

# Three New Non-reducing Polyketide Synthase Genes from the Lichen-Forming Fungus *Usnea longissima*

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**Abstract** *Usnea longissima* has a long history of use as a traditional medicine. Several bioactive compounds, primarily belonging to the polyketide family, have been isolated from *U. longissima*. However, the genes for the biosynthesis of these compounds are yet to be identified. In the present study, three different types of non-reducing polyketide synthases (UIPKS2, UIPKS4, and UIPKS6) were identified from a cultured lichen-forming fungus of *U. longissima*. Phylogenetic analysis of product template domains showed that UIPKS2 and UIPKS4 belong to group IV, which includes the non-reducing polyketide synthases with an methyltransferase (MeT) domain that are involved in methylorcinol-based compound synthesis; UIPKS6 was found to belong to group I, which includes the non-reducing polyketide synthases that synthesize single aromatic ring polyketides, such as orsellinic acid. Reverse transcriptase-PCR analysis demonstrated that UIPKS2 and UIPKS4 were upregulated by sucrose; UIPKS6 was downregulated by asparagine, glycine, and alanine.

**Keywords** Fungal non-reducing polyketide synthase, Lichen forming-fungi, mRNA expression, *Usnea longissima*

Lichens are stable and self-supporting symbiotic organisms between fungi (mycobiont) and photoautotrophic algal partners (photobiont), namely green algae and/or cyanobacteria [1]. Lichen-forming fungi (LFF) have been found to produce a wide array of secondary metabolites. These secondary metabolites have shown an impressive range of biological activities, including antibiotic, antifungal, anti-protozoan, anti-human immunodeficiency virus, anticancer, and antioxidant activities [2-5]. Accordingly, LFF show huge potential value for discovery of new drugs [6-9].

Fungal polyketide synthases (PKSs) primarily belong to iterative type I PKSs, which have  $\beta$ -ketoacyl synthase (KS),

acyl transferase (AT), and acyl carrier protein (ACP) domains. Specific iterative type I PKSs may also contain starter units: ACP transacylase (SAT), product template (PT), ketoreductase, dehydratase (DH), enoyl reductase, thioesterase (TE), or C-methyltransferase (CMeT) domains. Type I fungal PKSs have been divided into three classes according to the variation in these optional processing domains: aromatic or non-reducing PKS (NR-PKS), partially reducing PKS, and highly reducing PKS [10, 11]. The NR-PKSs can be divided into five classes according to the PT domains that are embedded in the multidomain NR-PKSs, which mediate the regioselective cyclization of the highly reactive polyketide backbones and dictate the final structure of the products [12].

Most unique lichen secondary metabolites, such as depsides, depsidones, depsones, and dibenzofurans, are derived from the polyketide pathway [13]. Among them, depsides consist of two or occasionally three aromatic rings joined by ester linkages; the depsidones have an extra ether linkage between the rings. Lichen depsides and depsidones can be grouped into either a  $\beta$ -orcinol or orcinol series, depending on the presence of a CH<sub>3</sub> on the C<sub>3</sub> carbon of their rings [14]. Their non-reduced backbones indicate that the corresponding PKSs belong to a subgroup of NR-PKSs [11]. The PKS genes of  $\beta$ -orcinols are similar and could belong to the NR-PKS group, with a methyltransferase (MeT) domain [14, 15]. To date, only a few lichen PKSs, mostly NR-PKSs, have been analyzed [14-19].

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In a previous study, we reported two PKSs, a NR-PKS and a reducing PKS, from *U. longissima* [20, 21]. In the present study, we report three additional novel NR-PKSs, which may be involved in the biosynthesis of orcinol and  $\beta$ -orcinol depside or depsidone.

## MATERIALS AND METHODS

**PCR amplification of the KS domain.** *U. longissima* LFF used in this study had been previously identified and deposited at the Korea Lichen and Allied Bioresearch Center, Sunchon National University [20]. The LFF was grown and maintained in malt-yeast (MY) medium (Difco, Detroit, MI, USA). Total DNA was isolated from the *U. longissima* LFF using a DNeasy Plant Mini Kit (Qiagen, Carlsbad, CA, USA). A series of degenerate primers (Table 1) were used for KS fragment amplification, using touchdown PCR cycling conditions. The PCR product was subcloned into the pGEM-T vector (Promega, Madison, WI, USA). Colonies with the putative inserted fragment were picked for sequencing.

