Bioproduction of Depsidones for Pharmaceutical Purposes

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1. Introduction

Lichens are intimate and long-term specific symbioses of photosynthetic algae or cyanobacteria and heterotrophic fungi joined to form a new biological entity different from its individual components (Galun & Kardish, 1995). Mainly chlorolichens produce unique phenolic substances, depside, depsidones and some dibenzofurans, such as usnic acids (Huneck & Yoshimura, 1996), that are not produced by other fungi and plants, and that show some biological activities with interesting applications from a pharmacological point of view, mainly as antiviral agents or for dermatological treatments. Although semi-synthetic organic processes have been attempted for the production of depsidones (Elix et al., 1987), they results very tedious, expensive and inappropriate for industrial applications. Alternatively, extraction from lichen thalli collected in nature implies a very high rate of biomass destruction which cannot be balanced because of the very slow rate of growth of these organisms. This last condition also invalidates the use of transgenic specimens. Thus, the use of bioreactors using very small amounts of lichen biomass seems to be as yet the most viable alternative to rapid and efficient production of depsidones.

Depsidones are organic compounds consisting of two phenolic acids linked together by both ester and ether bonds. So far, type I polyketide synthases (PKSs) are the suggested catalysts for the biosynthesis of these lichen compounds (Muggia & Grube, 2010). It is generally accepted that depsidones come from depsides, formed by two molecules of orsellinic acid derivatives linked by an ester bond, which means that the formation of ether was carried out on previously formed depsides. Thus, despide and depsidones are closely related and biogenetically they all seem to belong to only one group of chemical structures. It seems to be therefore definite that they are evolved from the same primary compound, the variations being brought about by processes of oxidation and reduction, and other simple reactions (Seshadri, 1944).

Polyketide synthases, also known as PKSs, are a family of enzymes or enzyme complexes that produce polyketides, a large class of secondary metabolites in bacteria, fungi, plants, and a few animals lineages. The biosyntheses of polyketides share striking similarities with fatty acid biosynthesis (Khosla et al., 1999; Jenke-Kodama et al., 2005). The pathway is

started by an acetyl-CoA carboxylase which produces malonyl-CoA, the main substrate of PKSs. These enzymes assemble structurally diverse products from simple acyl-CoA substrates by using a catalytic cycle involving decarboxylative Claisen condensations and variable modifications, such as reduction and dehydration. The PKS genes for a certain polyketide are usually organized in one operon in bacteria or in gene clusters in eukaryotes. Each type I polyketide-synthase module consists of several domains developing specific, catalytic functions, separated by short spacer regions. From N- to C-terminus, domains are arranged as: acyltransferase (AT), acyl carrier protein (ACP), keto-synthase (KS), ketoreductase (KR), dehydratase (DH), enoylreductase (ER), O- or C- methyltransferases (MT), sulfhydrolase (SH) and thioesterase (TE). Further optional accessory domains are represented by cyclase (CYC) (Fujii et al., 2001) and methyl transferase (MT) (Hutchinson et al., 2000) activities. In contrast to "post-PKS" O- and N-methylation reactions, which are catalyzed by distinct enzymes after polyketide assembly, methylation of the polyketide carbon backbone takes place during chain formation by means of the intrinsic fungal C-MT domains (Nicholson et al., 2001).

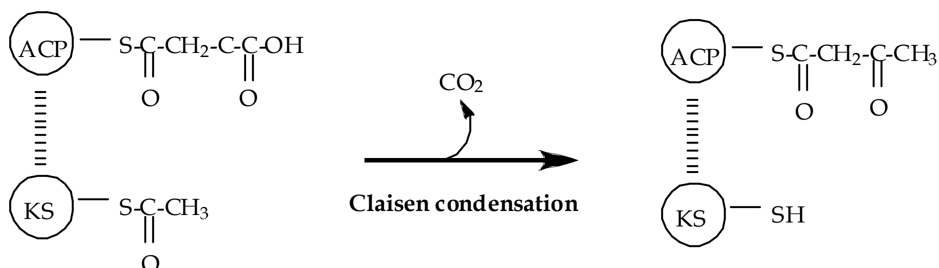

Fig. 1. Claisen condensation catalyzed by PKSI in which a 4C units suffers decarboxylation before receiving a metyl group of an acetate residue bound to KS subunit.

The occurrence of the Claisen condensation (Fig. 1.) is the main step in polyketide biosynthesis (Schümann & Hertweck, 2006) since it permits the production of a phenolic acid with a methyl group as substituent, such as orsellinic acid or 6-methylsalicylate, whereas the absence of this condensation reaction only produces polihydroxy derivatives such as tetrahydroxynaphtalene. After Claisen condensation, a new acetyl-CoA molecule binds on KS subunit to be newly transferred on elongating chain to form a final, lineal precursor of 8C before cyclisation (Fig. 2).

Products of PKS1 action are orsellinic acid or methyl-3-orsellinate when PKS contains an intrinsic methyl transferase subunit, although many derivatives can be synthesized by introducing into orsellinate molecule several chemical motifs through post-PKS modifications defined as tailoring reactions by Rawlings (1999). In some occasions, organic procedures of semi-synthesis or synthesis are used as good models to the experimental approach to the biosynthesis of lichen compounds. However, the biosynthetic pathway is very different from the synthetic way of methyl-3-orsellinate production since methyl-3-orsellinate can be generated from methyl-3-orcinol through the nitration of aryl function to be then reduced to amine group. This last derivative could be the substrat of a diazotonization for the corresponding diazonium salt. After this, a cyanide group could be introduced by nucleophilic substitution using potassium cyanide and then, the carboxyl

function could be produced after hydrolysis (Xavier Filho et al., 2004). For other derivatives, organic synthesis has been successfully used to a model to elucidate the biosynthetic way of polyketide production.

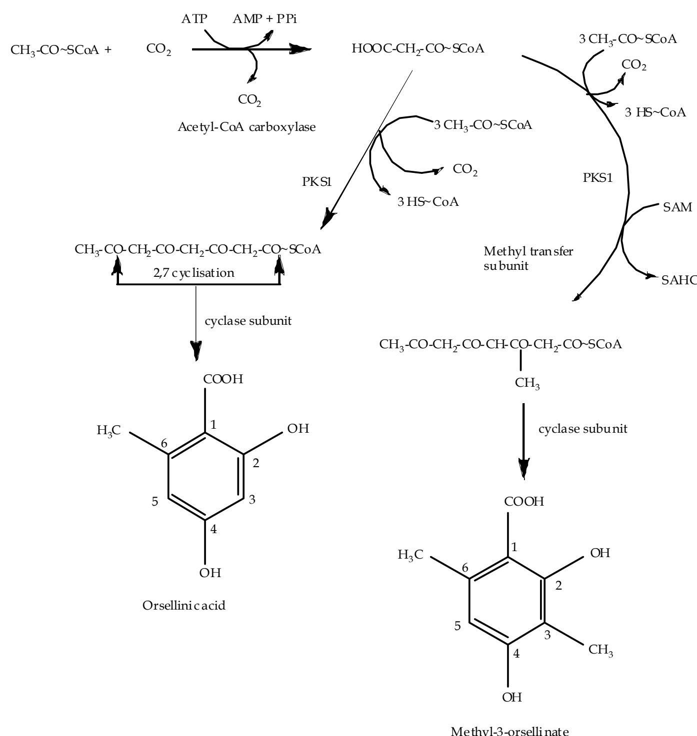

Fig. 2. Orcinol (left) or β-orcinol (right) cyclisation catalyzed by the cyclase subunit of two different PKS without or with methyl transferase subunit.

