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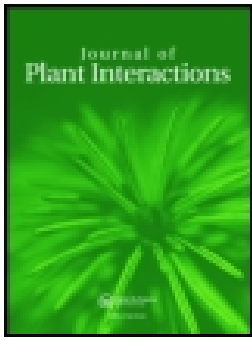
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## Endophytes: exploiting biodiversity for the improvement of natural product-based drug discovery

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## ORIGINAL ARTICLE

### Endophytes: exploiting biodiversity for the improvement of natural product-based drug discovery

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Endophytes, microorganisms that colonize internal tissues of all plant species, create a huge biodiversity with yet unknown novel natural products, presumed to push forward the frontiers of drug discovery. Next to the clinically acknowledged antineoplastic agent, paclitaxel, endophyte research has yielded potential drug lead compounds with antibacterial, antiviral, antioxidant, insulin mimetic, anti-neurodegenerative and immunosuppressant properties. Furthermore, while being implicated in livestock neurotoxicosis, some endophyte-produced alkaloids have been shown to display insecticidal activity. The endophyte-host relationship is postulated to be a 'balanced antagonism'. Moreover, the plausibility of horizontal gene transfer (HGT) hypothesis is taken into account. Knowledge of the genetic background of endophytic natural product biosynthesis is discussed on the basis of loline alkaloids, ergopeptines, lolitrems and maytansinoids. The current dynamic progress in genomics will contribute to a better understanding of endophytic microbes and to further exploiting them as a source of pharmaceutically relevant compounds.

**Keywords:** endophyte; natural product; drug discovery; plant-microbe interactions

#### Introduction

*Endophyte* (Gr. *endon*, within; *phyton*, plant) – the term was first coined by de Bary (de Bary 1866) and has become deeply embedded in the literature ever since. At present, endophytic organisms are defined as 'microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects' (Stone et al. 2000). First reports describing these microbes date back to the turn of the nineteenth and twentieth century (Freeman 1904). The most frequently encountered endophytes are representatives of the fungi; however, the existence of many endophytic bacteria has been documented as well.

Of the nearly 300,000 plant species inhabiting our planet, each individual one is host to several to hundreds of endophytes (Tan and Zou 2001), creating an enormous biodiversity: a myriad of undescribed species, a rich source of novel natural products therefrom and an unknown genetic background of all the interdependencies thus implied.

Several reviewers addressed the issue (e.g. Tan and Zou 2001; Strobel and Daisy 2003; Owen and Hundley 2004; Schulz and Boyle 2005; Gunatilaka 2006), shedding light onto selected aspects of the nature of endophytes. The aim of the hereby presented study is to render a comprehensive overview of the current knowledge on the subject. Considerable emphasis is put on the role and potential of metabolic engineering (Tyo et al. 2007) and combinatorial biosynthesis (Floss 2006), as well as on their impact on exploiting the astounding diversity of the endophytic world for its pharmaceutical potential.

#### Endophytes as biological factories of functional metabolites

##### *Endophytic natural products as drugs and novel drug leads*

It seems impossible to underestimate the impact of natural products on the drug discovery process. Even with lack of support from the major pharmaceutical companies, arguing that the natural product screening paradigm established in the late 1980s and early 1990s was no longer compatible with the HTS (high-throughput screening) approach, the significance of secondary metabolites as a source of novel drugs and drug-leads is still alive and well. Over 20 natural-product-derived drugs have been launched onto the worldwide market from 2001–2005 and around 140 have undergone various stages of clinical development in all major therapeutic areas (Butler 2005; Lam 2007).

The recent development and implementation of new technologies offers unique opportunities in the screening of natural products and will re-establish them as a major source for drug discovery. Improved natural product sourcing, aiming at narrowing the focus from all available sources to a single most prolific and reliable one, points to microorganisms as the ultimate, readily renewable, reproducible and inexhaustible source of novel structures bearing pharmaceutical potential. It is believed that up to 99% of microorganisms have yet to be discovered (Davis et al. 2005). This undefined richness of microbial world encompasses the plethora of endophytic entities occupying utterly millions of unique

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biological niches (higher plants) in various, many a time unusual, environments (Strobel 2006).

Functional metabolites of endophytic origin have already demonstrated a considerable potential to impact the pharmaceutical arena (Tan and Zou 2001; Strobel 2003; Strobel and Daisy 2003; Strobel et al. 2004; Gunatilaka 2006). A few examples are presented in this section, with the focus on their presumed therapeutic significance.

As the anti-infective branch is experiencing a shortage of lead compounds progressing into clinical trials, new antibacterial templates with novel mechanisms of action should have advantages over known antibiotics, especially in the fight against multi-drug resistant bacteria and emerging pathogens. Guanacastepene (Figure 1), a novel diterpenoid produced by a fungus isolated from the branch of *Daphnopsis americana* growing in Guanacaste, Costa Rica, might prove a representative of a potentially new class of antibacterial agents, showing activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (Singh et al. 2000).

Although apparently the potential for the discovery of endophytic compounds having antiviral activity is in its infancy, probably due to absence of appropriate antiviral screening systems in most compound discovery programmes, some promising fungal metabolites have been found. Initially, two novel tridepside human cytomegalovirus (hCMV) protease inhibitors, cytonic acids A and B were isolated from *Cytonaema* sp. inhabiting the internal tissues of *Quercus* sp. (Guo et al. 2000). Further studies of the microbial flora characteristic of oak trees resulted in the isolation of a potentially valuable fungal specimen from the leaves of *Quercus coccifera*. This endophyte proved to be a synthesizer of hinnuliquinone (Figure 2) – a potent inhibitor of the HIV-1 protease (Singh et al. 2004).

Two isobenzofuranones, pestacin and isopestacin (Figure 3), with structural similarity to the flavonoids – a well-established group of free-radical-scavengers, proved to exceed the anti-oxidant activity of trolox (a vitamin E derivative) by at least one order of

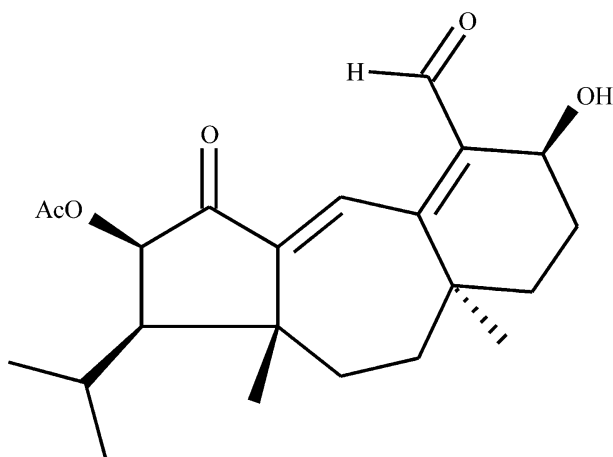


Figure 1. Guanacastepene.

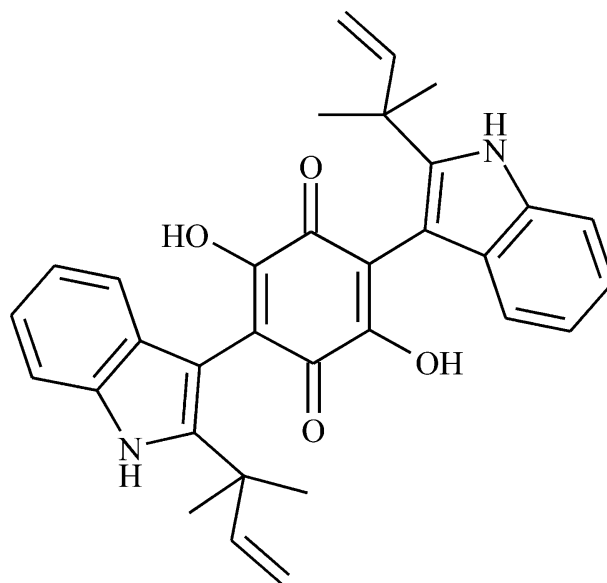


Figure 2. Hinnuliquinone.

magnitude, as measured by the total oxyradical scavenging capacity (TOSC) assay. These new potent antioxidants were obtained from *Pestalotiopsis microspora*, an endophyte of *Terminalia morobensis* inhabiting the Sepik River drainage of Papua New Guinea (Strobel et al. 2002; Harper et al. 2003).

*Pseudomassaria* sp., a fungal endophyte recovered from leaves of an undetermined plant collected near Kinshasa, Democratic Republic of Congo, was shown to be a biofactory of nonpeptidic insulin mimetic, L-783,281 (Figure 4). Its discovery prompted quite a revolutionary notion in the therapy of diabetes, namely: an orally administered activator of the human insulin receptor (Zhang et al. 1999).