**Genomic library screening.** The specific primers (Fnrks57 and Rnrks57; Flc3551 and Rlc3551; Fmtc141 and Rmtc141; shown in Table 1) were designed according to the obtained KS domain fragment sequence for digoxigenin (DIG) labeling. The DIG-labeled DNA probe was generated using a DIG-High Prime Labeling Mix (Roche Applied Sciences, Basel, Switzerland). The genomic libraries were constructed using a CopyControl HTP Fosmid Library Production Kit (Epicentre, Madison, WI, USA), according to the manufacturer's instructions. The *U. longissima* genomic library colonies ( $3 \times 10^3$ ) were transferred onto Nytran membranes (GE

Healthcare, Buckinghamshire, UK) and fixed for hybridization, following the manufacturer's instructions. The membranes were hybridized with the DIG-labeled probe at 42°C, and positive signals were detected using a DIG Detection Starter Kit I (Roche Applied Sciences).

**DNA sequencing and analysis.** Sequence of the Fosmid clone, including the putative PKS fragment, was determined by Genotech Co. Ltd. (Seoul, Korea). The potential open reading frame (ORF) was scanned with FGENESH [22] using *Aspergillus* as the matrix (<http://linux1.softberry.com>). The putative function of these ORFs was determined using a basic local alignment search tool (BLAST). The conserved domain was confirmed using the CDD-Search/PRS-BLAST (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>).

**Phylogenetic analyses.** The PT domain of the NR-PKS from *U. longissima* was aligned with 35 fungal NR-PKS sequences retrieved from GenBank. The alignment was analyzed using ClustalW embedded in the MEGA 4.0.2 program (<http://www.megasoftware.net/>). To obtain a confidence value for the aligned sequence dataset, bootstrap analysis with 100 replicates was performed using the MEGA 4.0.2 program. A phylogenetic tree was constructed using the minimum evolution method.

**Reverse transcription-polymerase chain reaction.** Mycelia of *U. longissima* LFF (100 mg) were ground into powder after freezing in liquid nitrogen. RNA was then extracted using an RNeasy Plant Mini Kit (Qiagen). SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was used for first-strand cDNA synthesis. The full cDNA of *U. longissima* PKS was amplified using

**Table 1.** Primers used in this work

Purpose	Name	Sequence	
Degenerate primers for fragment amplification of non-reducing PKSs M = A + C; N = A + C + G + T; R = A + G; Y = C + T	NR3KS-F (forward)	5'-CTA CGA CGC CTT CGA CCA YMR NTT YTT-3'	
	NR3KS-R (reverse)	5'-GGG GTG CCG GTG CCR TGN GCY TC-3'	
	Fcmet (forward)	5'-ATT GGT GCT GGA ACA GGT-3'	
	RcmetL2 (reverse)	5'-AAC CCG GAA GCT GGC CAA AT-3'	
	LC3 (forward)	5'-GCN GAR CAR ATG GAY CCN CA-3'	
	LC5c (reverse)	5'-GTN GAN GTN GCR TGN GCY TC-3'	
<b>Specific primers</b>	<i>UIPKS2</i> DIG-labeling and expression detection	Fnrks57 (forward)	5'-GGG TAG CCA ACG ACG ACTA-3'
		Rnrks57 (reverse)	5'-TCC TTG ATT TAC GGC AGA GC-3'
	<i>UIPKS4</i> DIG-labeling and expression detection	Flc3551 (forward)	5'-TTA CGA AGC GTT GGA GAT GT-3'
		Rlc3551 (reverse)	5'-GGT CAT TAT CCC TGA GAG CA-3'
	<i>UIPKS6</i> DIG-labeling and expression detection	Fmtc141 (forward)	5'-TTG GCG TTC CAT CAA TGA GT-3'
		Rmtc141 (reverse)	5'-AGG GTC CAC ACT TGC CTC TT-3'
	<i>UIPKS2</i> complete cDNA	UIPKS2-F0 (forward)	5'-ATG GCT CAT GAA AGT ATT GG-3'
		UIPKS2-R0 (reverse)	5'-CTA TCT CCA TTG GCT CGA AT-3'
	<i>UIPKS4</i> complete cDNA	UIPKS4F0 (forward)	5'-ATG ACG CTA CCC TCG CTG AT-3'
		UIPKS4R0 (reverse)	5'-TTA ATT CAT CCC CAA CTG CCT-3'
	<i>UIPKS6</i> complete cDNA	UIPKS6F0 (forward)	5'-ATG GCT TCC CAA GTT CTA CT-3'
		UIPKS6R0 (reverse)	5'-TCA ATT TTC ATC CGC GAT AGC T-3'

DIG, digoxigenin.