Based on the presumed catalytic domains required for the synthesis of the key intermediates β-orsellinic acid and methylphloroacetophenone, two pairs of degenerate primers were designed to target specifically the β-ketoacylsynthase (KS) and C-methyltransferase (CMeT) domains of fungal non-reducing polyketide synthase (NR-PKS) genes with CMeT domains. These primers were used to explore the genome of the lichen *Xanthoparmelia semiviridis*, which produces β-orcinol depsidones and usnic acid.

One of the two KS domains amplified from genomic DNA of field-collected *X. semiviridis* was used as a probe to recover the candidate PKS gene. A 13 kb fragment containing an intact putative PKS gene (xsepks1) of 6555 bp was recovered from a partial genomic library (Chooi et al., 2008).

Type III polyketide synthases do not use the acyl carrier protein domain and a simple homodimer of 40-45 kDa proteins performs the complete series of decarboxylation, condensation and cyclisation reactions (Parsley et al., 2011). Type III PKSs are represented by the gene families of chalcone synthases (CHSs) and stilbene synthases (STSs), which until recently have been regarded as typical for plant secondary metabolism (Figure 3). The

discovery of type III PKS genes in a screening of more than 50 fungal genomes and their presence in lichen mycobionts suggest that these polyketide synthases are widely distributed in ascomycetes (Muggia & Grube, 2010). Recent evidence supports the hypothesis of an ancient horizontal gene transfer of type I PKS genes from bacteria to fungi (including lichen mycobionts). Schmitt & Lumbsch (2009) suggested that homologues of these genes could be involved in the production of orsellinic acid, due to their relationship with enzymes producing 6-methylsalicylic acid in bacteria and fungi.

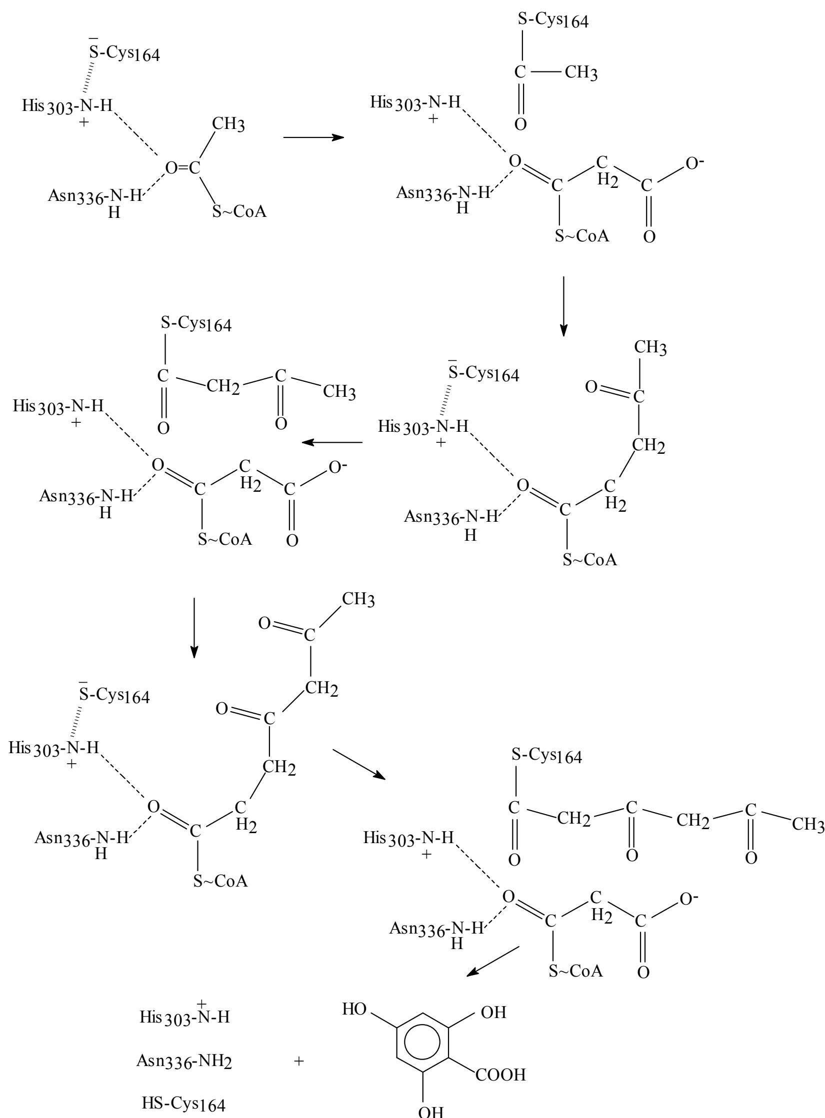

Fig. 3. Biosynthesis of a polyketide chain catalyzed by PKS III.

2. Chemical modifications of orsellinate units

A haematommoyl alcohol dehydrogenase has been described for the first time in a lichen species, *E. prunastri*. The enzyme catalyzes the oxidative production of haematommic acid, a phenolic subunit of the depside atranorin, from haematommoyl alcohol using NAD^+ as a cofactor. The enzyme is also able to reduce the haematommic acid subunit in the atranorin molecule using NADH as an electron donor. However,the affinity of the enzyme for haematommic acid is about three times higher than that found for the depside. The enzyme has been located, using adequate histochemical techniques, in the fungal medulla of the thallus whereas it is completely absent from the algal cells. Synthesis of the depside was enhanced by oxygen and NADH. This enhancement suggested the participation of an oxydase and an alcohol dehydrogenase to produce an aldehyde-substituted phenolic acid as the most probable precursor of atranorin (Fig. 4). The participation of both enzymes has been confirmed by loading immobilisates of living lichen cells with sodium azide (inhibitor of several metallo-oxydases) and pyrazole (an inhibitor of alcohol dehydrogenase), that impede the production *in vivo* of atranorin (Millanes et al., 2003).