Moreover, this intriguing endophytic metabolite was reported capable of stimulating the *Trk* family of tyrosine kinase receptors, leading to the activation of multiple signalling cascades, culminating in neuroregenerative effects, including neuronal survival and neurite outgrowth (Wilkie et al. 2001). Although the cytotoxicity of the compound seems to preclude its direct therapeutic application, it is a prototype for small molecule insulin and neurotrophin mimetics, and may lead to the development of pharmaceutically significant compounds for the treatment of diabetes and neurodegenerative disorders.

Following the track of search for new immunosuppressants from endophytes resulted in the isolation of subglutinol A (Figure 5) and B. These diterpene pyrones from *Fusarium subglutinans*, harbored by the perennial twining vine *Tripterygium wilfordii*, showed substantial immunosuppressive activity while causing none of the detrimental cytotoxic effects characteristic of cyclosporine A (Lee et al. 1995; US Patent 5648376, 1997).

Amongst the bioactive natural products of endophytic origin described to date, the ones notable for their antitumor activity seem to have drawn quite

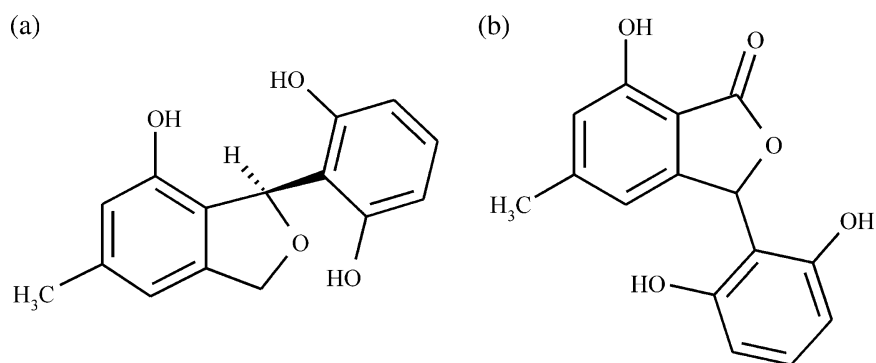


Figure 3. Pestacin (a) and Isopestacin (b).

an unequivocal attention, with paclitaxel (Taxol) as the most striking example (Figure 6).

Since its discovery in the 1960s, through structure elucidation completed in 1971 and FDA approval in 1992, this highly functionalized diterpenoid natural product has evolved to become a blockbuster drug with commercial sales of well over \$3 billion in 2004 (Croteau 2005). Since it was primarily obtained from the inner bark of *Taxus brevifolia*, a relatively rare and slow-growing tree, the supply of this potent antineoplastic agent was soon to become the issue of scarcity, as even early estimations indicated that the demand for paclitaxel might exceed 300 kg, which would amount to 750,000 yew trees per year (Stierle et al. 1995). The supply crisis, as well as the ecological implications resulting in the plant-endangered status, prompted the search for paclitaxel-producing microorganisms among the endophytic fungi of *Taxus*. While the 1990s brought quite an abundance of reports on paclitaxel-producing endophytes (e.g. Stierle et al. 1993; Strobel et al. 1996, 1997; Li et al. 1998a, b; Noh et al. 1999; Zhou and Ping 2001), no conclusive follow-up data concerning fungal metabolite profile or genetic background of the biosynthetic pathway leading to paclitaxel in fungi is available at present. While the pursuit of the idea of a microbial paclitaxel source, providing for an inexhaustible supply of this antineoplastic blockbuster and novel taxanes, is being hampered by obtaining disappointingly low yields, the active ingredient is still mainly derived via chemical semisynthesis from the advanced taxoid, 10-deacetylbaccatin III, readily available from

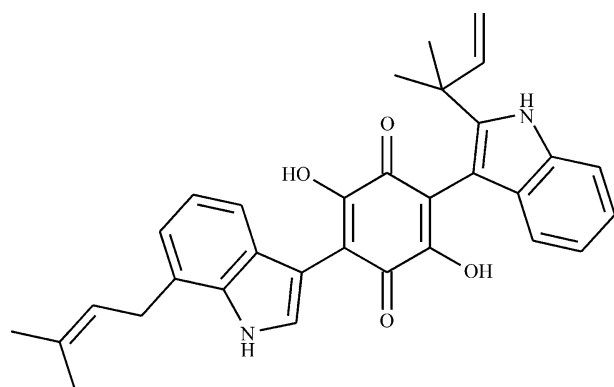


Figure 4. L-783,281.

the needles of the European yew tree, *Taxus baccata*, being a renewable source and, to a lesser extent, by means of plant cell culture methods (Leistner 2005; Frense 2007). Nonetheless, the exciting progress that has been made in the elucidation of biosynthetic route leading to paclitaxel *in planta* due to fundamental works of Croteau and his co-workers (Walker and Croteau 2001; Jennewein et al. 2004a, b; Croteau 2005; DeJong et al. 2006; Nims et al. 2006), as well as the recent advances in microbial genomics (Zazopoulos et al. 2003; Stephanopoulos et al. 2004; Keller et al. 2005; van Lanen and Shen 2006) and combinatorial biosynthesis (Floss 2006; Nguyen 2006; Klein-Marcuschamer et al. 2007), might still revive and boost the interest in endophytic paclitaxel synthesizers.

#### ***Agricultural aspects: endophytic insecticides vs. tremorgenic mycotoxins***

As the world becomes wary of ecological damage done by synthetic insecticides, endophytic research

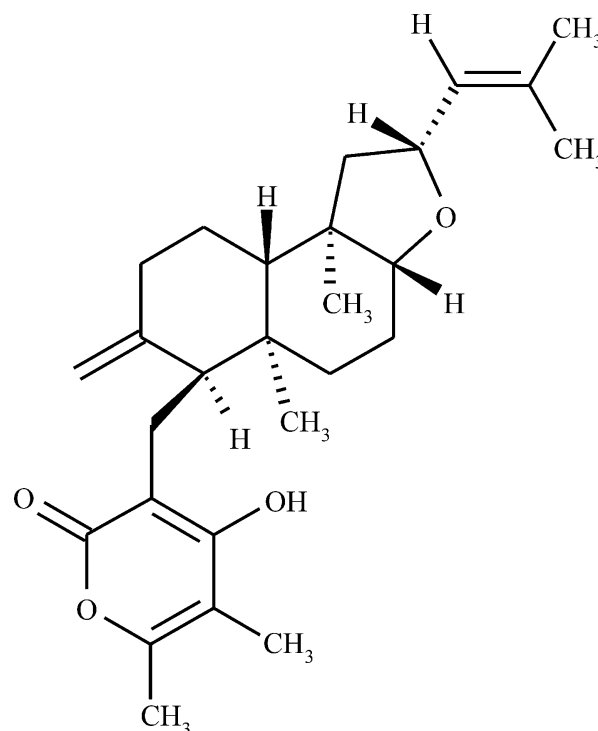


Figure 5. Subglutinol A.

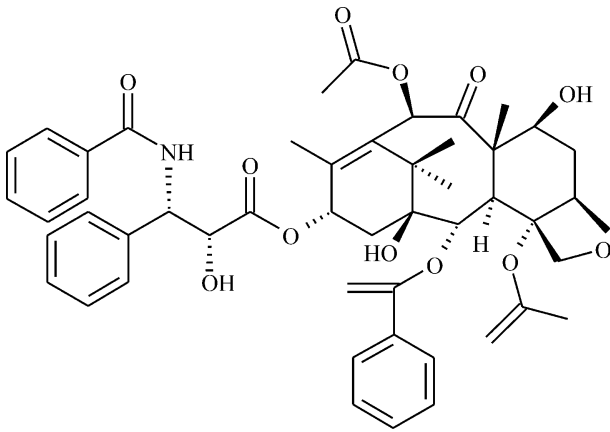


Figure 6. Paclitaxel.

continues for the discovery of powerful, selective and safer alternatives (Strobel and Daisy 2003).

From 1930–1970 millions of hectares in the temperate transition zone of the south central and southeastern USA were converted from revegetation shrubs and weeds to pastures of tall fescue (*Festuca arundinacea*). This grass was superior forage to native flora, but it was soon noticed that the expected livestock productiveness was not being met. An associated complex of symptoms episodically suffered by grazing livestock was termed ‘tall fescue toxicosis’ and consequently led to considerable economic losses to the US beef industry (Hoveland 1993; Thompson and Stuedeman 1993). Simultaneously, the livestock grazing on perennial ryegrass (*Lolium perenne*) pastures in New Zealand was afflicted by ‘ryegrass staggers’ (Gallagher et al. 1984; Bush et al. 1997). These problems spurred intense research that led to the isolation and identification of two respective fungal endophytes: *Neotyphodium coenophialum* and *Neotyphodium lolii*, both phylogenetically related to the ergot fungus *Claviceps purpurea* (Kuldau et al. 1997). Thus, the said endophytes were implicated in the associated livestock toxicoses, but both also greatly enhanced fitness of their plant hosts under biotic and abiotic stresses. Further investigation of the genus *Neotyphodium* (sexual state: *Epichloë*) proved its representatives capable of synthesizing four groups of alkaloids. Of these, the ergot alkaloids and tremorgenic lolitrems cause neurotoxic effects on grazing or granivorous vertebrates (Steyn and Vleggaar 1985; Bacon et al. 1986). Peramine is an insect feeding deterrent and protects perennial ryegrass from the Argentine stem weevil, *Listronotus bonariensis*, a highly destructive insect pest in New Zealand (Rowan and Latch 1994; Tanaka et al. 2005). Lolines are potent insecticidal and anti-aphid agents, not being implicated in any mammalian toxicoses (Jackson et al. 1996; Wilkinson et al. 2000). Obviously, the last of the aforementioned groups of alkaloids should provide for the most promising candidates to aid the agricultural arena.