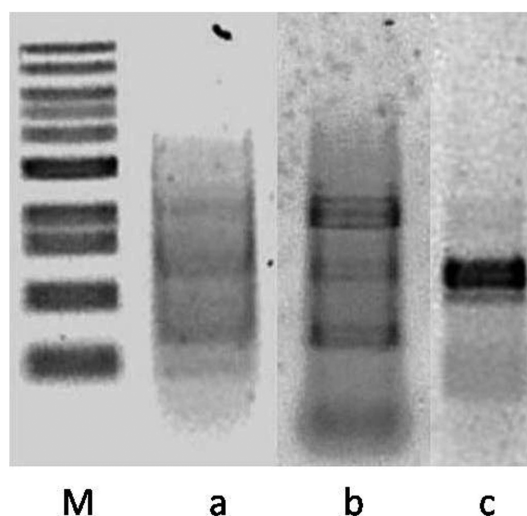
primers designed using the predicted mRNA sequence (Table 1).

### Expression patterns of NR-PKS transcripts in *U. longissima*.

*U. longissima* LFF were ground to homogeneity and incubated on MY solid medium. After 2 months of culture at 15°C, the mycelia were harvested, and then transferred into MY basal medium containing 2% or 10% (W/V) inositol, mannitol, sorbitol, sucrose, glucose, or fructose. Amino acid media were also prepared by adding 0.2% and 1% (W/V) glutamine, asparagine, glycine, or alanine to the MY basal medium. The *U. longissima* LFF were cultured for two months on the various media. RNA was extracted from the samples using an RNeasy Plant Mini Kit (Qiagen). One microgram of total RNA from each sample was used for first-strand cDNA synthesis, which was performed using SuperScript II reverse transcriptase (Invitrogen). Specific primers (Table 1) were used to detect NR-PKS gene expression, with tubulin as the control. All the PCR reactions were conducted using the following reaction mixtures: 1 µL cDNA, 2 µL primer mixture, 1 µL Accupower PCR PreMix (Bioneer, Daejeon, Korea), and 16 µL distilled water. Reverse transcription-polymerase chain reaction (RT-PCR) experiments were repeated three times for each of the three independent replicates.

## RESULTS

Isolation of the KS domain using degenerate primers. PCR of LFF genomic DNA with degenerate primers yielded several bands (Fig. 1), and the PCR products were subsequently cloned and sequenced. Of the clones, three contained a partial KS domain sequence (NRks57, LC3551, and MTc14). The NRks57 clone was obtained using degenerate primers NR3KSF and NR3KSR. The closest NRks57 homologue found in the GenBank database was a putative NR-PKS KS domain fragment from *Coccidioides posadasii* (87% identity; GenBank accession No. EFW20973.1). The LC3551 clone was obtained using degenerate primers LC3 and LC5c. The closest LC3551 homologue was a putative NR-PKS KS domain fragment from *Mycosphaerella populorum* (71% identity; GenBank accession No. EMF17516.1). The MTc14 clone was obtained using degenerate primers Fcmet and RcmctL2. The closest MTc14 homologue was a putative NR-PKS KS domain fragment from *Pertusaria pertusa* (54% identity; GenBank accession



**Fig. 1.** Polyketide synthase (PKS) fragment amplified with degenerate primers. Lane a, PCR products with primers LC3 and LC5c; lane b, PCR products with primers NRMeT-F and NRMeT-R; lane c, PCR products with primers NR3KS-F and NR3KS-R; lane M, 1 kb molecular weight marker.

No. ABQ11392.1). All the clones belonged to the group of NR-PKSs.

**Domain organization of PKS genes.** An initial *U. longissima* LFF genomic library was constructed in pEpiFOS (Epicentre). After screening of the genomic library, a clone containing a complete PKS fragment was chosen for further sequencing. A total of three PKSs, UIPKS2, UIPKS4, and UIPKS6, were obtained from the sequence analysis.