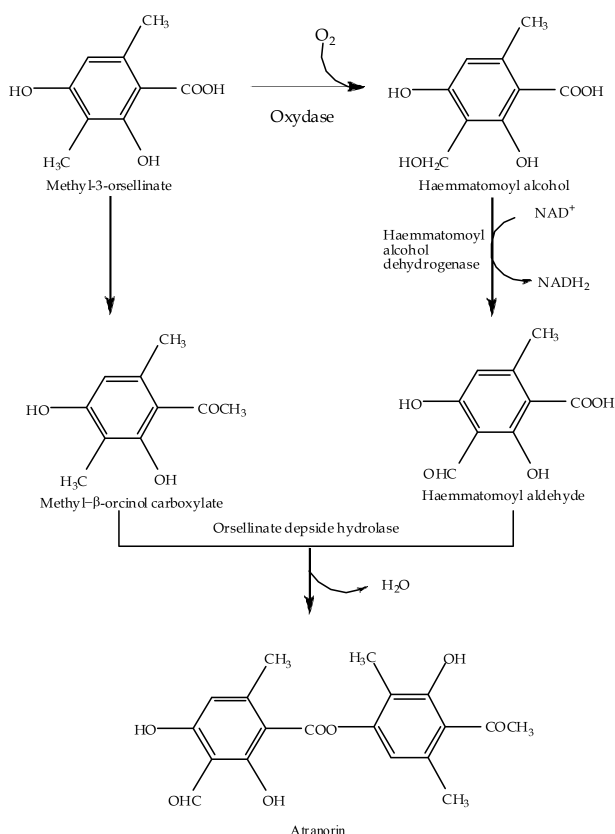

Fig. 4. Biosynthesis of atranorin, a depside of the β-orcinol series, requires the chemical modification of the precursor, methyl-3-orsellinate, by two successive oxidation reactions catalyzed by an O_2-dependent oxidase and a NAD^+-haemmatomoyl alcohol dehydrogenase.

3. Depside biosynthesis

Depsides are synthesized from their monocyclic precursors by an esterification reaction, and it is possible that several esterases exist. Mosbach & Ehrensvård (1966) found that *Lasallia pustulata* contained an esterase which hydrolyzed gyrophoric, umbilicaric and evernic acids. This orsellinate despide hydrolase has been purified and characterized. The molecular mass of a 135-fold purified enzyme was about 42 kDa, as determined by SDS-PAGE. This hydrolase was an exceptionally stable protein, withstanding incubation at 57 °C for 10 min without any loss of activity. In addition, it could be stored frozen for 6 months or at 25 °C for 4 days with practically no loss of activity. The K_m value for lecanoric acid, according to a michaelian kinetic, was estimated as about 56 μM (Schultz & Mosbach, 1971). The enzyme hydrolyzed gyrophoric, lecanoric and evernic acids but no hydrolysis was achieved when *iso*-evernic and digallic acids, or phenylbenzoate, were used as substrates. This indicated that a free hydroxy group in ortho position to the ester bond was required for activity.
A similar esterase has been purified from *Evernia prunastri*. Optimal temperature varied from 30 °C to 50 °C, optimum pH over 8 to 9 and its molecular weigh was estimated as about 120 kDa by SDS-PAGE. The K_m value for evernic acid was estimated at 21.3 mM. Orcinol, as well as both D- and L-usnic acids, behave as competitive inhibitors of the enzyme with K_i values of 0.72, 2.87 and 6.0 mM, respectively. Salicylic acid behaves as a noncompetitive inhibitor with a K_i value of 29.6 mM (González et al., 1984). The enzyme is extremely thermolabile in solution but it remains active at room temperature during several weeks after immobilization in polyacrylamide (García-Junceda et al., 1991a). In addition, orcinol was unable to inhibits immobilized hydrolase in a concentration range from 0.1 to 1.0 mM. *Evernia* hydrolase behaved as a constitutive protein (Vicente & Legaz, 1988) since increases in enzyme activity following incubation of thalli on buffer, 2% (w/v) bicarbonate or 35 μM evernic acid solutions were not impeded by 100 μM cycloheximide. A complete loss of enzyme activity was achieved after drying the lichen thallus at room temperature for 3h. It seems that water content was the major factor affecting hydrolase activity in this lichen species.

4. Depsidone biosynthesis

The existence of coupled depside-depsidone in the same lichen species, such as olivetoric-physodic acids in *Cetraria ciliaris* (Culberson, 1964) indicates that these compounds could be biogenetically related. In fact, it has been proposed that depsidones are formed by dehydrogenative coupling of depsides. Such a synthetic strategy was used when a naturally occurring depsidone was synthesized in the laboratory. In any case, the formation of monocyclic units seems to be a common pathway for both depsides and depsidones. This conclusion was derived from labelling experiments in which the distribution of radiactivity from $^{14}CO_2$ into the depsides evernic acid, atranorin and chloroatranorin, synthesize by *E. prunastri* (Blanco et al., 1984), and into depsidones, physodic and physodalic acids from *Hypogymnia physodes* (Fox & Mosbach, 1967), were almost identical and coincided with that expected from a conventional PKS. Several authors have recognized that the secondary structural differences between known depsides and depsidones need not necessarily have occurred after cyclisation. In other words, the attachment of a fumarate residue on 3-substituted carbon atom of methyl-3-orsellinate to produce the second monocyclic precursor of the depsidone, fumarprotocetraric acid, can be achieved on the monocyclic phenol, on

atranorin (the depside probable precursor of the depsidone) or even on the depsidone itself. Vicente et al. (1984) found that the time course of fumarprotocetraric acid production in *Cladonia sandstedei* floating on urea was inversely related to that ot atranorin accumulation. This may imply a biogenetical relationship between both compounds, but it does not provide any evidence about the structural change described above. However, norstictic or salazinic acids are produced by *C. sandstedei* thalli floating on ammonia, whereas fumarprotocetraric acid and atranorin are completely remobilized. This may be considered as evidence supporting the suggestion that depsidones can be modified after the formation of the ether bond.

To explain the occurrence of iso-structural depside-depsidone pairs, Elix et al. (1987) and Culberson & Elix (1989) suggested that the C-hydroxylation of a *p*-depside in the 5′ position would be followed by acyl migration and a subsequent Smiles re-arrangement of the formed *m*-depside would lead to orcinol-depsidones. This hypothesis has been used to explain the biosynthesis of grayanic acid by the mycobiont of *Cladonia grayi* in axenic culture (Culberson & Armaleo 1992, Armaleo 1995). Alternatively, García- Junceda et al. (1991b) have found that *Pseudevernia furfuracea*, containing the depsidone physodic acid, produces a depsidone ether hydrolase which is able to hydrolyze the ether bond of physodic acid to produce 5-hydroxyolivetoric acid. So far, the appearance of substituents at 6 and 6′ positions has not been studied at an enzymatic level. However, Pereira et al. (1999) found that *Cladonia clathrata* cells immobilized on kaolinite and supplemented with acetate are not able to produce fumarprotocetraric acid, the natural depsidone produced by living thalli, but some of their precursors or catabolites, protocetraric, hypoprotocetraric or 4-O-methylhypoprotocetraric acids, are alternatively accumulated. This has been interpreted as a requirement of a redox coenzyme to support the reducing coupling of a succinyl-CoA rest to the alcohol function in C3position to produce fumarprotocetraric acid (Figure 5).