As the forage grasses associated endophytes seem to constitute an extensively investigated and well documented microbial group (see further sections), they might prove to become a platform for the endophyte research to advance to the next level of modern microbial genomics and systems biology (Stephanopoulos et al. 2004; van Lanen and Shen 2006).

## Endophyte vs. host: the relationship

### *A conflict of interests?*

Over the years, despite the controversy it seemed to have aroused in the early 1990s (Wennström 1994), the term ‘endophyte’ has evolved from its original suggested definition, which described merely the location, to depict the nature of the association between the microbe and the host-plant (Wilson 1995). To truly define the interaction, however, seems to be quite a task. In fact, it can vary in a seamless manner from mutualism to parasitism, based on a fine-tuned balance between the demands of the invader and the plant response (Kogel et al. 2006).

A very elegant hypothesis has been proposed and extensively documented by Schulz and Boyle (2005), postulating the relationship to be a ‘balanced antagonism’ (Figure 7).

The said notion can be deciphered as equilibrium, under environmental, physiological and genetic control, that results in fitness benefits for both partners. On one hand, the theory depicts fungal endophytes as ‘masters of phenotypic plasticity’, able to infect as endosymbionts, to colonize cryptically, and finally to sporulate as pathogens or saprophytes. This creative variability implies evolutionary potential. On the other hand, it does not exclude the possibility of secondary metabolites being a contribution of the endophytic partner to a mutualistic relationship. A very interesting example of the protective power of an endophyte involves the aquatic plant *Rhynholacis penicillata* collected from a river system in Southwest Venezuela where the harsh aquatic environment subjected the plant to constant beating by virtue of rushing waters, debris, and tumbling rocks and pebbles. Such circumstances should provide ample opportunity for pathogenic oomycetes to invade the plant. Still, the plant population remained quite healthy. Upon extraction and investigation of the endophytic flora of *R. penicillata*, a potent anti-fungal bacterium, *Serratia marcescens*, was identified, which in turn was shown to produce oocydin A (Figure 8), a novel anti-oomycetous compound that obviously provided the plant with the requisite protection from the water molds (Strobel et al. 1999).

Recently, it has been hypothesized that endophytes may protect their host plants by scavenging the damaging reactive oxygen species (ROS) generated by the plant defence mechanisms in response to

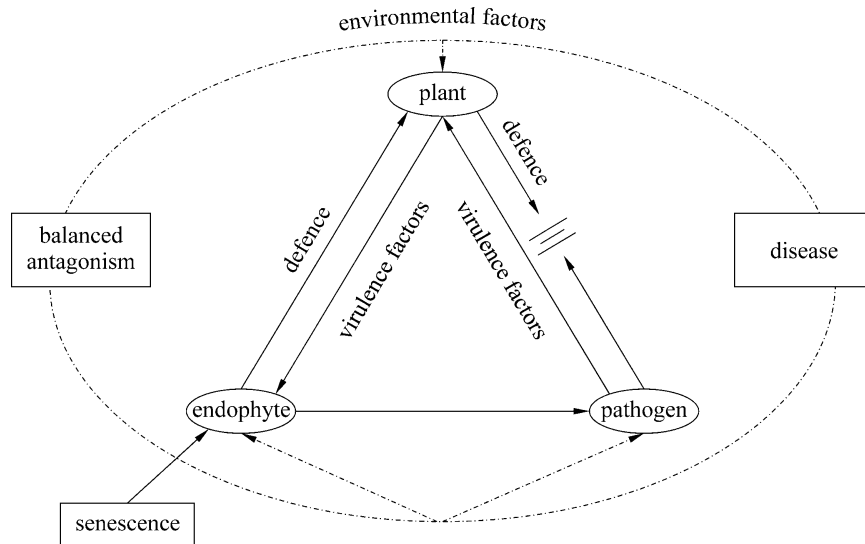


Figure 7. Balanced antagonism hypothesis (Schulz and Boyle 2005).

environmental stress (Rodriguez and Redman 2005; Tanaka et al. 2006). Thus, a strong activation of ROS due to biotic and abiotic stresses on part of the host followed by an equally rapid free-radical-scavenging response of the microbe might potentially be a prime mechanism in maintaining the delicate balance between the two.

To address the question asked in the title of this section: there is always a 'conflict of interests' at all stages of relationships between endophytic and plant partners (Smith and Read 1997). However, the development of tools for non-invasive observation of subcellular activities during the establishment of mutualistic interactions will provide a deeper understanding of the mechanism that balances virulence against defence; hostility against hospitality (Kogel et al. 2006).

#### *A relationship within a relationship*

While fungal viruses have been shown capable of modulating plant-fungal symbioses by regulating the hypovirulence of pathogenic fungi (e.g. Ahn and Lee 2001; Dawe and Nuss 2001), the effect of mycoviruses on mutualistic fungal endophytes has not been described. But one report of a mycovirus from the well-known endophytic microbe, *Epichloë festucae*, was recorded (Zabalgoeazcoa et al. 1998). Still, no decisive phenotypic traits have been associated with this virus since.

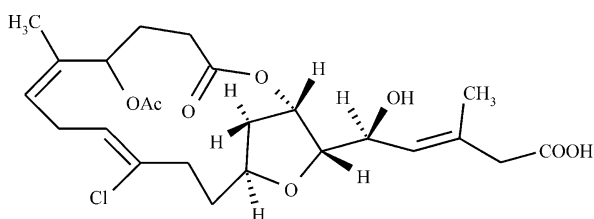


Figure 8. Oocydin A.

However, a very recent study reports a complex tripartite symbiosis involving a virus, a fungal endophyte and a plant (Márquez et al. 2007). Thus far it was believed that the mutualistic relationship between a tropical panic grass from geothermal soils, *Dichanthelium lanuginosum*, and the fungus *Curvularia protuberata*, allowed both organisms to grow at high soil temperatures in Yellowstone National Park (Redman et al. 2002). The in-depth investigation of this beneficial interaction showed, however, that the ability of the fungus to confer heat tolerance to its host plant is, in fact, related to a presence of yet another kind. To reflect its host of origin and its unique phenotype the responsible mycovirus was named *Curvularia thermal tolerance virus* (CThTV).

#### *A physical interaction merely? The plausibility of horizontal gene transfer (HGT) hypothesis*

While enthusiasts call it 'the essence of the phylogenetic process and the driving force in a new paradigm for evolution' (Doolittle 1999), sceptics describe it as no more than one of many phylogenetic anomalies (Kurland et al. 2003). Though highly controversial, the hypothesis of horizontal gene transfer seems quite seductive.

Hereby, two intriguing examples from the endophytic world are discussed. Both instances deal with the occurrence of identical natural products in unrelated taxa, namely: the host and the invader.

The presence of maytansinoids, potent cytotoxic agents, was first noticed in the Ethiopian shrub, *Maytenus serrata* (Kupchan et al. 1972). Further investigation recorded their occurrence not only in higher plants (Wani et al. 1973; Ahmed et al. 1981; Powel et al. 1982), but also in mosses (Sakai et al. 1988; Suwanborirux et al. 1990) and, remarkably, in gram-positive *Actinomycetes* (Higashide et al. 1977; Asai et al. 1978). One could assume that the biosynthesis of these unique natural products has

been repeatedly invented during evolution. However, the fact that approximately 48 genes are involved in the bacterial synthesis of maytansinoids (Yu et al. 2002) makes it highly unlikely. Similarly, the aforementioned ubiquity of paclitaxel occurrence in yews as well as in taxonomically distant fungal microbes raises questions. Therefore, it seems possible that in the course of evolution a lateral (horizontal) gene transfer took place between different, taxonomically unrelated species, thus explaining the distant distribution of the antineoplastic secondary metabolites mentioned above between pro- and eukaryotes.

Nevertheless, before invoking HGT, alternative and often equally plausible, explanations ought to be thoroughly considered. In case of maytansinoids, all evidence seems to point to them being ultimately produced by plant associated microorganisms. Maytansine, the unique parent compound (Figure 9), was found neither in cell suspension cultures from *Maytenus buchananii* (Kutney et al. 1981) nor in callus cultures raised from *Maytenus wallichiana* (Dymowski and Furmanowa 1990) and *Putterlickia verrucosa* (Pullen et al. 2003).