UIPKS2 was a 6,290-bp gene encoding a 1,788-amino acid protein. The presence of six introns was confirmed by RT-PCR (Table 2). Six catalytic domains were identified in UIPKS2 by conserved motif analysis. These domains were KS, AT, PT, two ACP, and a TE domain. The domains were in the following order: KS/AT/PT/ACP/ACP/TE (Table 2). Comparison of UIPKS2 with corresponding domains of other fungal PKSs showed conservation of active site residues in the KS (DTACSASAV), AT (GHSFGQ), PT (GHEVVDQTLCPAS), ACP1 (NIDSLATE) ACP2 (GVDSLLATE), and TE (GWSSGGHL) domains. UIPKS2 was deposited in GenBank with the accession No. JX232185.

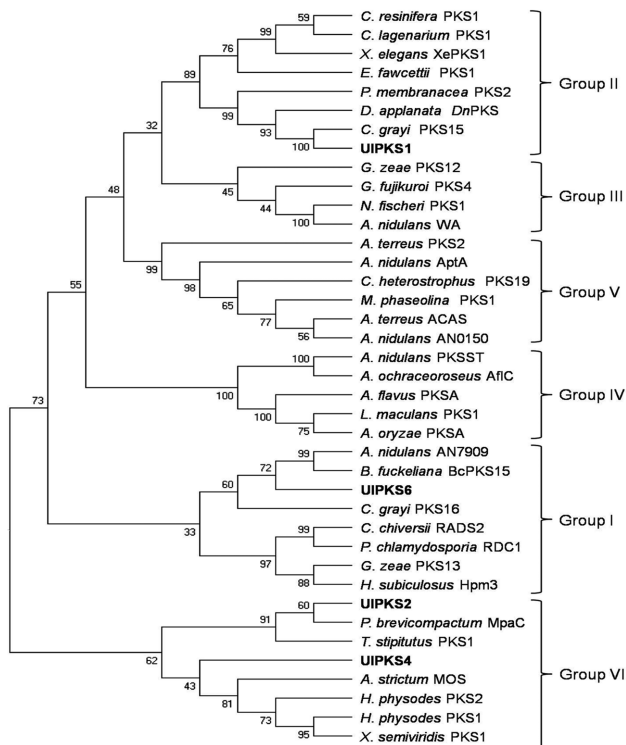
UIPKS4, which encoded a 2441-amino acid protein, was confirmed to have seven exons by RT-PCR (Table 2). UIPKS4

**Table 2.** PKS genes obtained

Gene name	Domain organization	Putative function	Intron position (relative to the start codon)
UIPKS2	KS-AT-PT-ACP-ACP-EST	Unknown	499~701, 1514~1613, 1792~1854, 2959~3043, 4076~4451, 5988~6051
UIPKS4	KS-AT-PT-ACP-MeT-EST	Involved in $\beta$ -orsenillic acid synthesis	480~756, 977~1011, 6133~6244, 6812~6863, 7233~7296, 7383~7443
UIPKS6	KS-AT-PT-DH-ACP-TE	Involved in orsenillic acid synthesis	305~357, 623~906, 1320~1357, 2305~2365, 2579~2632

PKS, polyketide synthase.

included six catalytic domains: KS, AT, PT, ACP, CMeT, and a TE domain. Comparison of UIPKS4 with corresponding