Depsidone ether hydrolase has been purified at homogeneity by ammonium sulfate precipitation, size-exclusion chromatography through Sephadex G-100 and ionic exchange chromatography in DEAE-Sephadex A50. Purified enzyme was immobilized in polyacrylamide and assayed for the hydrolysis of physodic acid. Both soluble and immobilized enzymes showed sigmoidal kinetics for substrate saturation, but their apparent K_m value, K'_m, decreased from 0.4 mM physodic acid for the soluble protein to 0.1 mM for the immobilized enzyme. The value of substrate interaction coefficient, n_H, showed to be 2.0 in both cases, as expected from the sigmodidal kinetics. The unusual decrease of the apparent K'_m value could be explained through a protein-protein interaction that would increase the allosteric acceleration for binding of the substrate to the enzyme molecule, since the inflexion point in the direct curves of substrate saturation, decreased from 0.3 mM physodic acid to 0.2 mM after immobilization. This probable interaction also was indicated by the fact that mixing two fractions obtained from DEAE-Sephadex column developing high and low enzyme activity, respectively, the mixture showed an hydrolase activity higher than the simple sum of the separate activities of each fractions. This protein-protein interaction could imply changes in the spatial structure of the enzyme which would increase its affinity for the substrate rather than an effective dimerization, since n_H value did not vary after immobilization (García-Junceda et al., 1991b).

The soluble enzyme showed an optimum temperature value of 30 °C and was completely inactivated at 60 °C. However, the optimal temperature for the immobilized enzyme was 35°C and retained about 40% of its activity at 70°C. Depsidone ether hydrolase remained almost completely active after 56 days of storage at room temperature.

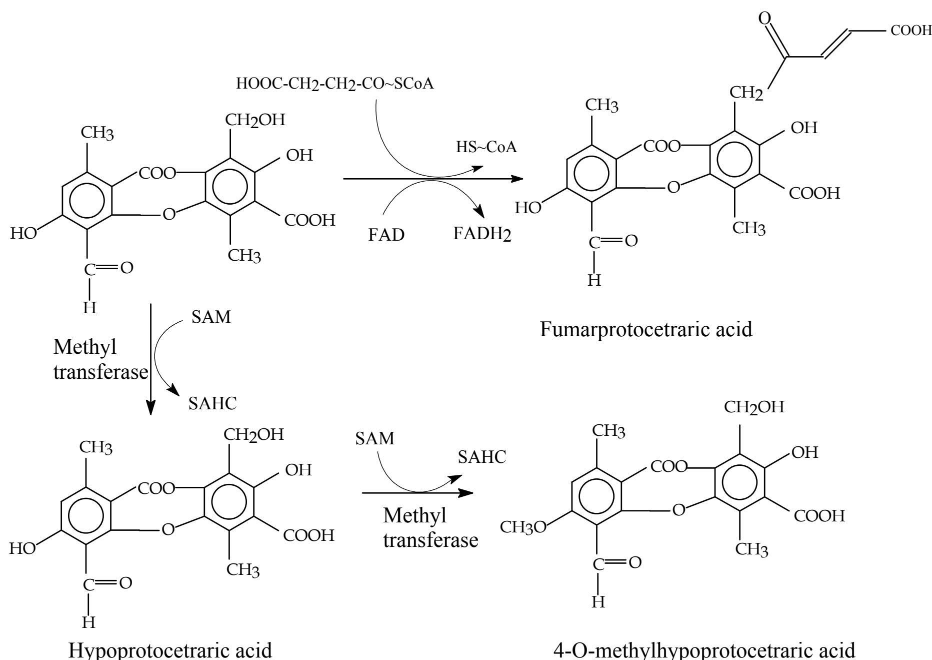

Fig. 5. Enzymatic addition of a fumarate residue, from succinyl-CoA, to the alcohol function at C3′ position of protocetraric acid to produce fumarprotocetraric acid. Alternatively, protocetraric acid could be used to the *in vivo* production of hypoprotocetraric and 4-O-methylhypoprotocetraric acids which are not substrate of the condensating reaction.

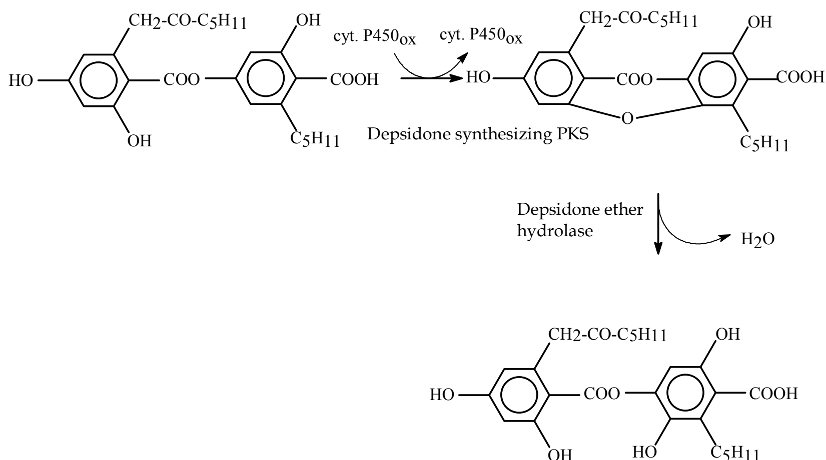

Fig. 6. CgrPKS16 protein, depending on cytochrome P450, catalyzes the oxidative production of a depsidone, physodic acid, from the corresponding depside, olivetoric acid. However, the catabolyzing enzyme depsidone ether hydrolase breaks the ether bond by hydrolysis, producing 5-hydroxyphysodic acid.

The natural occurrence of 5'-hydroxyolivetoric acid strongly suggests that depsidone could be produced by a single dehydration of the depside. This procedure is similar to that proposed by Sala et al. (1981) for the organic synthesis of some depsidones of *Buellia canescens.* However, Armaleo et al. (2011) described the first lichen PKS cluster likely to be implicated in the biosynthesis of a depside and a depsidone. Among the many PKS genes in *Cladonia gray*i they are four, named CgrPKS13 to16, potentially responsible for grayanic acid biosynthesis. CgrPKS16 protein domains were compatible with orcinol depside biosynthesis. Phylogenetically, CgrPKS16 fell in a new subclade of fungal PKSs uniquely producing orcinol compounds. In the *C. grayi* genome, CgrPKS16 clustered with a CytP450 and an O-methyltransferase gene, appropriately matching the three compounds in the grayanic acid pathway. Specifically, authors propose that a single PKS synthesizes two aromatic rings and links them into a depside, and the depside to depsidone transition requires only a cytochrome P450 (Figure 6).

5. Biotechnological production of depsidones

Several genes for lichen phenolics biosynthesis have been as yet identified and cloned. For example, the ketosynthase domains of putative polyketide synthase (PKS) genes from 15 species in the lichenized genus *Lecanora* as well as three representatives of other genera have been amplified and sequenced using conserved primers (Grube & Blaha, 2003). However thee low rate of cell division and the very low rates of vegetative growth have as yet impeded the production of transgenic specimens in which the production of a particular metabolite has been enhanced. Traditionally, biotechnological processes directed to overproduction of lichen phenolics have been related to cell cultures and cell immobilizations.