This is in line with the result of an in-depth search for the unique gene involved in maytansinoid biosynthesis, encoding for 3-amino-5-hydroxybenzoic acid (AHBA) synthase, in *Putterlickia verrucosa* cell cultures. An extensive PCR based homology screen gave negative results only (Pullen et al. 2003). These observations point to the conclusion that plants do not produce maytansinoids *ab initio*. However, an active role of the plant in an overall biosynthesis cannot be excluded, as it seems likely that the host converts a bacterially synthesized precursor into the final, biologically active compound. Secondly, it is possible that maytansine is only produced as a consequence of a pathogen attack on the plant. The plants may contain a biologically inactive bacterially produced precursor, which is only converted into the potent final product in response to a signal resulting from the attack. Alternatively, and more plausibly, the bacterial production of the maytansinoid pre-

cursor could be triggered by a plant signal in response to the pathogen aggression (Cassady et al. 2004).

On the contrary, the bio-formation of paclitaxel seems to be a genuine feature of the yew host, as ample evidence supporting the production of the diterpenoid by sterile cell suspension cultures of *Taxus* has been provided (e.g. Ketchum and Gibson 1996; Ketchum and Croteau 1998; Yukimune et al. 2000; Wu and Lin 2003; Naill and Roberts 2005; Khosroushahi et al. 2006; Vongpaseuth and Roberts 2007). This conclusion is further supported by the aforementioned work of Croteau and his associates who succeeded in the isolation of paclitaxel biosynthetic genes of plant origin. Interestingly, the taxadiene synthase gene has a long N-terminal targeting sequence for localization to and processing in the plastids, indicating that this gene is plant-derived rather than a fungal product (Koepp et al. 1995; Walker and Croteau 2001). Accordingly, an extensive PCR-based screen for taxadiene synthase gene in *Taxomyces andreanae*, the very first presumed endophytic taxane-producer (Stierle et al. 1993), failed to provide for any positive results (Staniek, unpublished data).

To sum up, the evidence for lateral gene transfer in eukaryotes remains largely anecdotal (Rosewich and Kistler 2000). However tempting and attractive, the HGT hypothesis has to give way to a more plausible alternative postulating the endophyte-host co-evolution.

#### Investigating the genetic background of endophytic biosynthetic pathways: identification, molecular cloning, genetic analysis and manipulation of endophytic gene clusters

Clusters of functionally related genes are a general feature of prokaryotic gene organization, but were believed to be much less prevalent in eukaryotes. However, the advent of recombinant DNA methodologies in the 1980s enabled dramatic progress in the genetics and biochemistry of fungal secondary metabolism. This rapid progress was facilitated by what is now considered a hallmark characteristic of secondary metabolic biosynthetic pathways – the grouping of pathway genes in a contiguous cluster (Keller and Hohn 1997; Keller et al. 2005).

#### The balansiaceous endophytes and their natural products

The balansiaceous endophytes form a unique group of closely related fungi with ecological requirements and adaptations distinct from those of other endophytes (Petrini 1996). They belong to the clavicipitaceous genera *Epichloë* and *Balansia*, and their anamorphs *Neotyphodium* and *Ephelis* (Schardl et al. 2004).

As previously stated, due to their ecological and economic impact, theirs seems to be the best studied group of endophytes. The following section focuses

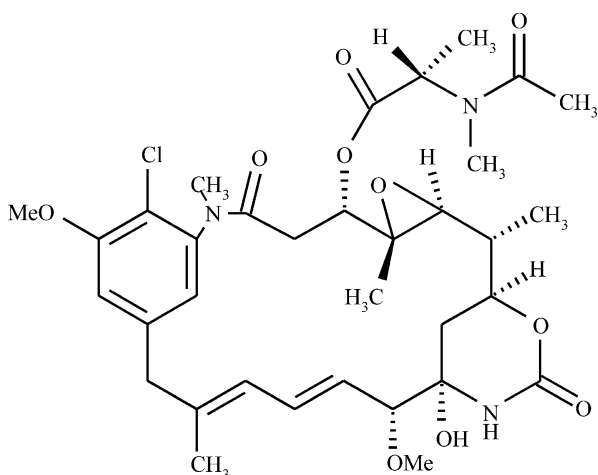


Figure 9. Maytansine



on the recent advances in dissecting the molecular mechanisms driving the biosynthesis of their secondary metabolites.

#### *Loline alkaloids (LA)*

Unique not only in structure, comprising a saturated 1-aminopyrrolizidine-ring system, with a highly strained ether bridge between C-2 and C-7 (Figure 10) (Petroski et al. 1989), but also due to their potent, broad-spectrum insecticidal activities (Jackson et al. 1996; Wilkinson et al. 2000), loline alkaloids have remained a biosynthetic enigma till very recently (Blankenship et al. 2005).

Establishing that the production of lolines in axenic cultures of *Neotyphodium uncinatum* can be regulated by culture conditions (Blankenship et al. 2001), which in turn suggests differential expression of genes involved in LA biosynthesis, prompted a notion that the isolation of the genes up-regulated during loline production could be a first step in identifying possible enzymes catalyzing their biosynthesis. The recent development of novel highly effective methods for identifying differentially expressed transcripts, enabled the application of suppression subtractive hybridization technique (Diatchenko et al. 1996, 1999) and provided for a successful identification of two genes, *lola* and *lolC*, associated with endophytic loline synthesis (Spiering et al. 2002).

These encouraging results spurred intense research on the molecular genetics of loline-alkaloid production, originating from a preliminary hypothesis of *lola* and *lolC* being clustered (Spiering et al. 2005; US Patent 7183098, 2007). The question was tackled with an impressive arsenal of up-to-date techniques and ultimately resulted in the identification of double homologs of nine genes similarly arranged into two clusters, *LOL-1* and *LOL-2*. Long-range (LA: long and accurate) PCR and parallel screening of a partial genomic library of *N. uncinatum* to ascertain the existence of two highly similar, yet distinct gene clusters. Extensive genome walking into unknown regions yielding a total of 25 kb sequenced from *LOL-1* and 16 kb from *LOL-2*. Gene-prediction searches with the FGENESH pro-

gramme (available from: <http://www.softberry.com/berry.phtml>) aided by RACE (rapid amplification of cDNA ends) and confirmed by reverse transcription-PCR based expression verification for identifying and mapping ORFs (open reading frames). Large-scale utilization of Basic Local Alignment Search Tool (NCBI) to ascribe the putative functions of the enzymatic entities encoded by the nine *lol* genes. And, last but definitely not least, the very first attempt of gene silencing by RNAi (RNA interference) performed on a mutualistic fungus, providing direct and indisputable evidence for involvement of *lolC* in loline biosynthesis.

All in all, an unprecedented endeavor leading to a final conclusion that the products of the *lol* genes thus identified may be sufficient for the biosynthesis of the entire loline-alkaloid three-ring structure in *Neotyphodium uncinatum*, from the primary precursor amino acids, L-proline and L-homoserine (Figure 11) (Blankenship et al. 2005; Spiering et al. 2005).

As spectacular as it may seem, the aforementioned accomplishment did not put a closure to solving the riddle of endophytic lolines. On the contrary, it contributed to further identification of single *LOL* gene clusters in *Epichloë festucae*, *Neotyphodium* sp. PauTG-1 and *Neotyphodium coenophialum* (Kutil et al. 2007).

To analyze the genetic architecture and to predict the evolutionary history of *LOL*, the five characterized clusters were compared by means of the coupled powers of phylogenetic approaches and other forms of sequence analysis. All *lol* genes anchored to the map for each species turned out to occur in strictly conserved order and orientation (Figure 12).

Furthermore, PhyloCon-based (Wang and Stormo 2003) comparison of the putative *lol* gene promoter regions yielded the identification of four motifs conserved across the genes in all five clusters, with each motif having significant similarity to known fungal transcription factor binding sites in the TRANSFAC database (Wingender et al. 1996). Conservation of these motifs further supported the hypothesis of the *lol* genes being co-regulated (Spiering et al. 2002, 2005).

Interestingly, the history of asexual *Neotyphodium* spp. includes multiple interspecific hybridization events (Tsai et al. 1994; Schardl 2001; Clay and Schardl 2002; Moon et al. 2004). Thus, comparing the clusters from three seed-transmitted *Neotyphodium* anamorphs and their sexual counterpart, *E. festucae*, allowed determining which *Epichloë* ancestors were the most likely contributors of *LOL* in these asexual loline synthesizers. Perhaps the most striking outcome of this comparative analysis was that three *Neotyphodium* species with a history of *Epichloë typhina* as an ancestor possess nearly identical copies of *LOL* clusters (*N. uncinatum* *LOL-2*, *N. coenophialum*, *N. sp.* PauTG-1). This is most remarkable in light of the fact that there are no known isolates of

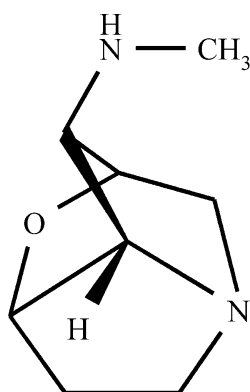


Figure 10. Loline.