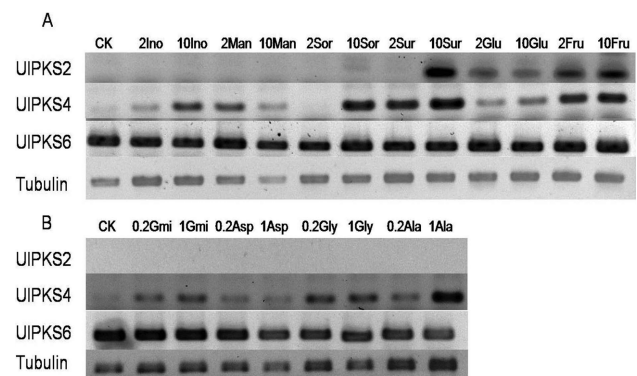


**Fig. 2.** Phylogenetic relationship of *Usnea longissima* non-reducing polyketide synthase (PKS) with other fungal PKSs. The available genomic sequence of *UIPKS2*, *UIPKS4* and *UIPKS6* were translated using FGENESH. The translated PKS was aligned with 35 fungal non-reducing PKS sequences retrieved from GenBank. *Arthroderma benhamiae* PKS1 (EFE29950.1), *Aspergillus flavus* PKSA (AAS90093), *A. nidulans* AptA (XP\_663604), *A. nidulans* AN0150 (XP\_657754), *A. nidulans* pksST (Q12397), *A. nidulans* WA (CBF74114), *A. nidulans* AN7909 (XP\_681178), *A. ochraceoroseus* AfIC (ACH72912), *A. oryzae* PKS1 (XP\_001816639.1), *A. oryzae* PKSA (BAE71314), *A. parasiticus* PKSA (AAS66004), *A. terreus* ACAS (XP\_001217072), *A. terreus* PKS1 (BAB88689.1), *A. terreus* PKS2 (BAB88752.1), *Cladonia grayi* PKS16 (ADM79459.1), *Chaetomium chiversii* RADS2 (ACM42403), *Cladonia grayi* PKS15 (ADX36087.1), *Cochliobolus heterostrophus* PKS19 (AAR90273), *Ceratocystis resinifera* PKS1 (AAO60166), *Colletotrichum lagenarium* PKS1 (BAA18956), *Dirinaria applanata* DnPKS (ACH72076), *Elsinoe fawcettii* PKS1 (ABU63483), *Gibberella zeae* PKS12 (AAU10633), *G. fujikuroi* PKS4 (CAB92399), *Hypomyces subiculosus* Hpm3 (ACD39762), *Hypogymnia physodes* PKS1 (AFO67255.1), *H. physodes* PKS2 (AFO67256.1), *Leptosphaeria maculans* PKS1 (AAS92537), *Macrostroma phaseolina* PKS1 (EKG11397.1), *Neosartorya fischeri* PKSP (XP\_001261235.1), *Ophiostoma piceae* PKSA (ABD47522), *Peltigera membranacea* PKS1 (AEE65374.1), *Pochonia chlamydosporia* RDC1 (ACD39770), *Talaromyces stipitatus* PKS1 (XP\_002339967.1), *Xanthoparmelia semiviridis* PKS1 (ABS58604.1), and *Xanthoria elegans* XePKS1 (ABG91136). The *U. longissima* non-reducing PKS clade obtained is marked in bold.

domains of other fungal PKSs showed conservation of active site residues in the KS (DTACFSSAV), AT (IGHSFGQ), PT (GHHAQFWNFHKS), ACP (GVDSLMMIE), MeT (LEVGAGTGG), and TE (GWSSGGQL) domains. The accession number for *UIPKS4* is JX232186.

*UIPKS6* is a 2072-amino acid protein encoded by a 6,780-bp gene. A search for conserved motifs revealed six catalytic domains, in the following order: KS/AT/PT/DH/ACP/TE (Table 2). *UIPKS6* has the following conserved active site residues: KS (ACSARTSYSL), AT (YGFHSAQ), PT (GHLVDNTAIC), DH (GLSHRLQRP), ACP (GLDSLMSIA), and TE (GGWSAGA). *UIPKS6* was deposited in GenBank with the accession No. JX232188.

**Phylogenetic analysis of NR-PKSs.** The amino acid sequence of the PT domain of the three NR-PKSs from *U. longissima* and several fungal NR-PKSs were used to generate multiple alignments and phylogenetic trees (Fig. 2). *UIPKS2* was grouped with *Penicillium brevicompactum* MpaC, which is involved in 5-methylorsellinic acid biosynthesis, and *UIPKS4* was grouped with *Acremonium strictum* MoS, which is involved in 3-methylorcinolaldehyde biosynthesis. These PKSs formed an independent clade, group VI. *UIPKS6* was grouped with *Cladonia grayi* PKS16 and *Aspergillus nidulans* AN7909, and these three sequences



