz, 1H, H-20), 5.08 (ddd, *J* = 13.1, 7.9, 5.5 Hz, 1H, H-2), 6.49 (brd, 1H, N*H*-Ac-Try), 6.86 (s, 1H, H-11), 7.38 (t, *J* = 7.3 Hz, 2H, H-7 and H-15), 7.43 (t, *J* = 7.6 Hz, 1H, H-8), 7.57 (d, *J* = 7.6 Hz, 1H, H-17), 7.57 (t, *J* = 7.3 Hz, 1H, H-16), 7.67 (d, *J* = 7.6 Hz, 1H, H-6), 7.82 (d, *J* = 7.6 Hz, 1H, H-14), 8.50 (brs, 1H, NH-Abz), and 8.62 (d, *J* = 8.1 Hz, 1H, H-9); $^{13}C$ NMR: 23.0, 24.7, 26.3, 29.3, 47.7, 51.0, 59.8, 116.4, 117.2, 118.3, 123.7, 124.5, 125.6, 126.1, 126.3, 128.1, 129.6, 131.7, 132.5, 133.4, 135.5, 166.2, 167.4, 170.1, and 172.4; HRESIMS *m/z* 445.1870 $[M + H]^+$ (calcd for $C_{25}H_{25}N_4O_4$ 445.1876).

### 3.4. *Acid hydrolysis of psychrophilin E (1)*

The compound (0.5 mg) was refluxed at 95°C for 6 h with 6 N methanolic HCl. After cooling, the sample was evaporated under reduced pressure to dryness. Then, the remaining residue was dissolved in 1.0 mL of methylene chloride and 100 μL of TFAA and then it was refluxed at 60°C for 20 min. The sample was then cooled and the remaining liquid was evaporated at room temperature. The residue obtained was dissolved in methylene chloride for chiral gas GC analysis. The same procedure was conducted for an unequal known mixture of D- and L-proline standards. The configuration of proline was determined by an isothermic GC analysis using CP-chirasil Dex CB column at 120°C over 15 min. Retention times (in min) for the standards were proline, *R*, 7.12 min, and *S*, 7.24 min. Analysis of the derivative gave a retention time of 7.24 min, determining an *S* configuration for the proline residue.

### 3.5. *Cell proliferation assay*

Anti-proliferative activity was tested *in vitro* against human chronic myelogenous leukaemia (K562), human colon carcinoma (HCT116), human ovarian cancer (A2780) and cisplatin-resistant human ovarian cancer (A2780CisR) cell lines using a microplate-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and compared with that of untreated controls as previously described (Müller et al. 2004). All experiments were carried out in triplicate using cisplatin (CDDP) as positive control.

## 4. Conclusions

Psychrophilins A–D were all reported from marine-derived psychrotolerant fungal species of the genus *Penicillium*, cultivated in the dark at 20–25°C, psychrophilin E (**1**) was obtained from a mixed culture of two marine alga-derived fungal strains of the genus *Aspergillus* which were cultivated at 28°C. In addition, psychrophilin E (**1**) exhibited acetylation of α-amino group in tryptophan residue into an *N*-acetyl moiety, which was oxidised in psychrophilins A–D into a

nitro group (Dalsgaard et al. 2004a, 2004b; 2005). Furthermore, LC–MS screening of the fungal extract and purified fractions did not reveal the existence of other detectable psychrophilin derivatives which implies that this co-culture may have played a role in modifying the biosynthetic pathway to acetylation rather than oxidation of α-amino group in tryptophan residue.

Interestingly, the additional methoxy group in **5** potentiated its activity towards HCT116 and K562 cell lines (Table 1) as compared with sterigmatocystin (**4**) with $IC_{50}$ values of 4.4 and 13.4 μM with **5** and 10.3 and 57.0 μM with **4**, respectively. These results may strengthen the significance of 5-methoxy group as a functional group important for anti-proliferative activity of compound (**5**).

## Supplementary material

Supplementary material relating to this article is available online alongside Table S1 and Figures S1–S15.

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