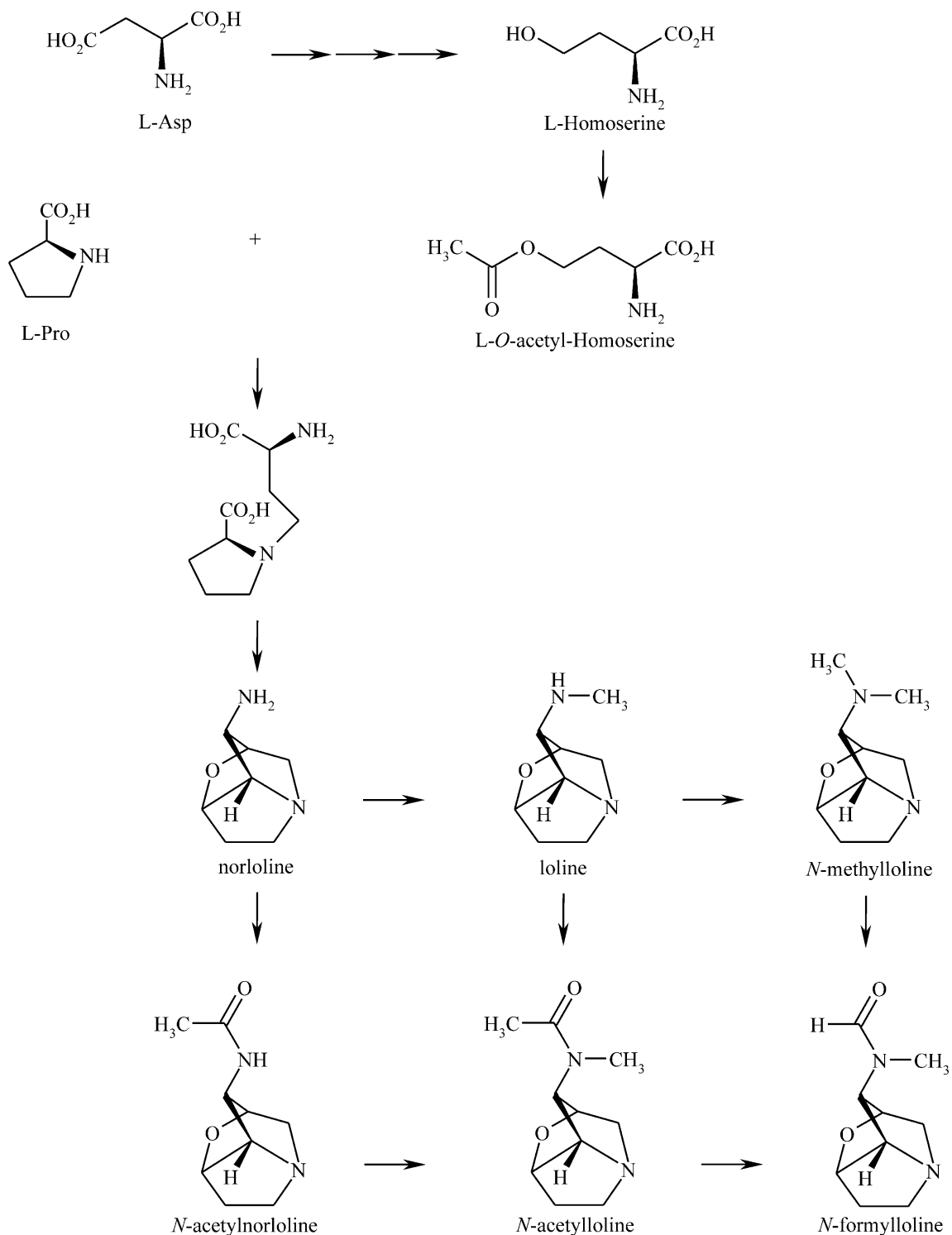


Figure 11. The loline alkaloid biosynthetic pathway (Blankenship et al. 2005).

the extant species *E. typhina* which express loline alkaloids (Leuchtmann et al. 2000). Thus, these data support a model of evolution in which the polymorphism in loline alkaloid production phenotypes among endophytic species is likely due to the loss of the trait over time (Kutil et al. 2007).

#### Ergopeptines

Ergot alkaloids are produced by ascomycetous fungi from discontinuous taxonomic groupings, including

plant-associated fungal genera from the family *Clavicipitaceae* and some members of the order *Eurotiales*, including the human pathogen *Aspergillus fumigatus* (Panaccione 2005). Due to their long biotechnological tradition, with manifold applications in therapy (Tudzynski et al. 2001), the physiology and biochemistry of ergopeptine formation have been studied in minute detail (Floss 1976; Socic and Gaberc-Porekar 1992; Gröger and Floss 1998). Nevertheless, an uncomfortable premonition of scar-

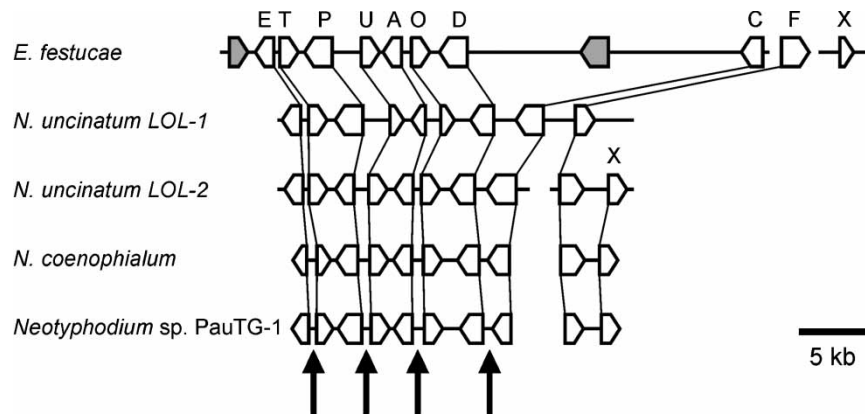


Figure 12. Comparative maps of *LOL* gene cluster(s) from four endophyte species; the shaded boxes in the *E. festucae* map indicate regions with homology to *pol* proteins; disconnected contig lines indicate genes that are sequenced, but not anchored to other *lol* genes; the contig of sequence known for this region in *E. festucae* continues beyond the boundaries shown; arrows along the bottom of the diagram indicate locations of putative promoter regions analyzed in the motif analysis (Kutil et al. 2007).

city in knowledge about the genetics of the biosynthetic route leading to these valuable alkaloids seemed to have arisen.

Yet again, the dynamic progress in the field of molecular genetics proved invaluable. The first step towards revealing the molecular background of ergopeptine biosynthesis was taken: the gene coding for dimethylallyl tryptophan synthase (*DMATS*) – the committed enzyme of the synthetic process in question (Figure 13a) – was cloned via reverse genetics (Tsai et al. 1995), identified by a differential cDNA screening approach (Arntz and Tudzynski 1997) and characterized initially for *Claviceps fusiformis*, subsequently for *Claviceps purpurea* (Tudzynski et al. 1999), and finally for the phylogenetically distant *Aspergillus fumigatus* (Coyle and Panaccione 2005; Unsöld and Li 2005; Li and Unsöld 2006).

Fuelled by the conviction of secondary metabolite pathway genes clustering in fungal genomes (Keller and Hohn 1997), large-scale studies have been taken up, resulting in the identification of gene clusters for ergot alkaloid biosynthesis in *C. purpurea* (Tudzynski et al. 1999; Correia et al. 2003; Haarmann et al. 2005, 2006) and *A. fumigatus* (Coyle and Panaccione 2005; Unsöld and Li 2005; Li and Unsöld 2006); they contain 13 and 14 genes, respectively. It has been recently hypothesized that the fundamental ability to synthesize some type of ergot alkaloid was present in the most recent common ancestor of these *Ascomycetes*, accounting for the current phylogenetically discontinuous distribution of these secondary metabolites (Panaccione 2005). Thus, genes shared between the two clusters are presumed to be responsible for the early steps common to the two organisms (Coyle and Panaccione 2005).

The impressive achievements described hitherto, have been successfully conveyed into the realm of clavicipitaceous endophytic ergopeptine producers. *DMATS*, as a gene encoding for the enzymatic entity indispensable for ergot alkaloid production, was characterized for *Neotyphodium lolii* harbored by

perennial ryegrass. Once the putative endophyte homolog was identified by degenerate PCR, its function was tested by gene knockout. Subsequent complementation experiments resulted in the ultimate confirmation of the postulated role of *DMATS* in the biosynthetic process in question (Wang et al. 2004). Analogous experimental procedure allowed for the characterization of two consecutive genes, displaying the capacity to encode nonribosomal peptide synthetases (NRPS), namely: *lpsA* (Pannaccione et al. 2001, 2003) and *lpsB* (Tanaka et al. 2005; Fleetwood et al. 2007), playing a vital role in the ergopeptine formation (Figure 13b) (Riederer et al. 1996; Walzel et al. 1997; Correia et al. 2003). The ultimate objective: to determine whether ergot alkaloid biosynthetic genes were clustered in *Neotyphodium lolii* was pursued by using chromosome walking and Southern blot analysis and successfully attained (Fleetwood et al. 2007).