**Fig. 3.** Gene expression of non-reducing polyketide synthase (PKS) from *Usnea longissima* with different carbon sources (A) and amino acids (B). A, Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *UIPKS2*, *UIPKS4*, and *UIPKS6* gene expression with different carbon sources. The numbers in front of the carbon sources represent its concentration, e.g., 2Ino and 10Ino for 2% and 10% inositol, respectively. The names of the carbon sources are abbreviated: Man for mannitol, Sor for sorbitol, Sur for sucrose, Glu for glucose, and Fru for fructose. CK is the control malt-yeast (MY) medium; B, RT-PCR analysis of *UIPKS2*, *UIPKS4*, and *UIPKS6* gene expression with different amino acids. The numbers in front of each amino acid source represent its concentration, e.g., 0.2Gmin and 1Gmin for 0.2% and 1% glutamine, respectively. The names of the amino acid sources are abbreviated: Gmi for glutamine, Asp for asparagine, Gly for glycine, and Ala for alanine. CK is the control MY medium.

formed an independent clade that is closely related to the NR-PKSs, group I [12]. These PKSs synthesize compounds containing a single aromatic ring, similar to orsellinic acid synthase AN7909 from *A. nidulans* and *C. grayi* PKS16, which is involved in orcinol depside biosynthesis.

**Expression patterns of PKS transcripts.** To confirm the functionality of the genes in the production of metabolites in intact LFF, *U. longissima* LFF were transferred to a media containing various carbon sources and amino acids. The expression of UIPKS2, UIPKS4, and UIPKS6 was evaluated under various nutrient conditions using RT-PCR. As shown in Fig. 3, although UIPKS2 was not expressed in most media, sucrose and fructose strongly induced UIPKS2 gene expression at higher concentrations (10%). UIPKS4 was expressed at a very low level in the basic MY medium. The amino acids glutamine, asparagine, and glycine had very slight effects on basal UIPKS4 expression, but alanine strongly upregulated UIPKS4 expression at higher concentrations (1%). Furthermore, inositol, mannitol, and glucose only slightly induced UIPKS4 gene expression, but sorbitol, sucrose, and fructose strongly induced UIPKS4 gene expression at higher concentrations (10%). UIPKS6 was expressed at high levels in the basic MY medium, in media with a carbon source (inositol, mannitol, sorbitol, sucrose, glucose, and fructose), and in glutamine media. Asparagine, glycine, and alanine slightly downregulated UIPKS6 expression.

## DISCUSSION

*U. longissima* has a long history of use as a traditional medicine in China, India, Turkey, Canada, and Europe [23]. Several bioactive compounds that are synthesized via the polyketide pathway have been isolated from *U. longissima* (Fig. 4) [24, 25], including  $\beta$ -orcinol-type despides: atranorin, diffractaic acid; a  $\beta$ -orcinol-type depsidone: salazinic acid; an orcinol-type despide: evernic acid; a dibenzofuran: usnic acid; and an anthraquinone: longissimausnone. Similar to other lichens, *U. longissima* is a stable and self-

supporting symbiotic organism between a fungus and its photoautotrophic algal partners, and its secondary metabolites are mainly produced by the fungal component. However, in nature, lichens are composed of not only the mycobiont, algae, and cyanobacteria, but also include endolichenic fungi and lichenicolous fungi [26, 27]. The mixture of symbiotic organisms may provide imprecise information during the genetic analysis of LFF. Therefore, we isolated and cultured the LFF of *U. longissima*, using the Yamamoto method [28], to acquire genetic information.

In the present study, we investigated three NR-PKS genes from the LFF of *U. longissima*. Three entire NR-PKS genes (UIPKS2, 6,290 bp; UIPKS4, 7,927 bp; and UIPKS6, 6,780 bp) were identified via the screening of a Fosmid genomic library. UIPKS4 domain analysis revealed the presence of KS, AT, PT, ACP, MeT, and TE domains. The domain architecture of the UIPKS4 protein has also been identified in other non-lichenized fungi, such as *Penicillium brevicompactum*, in which MpaC is involved in 5-methylorsellinic acid synthesis [29], and *Acremonium strictum*, in which MOS is involved in methylorcinol synthesis [30]. All of the identified genes had the domain organization KS-AT-PT-ACP-MeT-TE. Phylogenetic analyses using the amino acid sequence of the PT domains revealed that both UIPKS2 and UIPKS4 belonged to group VI (Fig. 2), which also included *Penicillium brevicompactum* MpaC, *Acremonium strictum* MOS, and *Xanthoparmelia semiviridis* PKS1. All of these genes possessed a MeT domain.  $\beta$ -Orcinol lichen compounds require a NR-PKS with a CMeT domain [14, 15]. This suggested that UIPKS4 may be involved in  $\beta$ -orcinol depside or depsidone synthesis. However, domain analysis of UIPKS2 revealed the presence of the KS, AT, PT, ACP, and TE domains, but no MeT domain, which may have been lost during the course of evolution [11].