Aposymbiotically grown mycobionts can also produce these secondary metabolites in culture. However, they also frequently biosynthesize different polyketides than those of the natural lichen, or primary triacylglycerides and fatty acids (Adler et al. 2004; Molina et al. 2003; Reis et al. 2005). The obtained results are often difficult to interpret and factors favoring the production of lichen substances in culture have not been specified for several decades (Stocker-Wörgötter 2008). The production of lichen substances by aposymbiotically grown mycobionts frequently depends on culture conditions. Most experimental works indicate that these key conditions are related to composition of the nutrient medium (Zocher and Stocker-Wörgötter 2005) and stress parameters like osmotic stress (Kon et al. 1997; Hamada 1996), including slow desiccation of the medium (Stocker- Wörgötter 2002; Stocker-Wörgötter et al. 2004), temperature stress, or exposure to high light intensities like UV-C stress (Hager et al. 2008). Manipulations of the culture conditions have shown that a broader variation of metabolites can be obtained in culture than are actually found in natural lichen thalli (Hamada et al. 2001; Boustie and Grube 2005). A strain of the lichen mycobiont isolated from a thallus of *Parmotrema reticulatum* was cultured axenically on different media. Atranorin, the major cortical lichen depside, was produced when the colonies were grown over 5 and 10 months on solid LB medium, combined with a dessication treatment . Colonies grown on MEYE and MY10 with a desiccation treatment did not produce any lichen secondary metabolite. Mycobionts grown for 5 months on solid MEYE medium without a desiccation treatment produced triacylglycerides as the major metabolites, and the fatty acids were characterized as their methyl esters (Fazio et al., 2009).

Tissues from lichen cultures produce several diphenyl oxides, depsides and depsidones. Salazinic acid were detected in cultures of *Usnea flexilis*, *Usnea* sp., and *Ramalina* sp., and protocetraric was produced by *U. flexilis* (Yamamoto et al., 1998) in culture.

Immobilization of lichen cells using different natural or synthetic matrices is the usual procedure to produce lichen phenolics without an excessive use of biomass (Blanco et al., 2002). When cells of *C. verticillaris* were immobilized in calcium-alginate and supplemented with 1.0 mM acetate, a maximum of fumarprotocetraric acid was recovered from the medium at the 10th day. The appearance of this phenolic in the incubation medium could be due to the release of the pre-existent fumarprotocetraric acid from lichen thalli used to produce the immobilisate but a part of this phenolic might be really synthesized by the immobilized cells, since both FMN and CoA impeded, in some extent, the release of fumarprotocetraric released to the media. After this, the amount of fumarprotocetraric acid recovered from the media rapidly decreased for the 15th day and more slowly thereafter, to be practically nullified at the end of the experiment (90 days). Two main differences in the biosynthetic behaviour of *C. verticillaris* immobilisates and those of *C. clathrata*, previously described (Pereira et al. 1999), can be discussed. First, only traces of fumarprotocetraric acid were detected during the first days of immobilization of *C. clathrata* cells whereas *C. verticillaris* produced considerable amounts of the depsidone, although this ability was lost after ten days of beads storage. This loss could be possibly explained as a consequence of the lack of endogenous flavins required to bind succinyl-CoA to a precursor compound and to the impossibility of an effective uptake of exogenous FMN by immobilized cells. Second, *C. clathrata* releases protocetraric acid into the medium whereas this depsidone has not been detected as released from immobilized cells of *C. verticillaris*. However, cells permeabilized with 2-propanol and supplemented with acetate and FMN synthesized and released fumarprotocetraric acid continuously. This fact would confirm that cell permeabilization facilitates that flavin coenzyme enters lichen cells. Fumarprotocetraric acid was also continuously released from immobilized cells supplemented with acetate, but the recovering of the depsidone from bath media slowly decreased from the 7th day of immobilization when cells were stored on acetate only. The addition of 26 μM CoA to the medium containing 40 μM FMN did not enhance the production of fumarprotocetraric acid. This could indicate that CoA cannot get across cell membrane. Small amounts of 4-O-methylhypoprotocetraric acid were only released to the media in parallel to the highest production rate of fumarprotocetraric acid. However, 4-O-methylhypoprotocetraric acid is commonly accumulated by *Xanthoparmelia notata*, whereas hypoprotocetraric acid is a constant component of the chemosyndrom of *Ramalina hypoprotocetraric*a and *R. tumidula*. Release of atranorin from immobilized cells of *C. clathrata* was not influenced by the addition of FMN or CoA to the media, although its release progressively decreased to be nullified at 90 days (data not shown).

Recently, an enzyme capable to esterify an alcohol substituent of protocetraric acid with succinyl-CoA in a complex reaction coupled to dehydrogenation has been pre-purified in our laboratory. The enzyme has been isolated from thalli of *C. verticillaris* whereas protocetraric acid, used as substrate, was purified from *Ramalina farinacea*. The purity of the substrate was established by reverse phase HPLC. The enzyme converts protocetraric acid into fumarprotocetraric acid in a stoichiometric reaction dependent on FAD or FMN but it can also use NAD^+ and $NADP^+$ less efficiently. No reaction was achieved when succinyl-CoA was substituted by succinic acid and HS-CoA. The enzyme did not use hypoprotocetraric or 4-O-methylhyhypoprotocetraric acids as substrates of reaction (Figure

7). This implies that the carboxyl substituent in *meta* position with respect to the alcohol residue, which received the esterifying 4C-organic acid, is absolutely required to the correct formation of the enzyme-substrate complex. Apparently, *C. verticillaris* thalli develop esterifying enzyme activity higher in light than in the dark, since in light fumarprotocetraric acid accumulates in the medulla at high concentration values. Nevertheless, this fact does not imply a positive photo-regulation of the enzyme production but the highest supply of phosynthates from the algal cells to the mycobiont, since enzymes for depsidone production are always fungal proteins. In this way, Armaleo et al. (2008) found that light distribution correlated directly with the amount of atranorin and inversely with that of norstictic acid. We interpret these findings to suggest that sunlight indirectly affects depside and depsidone metabolism by influencing thallus temperature and water potential in the lichen *Parmotrema hypotropum*. *P. hypotropum* accumulates the depside atranorin in the cortex and the depsidone norstictic acid in the medulla and around the algae. A direct correlation was observed between the yearly amount of light reaching the lichen and the amount of atranorin. In contrast, the amount of norstictic acid decreased with increasing light. Authors suggest that depside/depsidone accumulation in lichens is mediated by localized changes in temperature and water potential produced by light absorption within each thallus (Figure. 8).

Although some of the enzymes described here have been successfully immobilized in several inert matrices, such as orsellinate decarboxylase, orsellinate depside hydrolase and depsidone ether hydrolase, always were used for basic research activity (Blanco et al., 2002). Biotechnological production of depsidones requires a process economically sustainable and the previous purification of the adequate enzyme and the chemical preparation of their substrates are very expensive to be used at an industrial scale. However, the use of immobilized cells basically supplemented with acetate is a process sufficiently rapid, accurate and reproducible to produce depsidones at a semi-industrial level.