Interestingly, while gene sequence is relatively highly conserved between each of the three thus far identified ergot alkaloid gene clusters, there are several differences in gene order and the *N. lolii* cluster (*EAS*) seems to be more complex in structure and organization, as compared to its pathogenic counterparts (Figure 14). What is more, the BLASTN (NCBI) and MEME (Multiple EM for Motif Elicitation) (Bailey and Elkan 1994) analyses of the *EAS* cluster revealed that its genes are closely associated with transposon relics, including retrotransposons and autonomous as well as non-autonomous DNA transposons.

Finally, it has been shown that ergot alkaloids are specifically produced during biotrophic growth of *Epichloë* endophytes (Tanaka et al. 2005). Attendant with this, the *eas* genes proved all to be expressed *in planta*, while giving no evidence for expression under axenic culture conditions. These results suggest that specific plant environment may be required for the induction of ergopeptine biosynthetic genes in the fungal endophyte (Fleetwood et al. 2007).

Notably, ergot alkaloids have also been considered to constitute a chemotaxonomic signature of

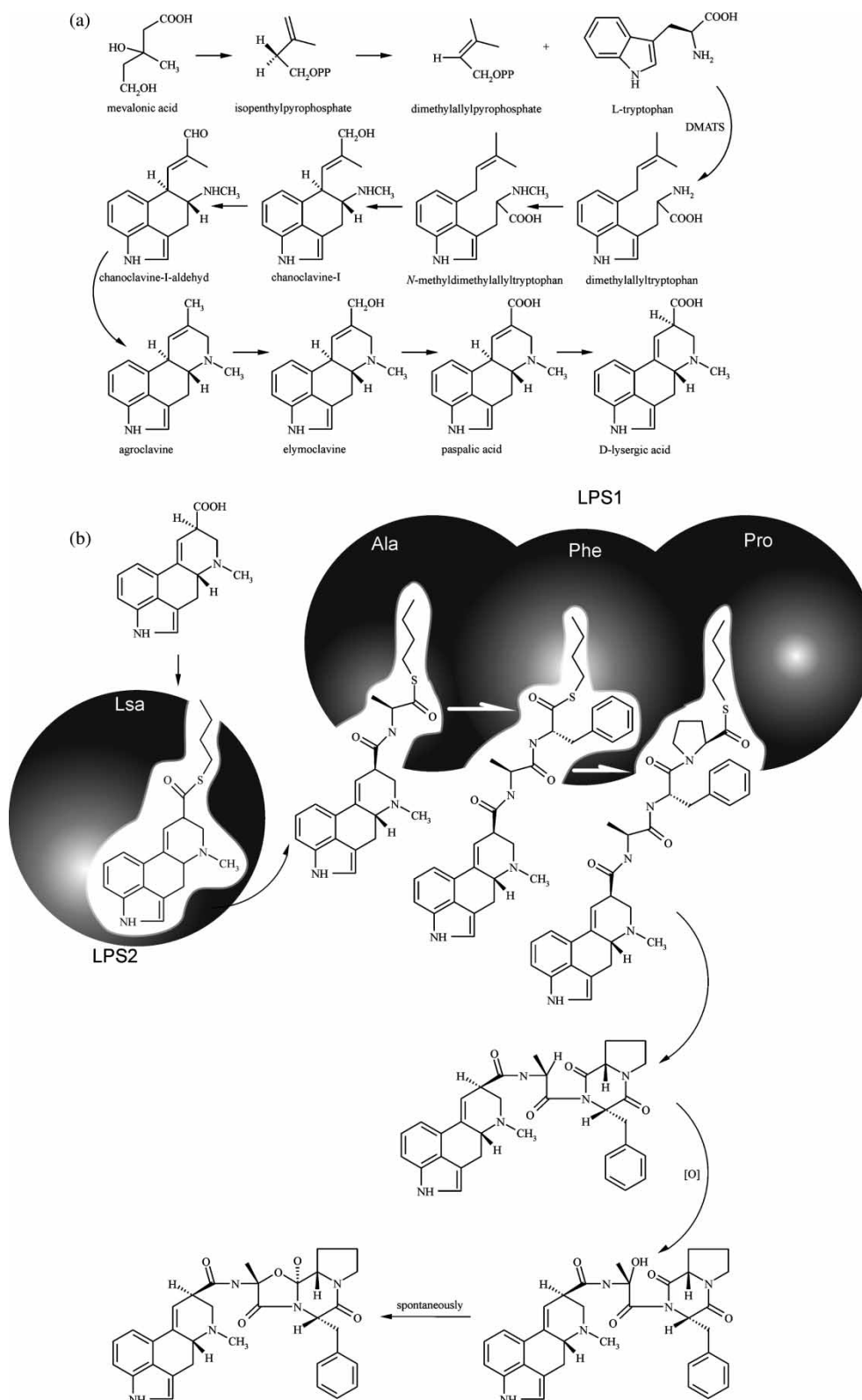


Figure 13. The ergot alkaloid biosynthetic pathway; biosynthesis of lysergic acid (a) and further ergopeptine formation, as exemplified by ergotamine (b) (Tudzynski et al. 1999, 2001).

*Convolvulaceae* plants. However, recent studies on *Ipomoea asarifolia*, a representative of this dicotyledonous plant family, indicated that the accumulation of the natural products in question depends, in fact, on the presence of a plant-associated clavicipi-

taceous fungus. Thus, it has been postulated that ergopeptines are not typical of the *Ascomycota*, the *Poaceae* or the *Convolvulaceae* alone, but, in contrast, they are very likely to prove a trait of a clavicipitaceous fungal taxon capable of colonizing certain

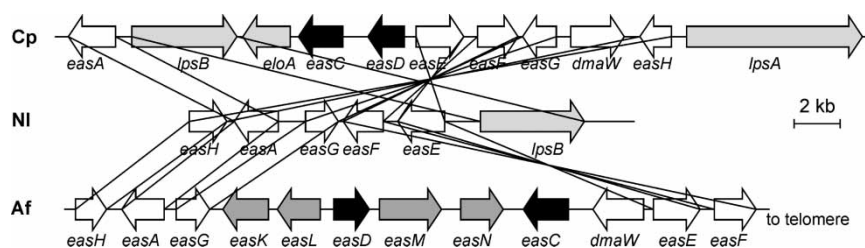


Figure 14. Comparative *eas* gene order among *C. purpurea* (Cp), *N. lolii* (Nl) and *A. fumigatus* (Af); black arrows, genes proposed to be required for ergot alkaloid synthesis, but not yet identified for *N. lolii*; light grey arrows, genes found in *N. lolii* and *C. purpurea*, but not in *A. fumigatus*; dark grey arrows, genes found only in *A. fumigatus*; *N. lolii dmaW*, *eloA* and *lpsA* are not shown, as their locations relative to *EAS* cluster are not known (Fleetwood et al. 2007).

higher plants in mutualistic symbiosis (Kucht et al. 2004; Steiner et al. 2006).

### Lolitrems

Indole-diterpenes are a large, structurally diverse group of natural products commonly found in filamentous fungi of the genera *Penicillium*, *Aspergillus* and *Claviceps* (Mantle 1987; Parker and Scott 2004). Many of these compounds are potent mammalian tremorgenic mycotoxins (Steyn and Vleggaar 1985) and fall into four major structural classes, namely the penitremes, janthitrems, paspalitrems and lolitrems (Figure 15) (Mantle and Weedon 1994). They all share a common structural core comprised of a cyclic diterpene skeleton derived from geranylgeranyl diphosphate (GGPP) and an indole moiety derived from tryptophan, while different patterns of prenylation, hydroxylation, epoxydation and acetylation, and differences in ring stereochemistry around the basic indole-diterpene ring structure, are to be accountable for their structural diversity (Parker and Scott 2004). The best known of these fungal tremorgens are the lolitrems produced by *Epichloë* endophytes in association with temperate forage grasses (Gallagher et al. 1984).

The history of unravelling the molecular genetics underlying endophytic lolitrem production seems to encompass some of the elements of both the aforementioned biosynthetic puzzles of microbial alkaloids.