Phylogenetic analyses using the amino acid sequence of the PT domains revealed a close relationship between UIPKS6 and the NR-PKS from *Cladonia grayi* (38% sequence identity; accession No. ADM79459.1), followed by *A. nidulans* (35% sequence identity; accession No. XP\_681178). All of these

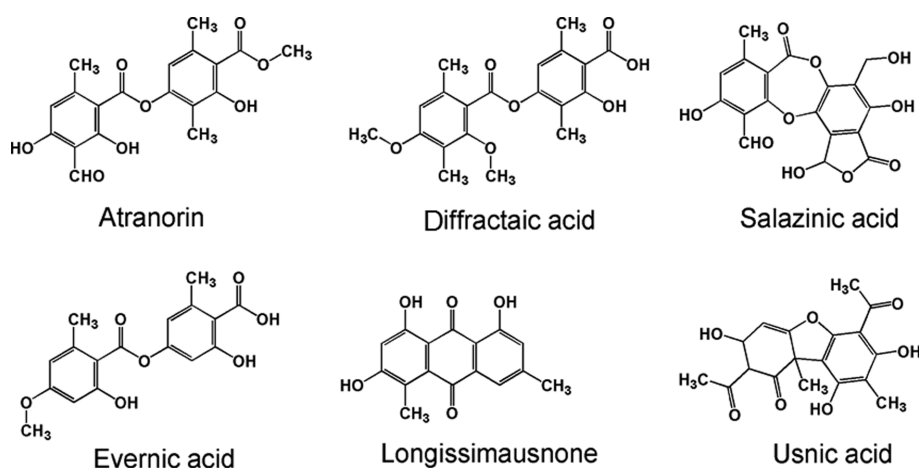


Fig. 4. The structures of the bioactive substances isolated from *Usnea longissima*.

PKSs fell into group I (Fig. 2). Group I included the *C. grayi* PKS16, involved in the production of depside grayanic acid [11], orsellinic acid synthase AN7909 from *A. nidulans* [31], and the NR-PKSs that are involved in the synthesis of orcinol-based resorcylic acid lactones, including the zearalenone PKS13, the hypothemycin Hpm3 [32], and the radicicol RDC1/RADS2 [33]. However, comparison of the domain organization of the group I PKSs showed that *UIPKS6* possessed an extra DH domain, suggesting that *UIPKS6* is a novel NR-PKS producing orcinol-based metabolites.

Carbon and nitrogen sources influence secondary metabolism in cultured LFF [18, 34, 35], and also influence the expression of biosynthetic genes. We analyzed gene expression in different nutrient conditions. Expression patterns showed that carbon-source media (inositol, mannitol, sorbitol, sucrose, glucose, and fructose) did not alter basal *UIPKS6* expression, but asparagines, glycine and alanine, slightly downregulated *UIPKS6* expression. *UIPKS4* expression varied across media. Glutamine, glycine, inositol, mannitol, and glucose slightly upregulated *UIPKS4* expression. Sorbitol (10%), sucrose (10%), and alanine (1%) strongly induced *UIPKS4* expression. *UIPKS2* was not expressed in amino acid media and most carbon source media (inositol, mannitol, and sorbitol), and was only expressed strongly in sucrose medium (10%). *UIPKS2*, *UIPKS4*, and *UIPKS6* showed different expression patterns, but all were strongly expressed by high concentration (10%) of sucrose. This response is similar to the role of sucrose in the osmotic activation of the acetate-polymevalonate pathway leading to polyketide production (divaricatic acid) in *Evernia esorediosa* and *Ramalina subbreucuscula* [34]. It suggests that sucrose and high osmotic culture conditions can activate the PKS pathway in LFF.

We reported three novel NR-PKSs from *U. longissima*, and predicted their function according to their domain composition and phylogenetic relationships. As no transformation system is currently available for LFF, future studies will focus on the heterologous expression of these NR-PKSs in other well-studied filamentous fungi, such as *Aspergillus* sp., to determine their functional characterization. Such studies will provide a basis for the sustainable application of lichen secondary metabolites in the pharmaceutical industry.

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