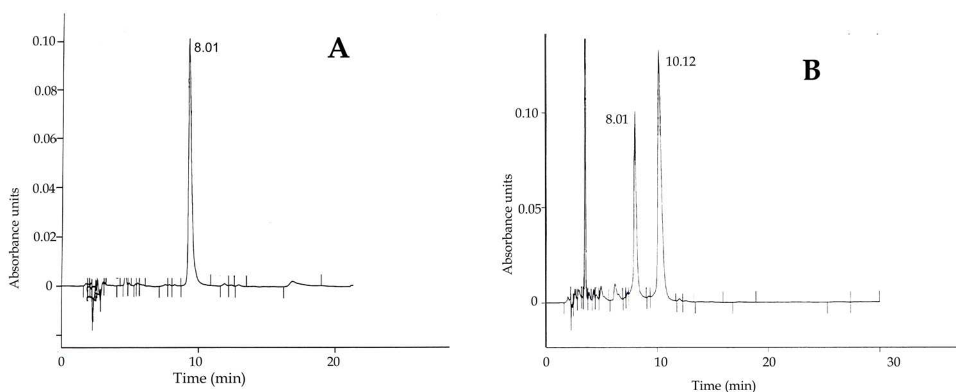

Fig. 7. Chromatographic traces in RP-HPLC of protocetraric acid, isolated from *Ramalina farinacea* (A) that eluted with a retention time value of 8.01 min, and phenolics extracted from a reaction mixture containing protocetraric acid and succinyl-CoA as substrates, the enzyme pre-purified from *Cladonia verticillaris*, and FAD as cofactor. Fumarprotocetraric acid elutes from the column with a retention time value of 10.12 min.

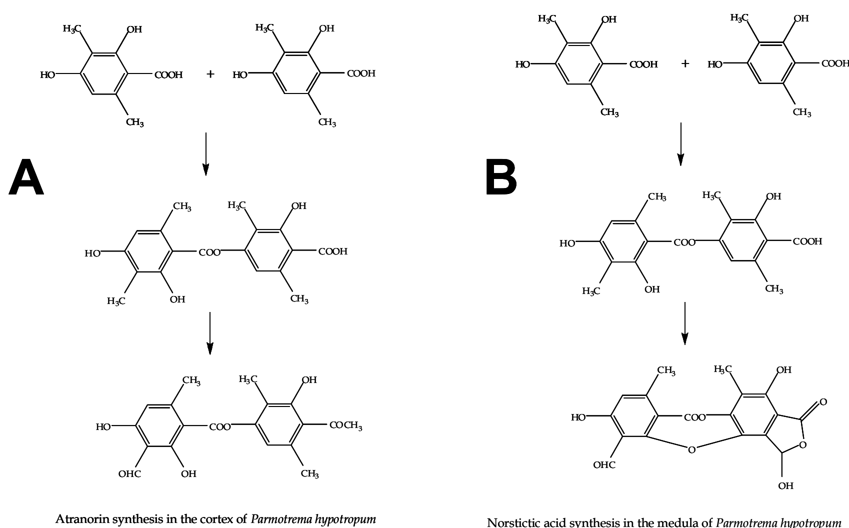

Fig. 8. Alternative production of atranorin (depside) and norstictic acid (depsidone) by thalli of *Parmotrema hypotropum*, according to Armaleo et al. (2008), as influenced by variable values of the ratio temperature/water potential (T/Ψ) along a day. When direct sunlight increases the temperature of the thallus and enhaces evapotranspiration (high T/Ψ values), atranorin is actively synthesized and deposited on the cortex (A). However, the decay of sunlight intensity decreases both thallus temperature and water loss (low values of T/Ψ) and, then, the depsidone norsticitc acid is actively produced and retained in the medulla (B).

6. Clinical and pharmaceutical applications

6.1 Depsidones as allergens

Parfums contains natural compounds used as fixing components. A fixing substance forms stable bonds, generally ester bonds, with those parfum components which easily volatilized, and then, the loss of these odoriferous substances to the air is delayed and they are retained on the skin for long time periods. One of the most popular fixing products if the oak moss, a complex mixture of phenols mainly extracted from three lichen species, *E. prunastri*, *Pseudevernia furfuracea* and *Usnea barbata* some of those containing depsidones (Huneck, 2001). In many cases, certain substances produced by lichens and included in cosmetic preparations cause skin photosensitization (Rademaker 2000). The same or similar substances become part of basic notes in perfumery and they can produce allergic reactions (Joulain & Tabacchi, 2009). Seven patients, from 2000, showed sensitivity to perfums containing oak moss as a part of the basic note. The substances mainly responsible for the allergic reaction are atranorin (4 patients), evernic acid (4 patients) and usnic acid (5 patients) as well as some depsidones, such as acids physodic/phisodalic acids, with low allergenic actitivy, or diffractaic and fumarprotocetraric acids, practically without allergic response. Atranorin is the compound that produces reactions of greater intensity. The clinical changes due to a severe reaction to the allergen were only observed in two patients and consisted of severe skinredness, swelling and interdigital sweating. In all other cases,

the reactions were weak, consisting of erythema and itching. These symptoms appeared associated with intermittent use for parfum (Thune et al., 1982).

6.2 Interactions with photodynamic astringents

The skin exposed to solar radiation leads to the formation of highly reactive intermediates such as singlets and triplets, oxygen singlet ($^1O_2$), hydroxyl radical ($OH^•$) etc, which can damage membranes by lipid peroxidation and oxidation of proteins. These free radicals and singlets also support the photosensitizing properties of some psoralens such as 8-methoxypsoralen, thus being able to act on such reactive intermediates in order to inhibit the photosensitizing action. The 8-methoxypsoralen is able to bind covalently to proteins such as lysozyme and bovine serum albumin (BSA), although the covalent conjugate with SAB, which is formed in the presence of oxygen under UV-irradiation, seems to follow an entirely different mechanism of photo-cycloaddition of the molecule to DNA. When the covalent bond is attempted in the presence of depsidones pannarin or 1'-chloropannarin, the photoprotective capacity of these phenols lichen can be explained on the basis of their ability for inhibiting the photobinding of psoralen to human serum albumin (Fernandez et al., 1998).

Human skin irradiation with both UV-A and UV-B increases the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and this contributes to inflammation, immunosuppression, gene mutation and carcinogenesis. The depsidone pannarin also acts as a potent dose-dependent agent for superoxide scavenging (Russo et al., 2008). Irradiation of pannarin, 1′-chloropannarin and atranorin with 366 nm light leads to significant hemolysis in a red cell suspension. However, their mechanism of action is different. Hemolysis induced by pannarin and 1′-chloropannarin increases in the presence of oxygen, whereas hemolysis induced by atranorin is higher in nitrogen-purged solutions. The effect of free radical scavengers, and the lack of effect of D_2O in the medium, suggest that the hemolysis induced by pannarin and 1′-chloropannarin is not mediated by $^1O_2$. Both the hemolytic and photohemolytic activities of the depsidones, particularly 1′-chloropannarin, increase when the temperature increases from 21 to 37 °C (Hidalgo et al., 1993).