Initially, employing *Penicillium paxilli* as a model experimental system to dissect the biochemistry of indole-diterpene biosynthesis (Young et al. 2001) (an obvious parallel can be drawn with the ergots and their model synthesizer: *Claviceps purpurea*) allowed for the isolation of three orthologous genes from the grass endophytes, *Neotyphodium lolii* and *Epichloë festucae*, and subsequent confirmation of their contiguous clustering (Young et al. 2005). While further attempts to clone additional linked *ltm* genes in the endophytes by such approaches as inverse PCR were thwarted by the presence of large blocks of highly repetitive retrotransposon sequences flanking both sides of the cluster, an alternative strategy combining suppression subtrac-

tive hybridization (a technique applied successfully in case of *LOL* characterization) with chromosome walking was taken up. As a result, two additional *LTM* gene clusters were identified and consequently proved to be linked with *LTM-1* (Young et al. 2006).

Ultimately, it has been shown that the genes for lolitrem biosynthesis in the endophytic anamorph *Neotyphodium lolii* form a complex genetic locus of at least three *LTM* gene clusters (Figure 16). What is more, the structure and organization of the said endophytic locus, as compared to the orthologous clusters from *Penicillium paxilli* (Young et al. 2001) and *Aspergillus flavus* (Zhang et al. 2004), has yet again proved to be of greater complexity (accordingly: *EAS* clusters of pathogenic vs. endophytic origin).

As sub-telomers are proposed to be 'a workshop of evolutionary genomic experimentation' (Wong and Wolfe 2005), it has been suggested that the elaborate nature of the cluster in question may be accounted for by its location in such a rapidly evolving region of the endophytic genome (Gardner et al. 2002; Kellis et al. 2003; Machida et al. 2005; Nierman et al. 2005), and consequently driven by recombination and mutational processes associated with Type I transposon elements (Kempken and Kück 1998) combined with the phenomenon of repeat induced point mutation (RIP) (Selker et al. 1987).

Thus, consecutive similarities between *LTM* and *EAS* loci seem to be brought to light. Firstly, the association of the genes composing both these endophytic clusters with the transposon relics appears to be of considerable importance (Young et al. 2006; Fleetwood et al. 2007). The abundance of these relic sequences adds to the evolutionary potential of both clusters. It might also suggest that the regulation at the level of chromatin may be of consequence for *ltm* and *eas* genes, since chromatin remodelling as a method of coordinate gene regulation has been proposed as a possible factor causing selection pressure for secondary metabolite genes to be clustered (Martienssen and Colot 2001; Volpe et al. 2002; Bok and Keller 2004; Keller et al. 2005; Lee et al. 2005). Secondly, all ten genes identified at the *LTM* locus, as well as each gene

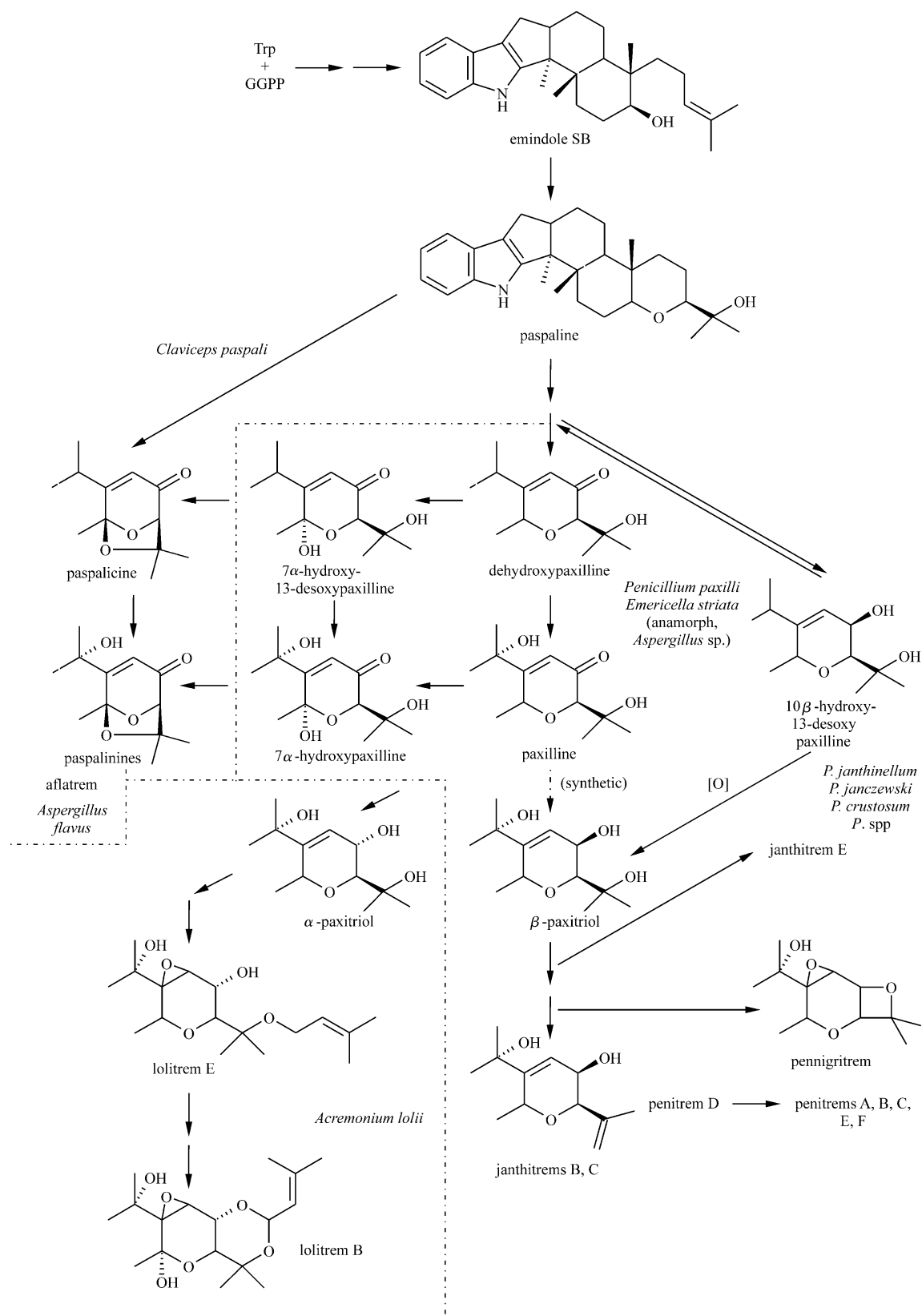


Figure 15. Proposed metabolic grid for indole-diterpenoid metabolites of particular species of *Acremonium*, *Aspergillus*, *Claviceps* and *Penicillium* linked according to sequential transformation in the diterpenoid moiety (Mantle and Weedon 1994).

in *EAS* cluster, have been shown to be highly expressed *in planta*, but only to a minimal extent or not at all – in axenic mycelial cultures, strongly suggesting that plant signalling is indispensable to induce their expression (Young et al. 2006; Fleet-

wood et al. 2007). However, the specific host stimuli required for the activation of the fungal genes remain to be identified.

Simultaneously, the aforementioned common traits underlying the ergopeptine and lolitrem biosyn-

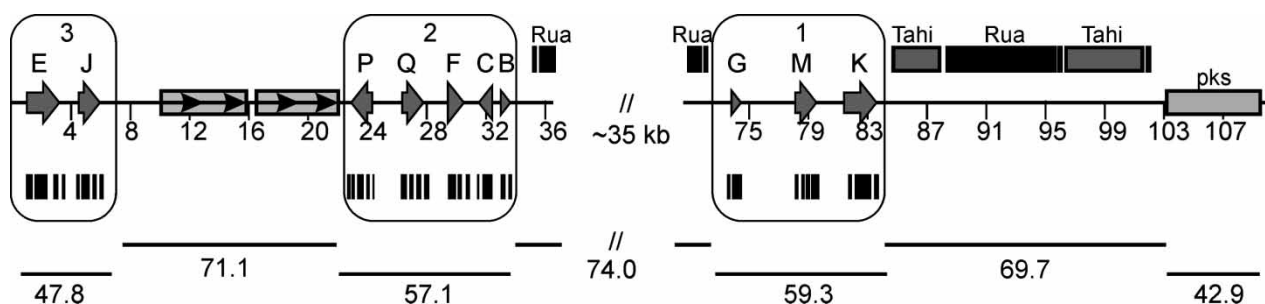


Figure 16. A physical map of the *N. lolii* LTM locus; the boundaries of the three *ltm* gene clusters are identified by boxes numbered 1–3; the genes, abbreviated to a single letter, are shown as arrows indicating the direction they are transcribed, with the exon structure shown as black blocks underneath each gene; the blocks between *ltm* clusters 2 and 3 show the positions of two imperfect direct repeats; the retrotransposon relics, Tahia and Rua, are identified by blocks above the sequence; the AT content of each region is indicated as a percentage at the bottom of the figure; the distance across the sequence, shown immediately below the genes, is represented in kb; the sequence containing *ltm* clusters 2 and 3 is separated from *ltm* cluster 1 by ~35 kb (Young et al. 2006).

thetic processes seem to emphasize the ultimate uniqueness of lolines.