6.3 Antibacterial activity

Lichens have been used for centuries in folk medicine for the treatment of infectious diseases. According to Ingolfsdottir (2002), *Usnea barbata* was used by Hippocrates to treat urinary disorders and *U. longissima* was used in China as an expectorant. Usnic acid isolated from *Cladonia arbuscula* shows high antibiotic activity against *Mycobacterium aurum*, a non-pathogen bacterium with similar sensitivity profile to *M. tuberculosis*. Lobaric acid and atranorin (depsides) isolated from *Stereocaulon alpinum*, salazinic acid (depsidone) from *Parmelia saxatilis*, and protoliquesterinic acid (a cycloaliphatic acid), isolated from *Cetraria islandica*, show four times lower activity against the same and other microorganisms than that detected for usnic acid (Ingolfsdóttir et al., 1997). Usnic acid (a dibenzofuran derivative) also shows antibiotic activity against some Gram-negative bacteria such *as Streptococcus pneumoniae* and *Enterococcus foecalis* and against many anaerobic bacteria such as *Bacteroides fragilis*, *Clostridium perfringens* and *Propionibacterium acnes* (Lauterwein et al., 1995). Some depsidones from *Ramalina farinacea* also develops anitimicrobial activity. Norstictic acid was active against *Aeromonas hydrophila* as well as the above microorganisms except *Yersinia enterocolitica*. Protocetraric acid showed activity only against the tested yeasts *Candida*

albicans and *Candida glabrata* (Tay et al., 2004). Basically, the antibiotic activity of lichen compounds lies to these actions that derive from its phenolic nature, such as membrane lipid peroxidation, uncoupling of oxidative phosphorylation, and inhibition of DNA duplication

Whereas usnic acid has received much attention from researchers as a potential antibiotic, depsidones have sometimes been also studied. Ingólfsdóttir et al (1998) reported that salazinic acid develops antimycobacterial activity. Honda et al. (2010) studied the antibiotic properties of several different depsidones, protocetraric, salazinic, norstictic, hypostictic acids, and five salazinic acid derivatives on *Mycobacterium tuberculosis*. They conclude that the activity against the microorganism depends on the physico-chemical parameters (lipophilicity and pKa) of each compound, that is a function of the substituents in each molecule evaluated and of its structural characteristics. A comparison among these shows that the more lipophilicis more active than the less lipophilic compound. Such features may result in intramolecular interactions among these groups, for example, by hydrogen bonds, which affect the lipophilicity and the pKa.

6.4 Cytotoxicity

Cell exposure to certain xenobiotics can cause cell damage or toxicity. The cytotoxic activity of different lichen polyphenols (9 depsidones, 3 depsides and one tridepside) isolated from various lichen species from South America was tested on cell cultures of rat lymphocytes (Correche et al., 2002). After addition of phenols, the cytotoxicity was analyzed by the addition of tritiated thymidine, a highly sensitive indicator of mitosis. The assay revealed that the majority of the metabolites exhibited a marked cytotoxic activity, in some cases even exceed that shown by colchicine, a potent inhibitor of the achromatric spindle. Comparing the rates of toxicity of the two groups of compounds (depsides and depsidones) also noted that the first turned out to be minor and therefore the depsidones such as salazinic, psoromic, fumatrotocetraric, lobaric, stictic, and variolaric acids could be potentially more harmful than the depsides divaricatic and diffractaic acids, as well as sphaerophorin (depside). In this assay, the toxicity index of depsidones ranged between 1.95 and 34.96. A comparative analysis of the toxicity index and the structure of these compounds showed some correlations. The presence in the ring of both aldehyde and hydroxyl groups positioned into carbons 3 and 4 respectively, was associated to a high rate of toxicity. Chemical species without such radicals showed significantly lower levels of toxicity. Depsides showed lower levels of toxicity that depsidones, reaching the value of cytotoxicity index to a maximum of 7.5. By comparing the chemical structure of these species to the degree of toxicity developed, a relationship between the presence of an acid group in C1 and a hydroxyl in C2 seems to be required but, as noted for atranorin, the occurrence of an aldehyde and an adjacent hydroxyl could explain their high cytotoxicity. It follows that the toxicity is in part conditioned by the ability to establish hydrogen bonds between adjacent radicals, as determinant of the biological response. However, (-)-usnic acid was the only compound to display a moderate cytotoxic activity on various cancer cell lines from a mixture of varios phenolics isolated from the lichen *Cladonia convoluta*, the depsidone 9'-(O-methyl)protocetraric acid and fumarprotocetraric acid. Usnic acid was also shown to induce apoptosis of murine leukaemia L1210 cells in a dose- and time-dependent manner (Bézivin et al., 2004).

By using a human melanoma cells (M14 cell line), Russo et al. (2008) found that sphaerophorin (depside) and pannarin (depsidone) showed a protective effect on plasmid DNA and exhibited a superoxide dismutase like effect. The data obtained from cell culture show that these lichen metabolites inhibit the growth of melanoma cells, inducing their apoptotic cell death, demonstrated by the fragmentation of genomic DNA and by a significant increase of caspase-3 activity, and correlated, at least in part, to the increase of ROS generation.

Alternatively, some non-lichenized fungi are also able to produce depsidones with cytotoxic activity. An endophytic fungus (*Botryosphaeria rhodina*), isolated from the stems of the medicinal plant *Bidens pilosa*, produces four depsidones, botryorhodines A–D, which exhibit potent cytotoxic and antiproliferative effects against several cancer cell lines, such as HeLa cells (Abdou et al., 2010). Ten depsidones, mollicellins, produced by the fungus *Chaetomium brasiliense*, showed also cytotoxic activity against KB, BC1, NCI-H187 lines, and five cholangiocarcinoma cell lines (Khumkomkhet et al., 2009).

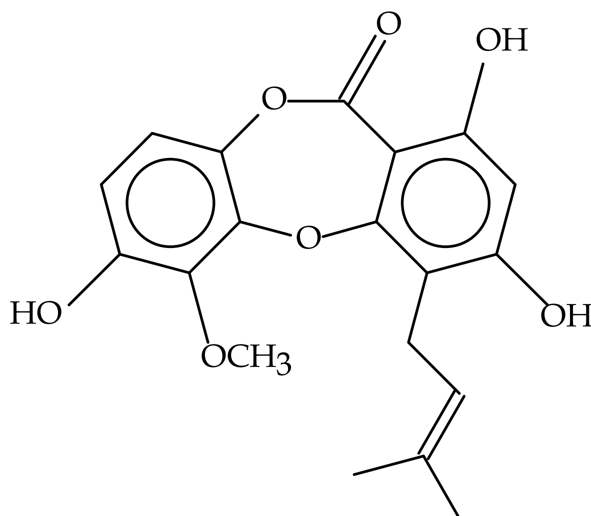

Fig. 9. Chemical structure of garcinisidone F fram *Garcinia assigu*.