### Maytansinoids from bacterial endophytes

As a matter of principle, hereby the elucidation of molecular background underlying the clustering of prokaryotic genes in the realm of endophytic secondary metabolism is shortly depicted. Thus, encompassing the diversity of microbial endophytic world, as well as acknowledging the early foundations set to boost the gene clustering investigation *per se* will be attempted.

The remarkable antitumor potency of maytansinoids, as well as the mystery surrounding their biosynthetic and evolutionary origins was reflected upon previously. It is noteworthy, however, that these 19-membered macrocyclic lactams are closely related to ansamycin antibiotics of microbial origin, such as rifamycin B and geldanamycin (Rinehart and Shield 1976). In fact, the aforementioned similarity stimulated a search for maytansinoid-producing microorganisms, ultimately leading to the isolation of ansamitocins (Figure 17) from the *Actinomycetes*, *Actinosynnema pretiosum* ssp. *pretiosum* and a mutant strain *Actinosynnema pretiosum* ssp. *auranticum* (Higashide et al. 1977; Asai et al. 1978).

The drive at the elucidation of microbial maytansinoids biosynthesis at the biochemical and genetic level was based upon the coupled forces of heterologous hybridization and PCR. The primary aim was to identify the gene encoding for the key enzyme in the assembly of 3-amino-5-hydroxybenzoic acid (AHBA) as the starter unit. Cloning by reverse genetics of the *AHBA synthase* from *Amycolatopsis mediterranei* (Kim et al. 1998) provided the necessary probe and set the pace for identification, isolation and sequencing of the consecutive entities embedded in the biosynthetic gene cluster in question. A total of 250 kb of contiguous DNA was cloned and mapped. The appropriate gene inactivations were carried out to address the essential role of the AHBA gene homologues and to define the boundary of the

biosynthetic locus (Yu et al. 2002). Notably, the ansamitocin synthesis genes (*asm*) turned out to be contained not in one, but two clusters in the *Actinosynnema pretiosum* genome (Figure 18). This separation of genes encoding the biosynthesis of a given antibiotic into two essential clusters is unprecedented in *Prokaryota*. Thus, it is proposed to be merely an inconsequential accident resulting from an evolutionary gene rearrangement which inserted 30 kb of non-essential DNA into the otherwise contiguous cluster (Yu et al. 2002).

Thus equipped with the essential genetic information and modern tools to manipulate the biosynthetic machinery, as well as urged by the extraordinary therapeutic potential, the research on microbial maytansinoids has recently entered a novel combinatorial stage. Two principal approaches are being explored. On one hand, the expression of all the *asm* genes, assembled into several cassettes in a heterologous host, such as *Streptomyces coelicolor* or *S. lividans* is being considered. This approach seems most promising in terms of elucidating the detailed biosynthetic process, but it has some major practical drawbacks, as the product titres are likely to be very low. On the other hand, it has been proposed to engineer mutations in the *asm* in the parent

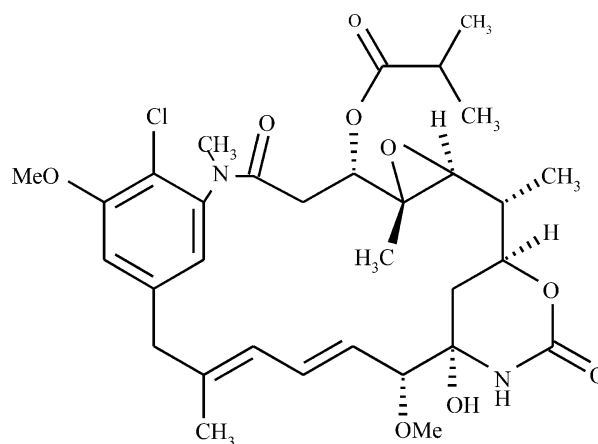


Figure 17. Ansamitocin P-3.

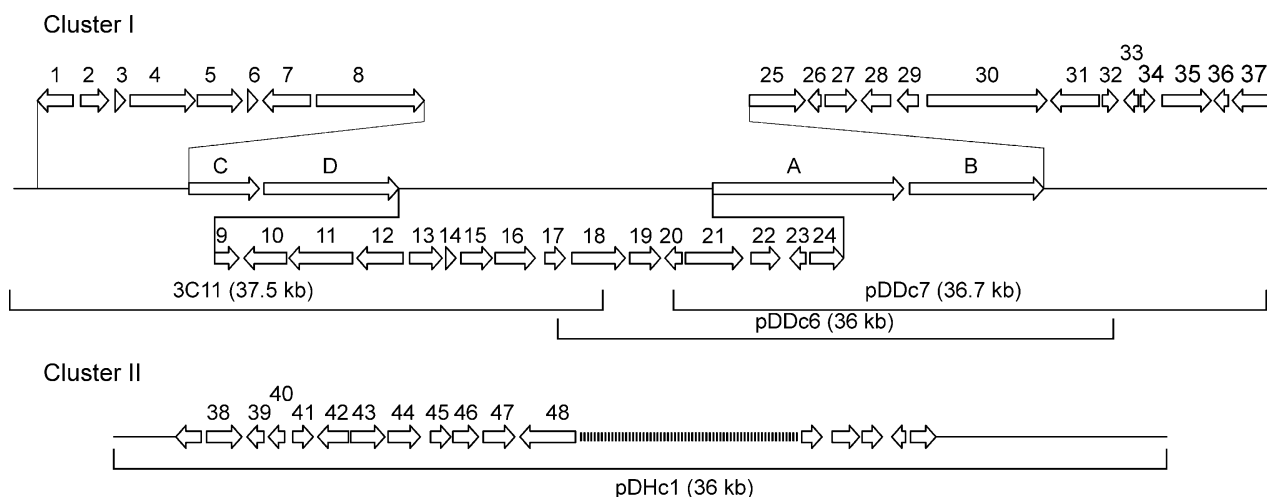


Figure 18. The ansamitocin biosynthetic gene cluster from *A. pretiosum*; asmABCD and asm19, polyketide synthase; asm22-24, asm43-45 and asm47, AHBA synthesis; asm13-17, methoxymalonate synthesis; asm7, asm10-12, asm19 and asm21, post-PKS modifications; asm2, asm8, asm18, asm29 and asm31, regulation (Floss 2006).

producer organism. This alternative approach has the advantage of all the regulatory elements and resistance mechanisms required for ansamitocin formation being present and functional (Cassady et al. 2004).

Undoubtedly challenging prospects, with substantial problems to be overcome and much more work needed, yet bearing a considerable potential for future drug discovery, optimization and production processes (Floss 2006).

In summary, the accomplishments thus described might prove to become a vital platform and a blueprint for detailed studies of endophytic microbes *en masse* – their biosynthetic networks, evolutionary implications, host interdependencies, ecological, pharmaceutical and industrial significance – in the modern era of metabolic engineering and combinatorial biosynthesis (Stephanopoulos et al. 2004; Floss 2006).

### Concluding remarks and future perspectives

**Biodiversity:** a precious source of novelty. Not only in terms of unravelling numerous mysteries of nature – discovering a plethora of yet undescribed species, their evolutionary backgrounds, genetics and ecology, as well as the richness of thus implied new, potentially valuable molecules; but also a revolution of thought – an expanded view promising to transform glimpses of reductionist research of the past years into snapshots of a dynamic world of systems biology, where cells grow, divide and produce, or organisms develop, differentiate and begin to deviate from the norm (Kate and Laird 2000; Stephanopoulos et al. 2004; Kayser and Quax 2007). Endophytic microbes seem to fit perfectly into this natural ‘warehouse’, only a small part of which we have been able to tap into so far.

The recent ‘genomics revolution’ has given momentum to considerable progress in the development of new technologies in bioscience, addressing specifically the arena of natural product biosynthesis. Whole-genome sequence mining (Lautru et al. 2005) and genome scanning as an alternative approach, providing an efficient way to discover natural product biosynthetic gene clusters without having the complete genome sequence (Zazopoulos et al. 2003); advances in microbial cell fermentation technology (Zengler et al. 2005; Weuster-Botzl et al. 2007) and metagenomics as a valuable alternative offering a cultivation-independent approach (Schloss and Handelsman 2005); ample successes in heterologous expression and metabolic engineering (among many others: Alper et al. 2005; Schmidt et al. 2005; Wenzel et al. 2005; DeJong et al. 2006; Julsing et al. 2006; Li et al. 2006; Lindahl et al. 2006; Nims et al. 2006; Ro et al. 2006), the latter being in fact perceived as a progenitor of functional genomics and systems biology (Stephanopoulos et al. 2004; Tyo et al. 2007) – to name only a few highlights.

As hereby reviewed, some of the aforementioned novel strategies have enabled penetration into the previously inaccessible natural-product resources: microbial endophytes. While one has to be mindful that the problem we set out to address is several orders of magnitude larger than those with which we are familiar, no one can deny the opportunities that now present themselves.

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