Some depsidones have been found in higher plants. For example, *Garcinia assigu* (Gutaceae) produces garcinisidone A-F the chemical structure of which is almost identical to those found in lichens (Fig. 9) . Garcinisidones B to F show inhibitory effects on the Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in Raji cells. Experiments examining the structure-activity relationship, focusing on the garcinisidone nucleus, demonstrated that the 9-prenyl substituent and the dimethylpyran ring on the A and/or B ring were not essential for the activity. In previous studies, it has been found that the prenyl side chain on xanthone, 7-methoxycoumarin, phenylpropanoid, and isoflavonoid nuclei plays an important role in anti-tumor-promoting activity. In view of the present findings taken together, garcinisidones with one or two prenyl side chains on the A-ring might be valuable anti-tumor-promoting agents effective against chemical induced carcinogenesis

6.5 Antioxidant activity

The depsidones norstisctic and fumarprotocetraric acids (Lohézic-Le Dévéhat et al., 2007) showed better superoxide anion scavenging activity (IC50 = 0.566 and 0.580 mM, respectively) than quercetin (IC50 = 0.754 mM). The antioxidant activity of lichenic metabolites, depsides and depsidones, was also assessed by their effects as inhibitors of rat brain homogenate auto-oxidation and β-carotene oxidation (Hidalgo et al., 1994). The results obtained in both systems indicate that lichenic metabolites afford a moderate protection in the micromolar concentration range. The largest effect was measured employing 1'-chloropannarin in the brain homogenate auto-oxidation, where a 66% protection was afforded at 1.7 μM. This protection is very similar to that elicited by addition of the reference antioxidant propylgallate (70% protection at 1.3 μM). Fumarprotocetraric acid from *C. verticillaris* possesed effective antioxidant activity at all concentrations assayed (Xavier Filho, personal communication). This antioxidant activity increased with the concentration used in the assay: 0,1 mg/mL of the product produced a 77,86% inhibition of the peroxidation of human plasma lipids (Fig. 10) and a 33,2% of the peroxidation of linoleic acid emulsion (Fig. 10).

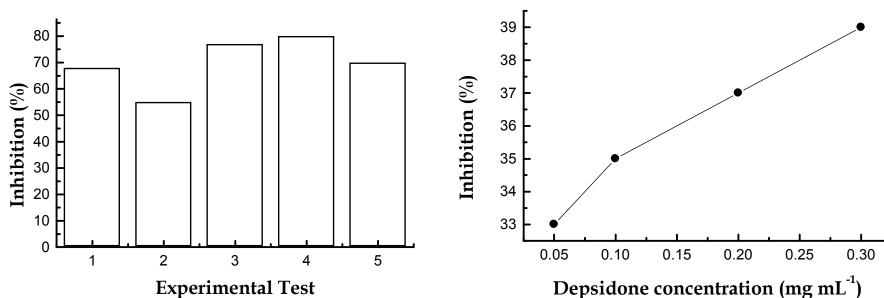

Fig. 10. Left: Inhibition of plasma's lipid peroxidation by 0.1 mg mL^{-1} fumarprotocetraric acid from *Cladonia verticillaris*. Number indicated different assays and number 5 is the average inhibition of four assays of plasma lipid peroxidation. Right: Inhibition of linoleic acid peroxidation by different concentrations of fumarprotocetraric acid.

6.6 Antiviral activity

Some depsidones showed to be active against HIV by inhibiting the viral integrase. During infection, integrase catalyzes two consecutive reactions. Initially, the enzyme processes the viral linear DNA by cutting two nucleotides from each 3 'end, leaving the 3'-OH end free. This is followed by the trans-esterification of phosphodiester bonds, one strand of DNA cutting and joining the 5 'end of the cut to the processed viral 3'-end. These two steps, called processing 3 'and band transfer of DNA can be measured in vitro using a purified recombinant integrase and a 21-mer duplex oligonucleotide corresponding to the end-HIV U5 LTR sequence (Vlietinck et al., 1998).

Using a 3D database of the National Institute of Cancer and the appropiate software (Chem-X), Neamati et al. (1997) have identified two potential architectures of active pharmacophores for virensic acid and other depsidones such as physodic acid. Both compounds, as well as granulatine (the methylester of virensic acid), exhibit high potential

for inhibition of viral integrase processing activity for both 3' processing and for the transfer belt. The common constant of both pharmacophores is to present a fixed distance of 8.37Å between the two neighboring hydroxyl to the carboxyl group of both phenolic units, a dimension possibly related to the topography of the domain receptor of depsidone.
The depside atranorin showed an activity slightly lower than that found for depsidones, but its oxime completely lost its inhibitory activity. Since integrase required to perform its catalytic action a divalent cation (Mg^{2+} or Mn^{2+}) as a metallic cofactor, it was thought that the chelating action of the lichen phenols could be the basis of its inhibitory activity of integrase, although there is still no clear demonstration for this assumption. It does seem to be specifically required that the polycyclic system was rigid and that when this stiffness decreases by introducing a substituent open-chain, significantly decreases the inhibitory activity.
Biotechnological processes consisting of the use of bioreactors with immobilized lichen cells is today the most accurate techniques for depsidone production. It has been used mainly to investigate the enzymatic pathways of depsidone biosynthesis and, in addition, it has been revealed as a very efficient method that produces high yields in product preparation. A bioreactor containing only 0.5 of lichen biomass is able to produce 3.0-5.0 g of the corresponding lichen products after two months with unappreciable loss of biosynthetic activity, never higher than 16 per cent. Moreover, bioreactor can be used to modify the chemical structure of particular phenolic molecules in order to increase a particular biological activity and a decrease of undesirable cytotoxic effect.

7. Acknowledgements

This work was supported by a grant from the Ministerio de Ciencia e Innovación (Spain), BFU2009-11983. We are indebted to Thalita Lucena, Eva M. Díaz, Elena Sánchez-Elordi, Borja Alarcón, Dr. Mara Sacristan, Prof. Eugênia C. Pereira and Prof. Lauro Xavier Filho for your help and criticism.

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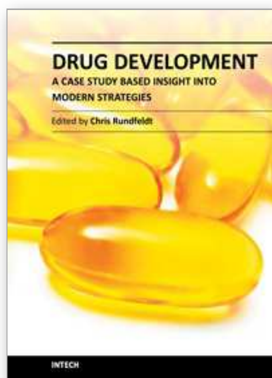

Drug Development - A Case Study Based Insight into Modern Strategies
Edited by Dr. Chris Rundfeldt

ISBN 978-953-307-257-9
Hard cover, 654 pages
Publisher InTech
Published online 07, December, 2011
Published in print edition December, 2011

This book represents a case study based overview of many different aspects of drug development, ranging from target identification and characterization to chemical optimization for efficacy and safety, as well as bioproduction of natural products utilizing for example lichen. In the last section, special aspects of the formal drug development process are discussed. Since drug development is a highly complex multidisciplinary process, case studies are an excellent tool to obtain insight in this field. While each chapter gives specific insight and may be read as an independent source of information, the whole book represents a unique collection of different facets giving insight in the complexity of drug development.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Maria Estrella Legaz, Roberto de Armas and Carlos Vicente (2011). Bioproduction of Depsidones for Pharmaceutical Purposes, Drug Development - A Case Study Based Insight into Modern Strategies, Dr. Chris Rundfeldt (Ed.), ISBN: 978-953-307-257-9, InTech, Available from: http://www.intechopen.com/books/drug-development-a-case-study-based-insight-into-modern-strategies/bioproduction-of-depsidones-for-pharmaceutical-purposes

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