Patrícia Alexandra Soares Sequeira

Degree in Biochemistry

Elucidate the biosynthesis and the functional role of a new class of antimicrobial peptaibiotics in *Neurospora crassa*

Dissertation to obtain the Master degree in Biochemistry for Health

Supervisor: Cristina Silva Pereira,PhD, ITQB NOVA
Co-supervisor: Isabel Martins,PhD, ITQB NOVA

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President: Doctor Pedro Matias
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Instituto de Tecnologia Química e Biológica António Xavier (Oeiras)

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Resumo

Os fungos são produtores abundantes de metabolitos secundários. Em particular, estes organismos estão envolvidos na produção de uma subclasse de péptidos não-ribossomais - peptaibióticos - que apresentam atividades antibióticas promissoras. Estudos anteriores demonstraram que algumas estirpes fúngicas são capazes de crescer em meios suplementados com líquido iónico e que esta suplementação leva a alterações no seu metaboloma. Além disso, em *Neurospora crassa*, o suplemento de meio de crescimento com cloreto de 1-etil-3-metilimidazólio ou cloreto de colina conduziu a níveis aumentados de 1-aminociclopropano-1-carboxilato deaminase, um enzima envolvido na produção do aminoácido raro ácido 1-aminociclopropano-1-carboxílico (ACC). Neste estudo, foi feita a suplementação do meio de crescimento com concentrações sub-letais de cloreto de colina para induzir um perfil metabólico diferencial em culturas de *N. crassa*. Os resultados mostraram uma maior diversidade de metabolitos peptídicos produzidos em culturas de *N. crassa* com suplementação de líquido iónico, em comparação com as condições controlo. Observou-se que alguns dos péptidos que foram semi-purificados contêm os aminoácidos ácido α-aminoisobutírico e/ou ácido 1-aminociclopropano-1-carboxílico, sendo que o primeiro é o aminoácido arquetípico mais abundante em peptaibióticos. A mistura de péptidos demonstrou um forte potencial anticancerígeno. As tentativas de caracterizar o mecanismo de biossíntese destes péptidos diferenciais revelaram um potencial *cross-talk* entre quatro genes *backbone* candidatos, que codificam enzimas envolvidos na produção de péptidos não-ribossomais. Em particular, dois desses genes, NC08441 e NCU04531, parecem estar associados e ter uma função preponderante. Este estudo constitui uma primeira demonstração da capacidade de *N. crassa* para produzir metabolitos peptídicos, incluindo peptaibióticos, sob o estímulo de líquidos iónicos específicos; no entanto, algumas das observações requerem mais investigação. Uma vez que estes metabolitos secundários possuem propriedades farmacológicas interessantes, a caracterização dos péptidos purificados será priorizada.

Palavras-chave: Metabolitos secundários, *Neurospora crassa*, peptaibióticos, sintetase não-ribossomal de péptido, atividade biológica

Abstract

Fungi are generally abundant producers of secondary metabolites. In particular, these organisms are involved in the production of a subclass of nonribosomal peptides - peptaibiotics - which display interesting antibiotic activities. Previous studies demonstrated that some fungal strains are able to grow in media supplemented with ionic liquid and this supplementation leads to alterations in their metabolic footprint. Moreover, in *Neurospora crassa,* growth media supplementation with either 1-ethyl-3-methylimidazolium chloride or cholinium chloride, led to increased levels of 1-aminocyclopropane-1-carboxylate deaminase, an enzyme involved in the production of the rare amino acid 1-aminocyclopropane-1-carboxylic acid (ACC). Herein, sub-lethal concentrations of the ionic liquid Cholinium chloride were used as growth media supplements to induce in *N. crassa* cultures a differential metabolic profile. Results showed a higher diversity of peptide metabolites produced by *N. crassa* grown in media supplemented with the ionic liquid compared to control conditions. Some of the ensuing semi-purified peptides contain α-aminoisobutyric acid and/or 1-aminocyclopropane-1-carboxylic acid, the first being the most abundant archetypal amino acid in peptaibiotics. In addition, the peptides mixture demonstrated a strong anti-cancer potential. Attempts to characterize the biosynthesis machinery behind the capacity to produce the differential peptides revealed a potential cross-talk between four candidate backbone genes coding for enzymes involved in the production of nonribosomal peptides; major role seems to be associated with NC08441 and NCU04531 genes coding for two of the backbone enzymes. Although some of these observations need to be validated and merit further investigation, this study constitutes a first demonstration of the capacity of *N. crassa* to produce peptide metabolites, including peptaibiotics under the stimuli of specific ionic liquids. Efforts to characterize the purified peptides will be made since these secondary metabolites carry interesting pharmacological properties.

Keywords: Secondary metabolites, *Neurospora crassa*, peptaibiotics, nonribosomal peptide synthetase, biological activity

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List of Abbreviations

ABI – Applied Biosystems

ACC - 1-Aminocyclopropane-1-carboxylic acid

ACN – Acetonitrile

ACP - Acyl carrier protein

Aib – α – Aminoisobutyric acid

aw – Water activity

AT - Acyltransferase

BGC – Biosynthetic gene cluster

cAMP - Cyclic adenosine monophosphate

CBC – CCAAT-binding complex

cDNA – Complementary deoxyribonucleic acid

DG18 – Dichloran- glycerol

DMATSs - Dimethylallyl tryptophan synthase

DNA – Deoxyribonucleic acid

DOSY - Diffusion ordered spectroscopy

[C_2mim]Cl - 1-ethyl-3-methylimidazolium chloride

ChoCl – Choline/Cholinium chloride

FGSC – Fungal Genetics Stock Center

HPLC – High performance liquid chromatography

IC50 - Half maximal inhibitory concentration

IL – Ionic liquid

KS- Ketoacyl synthase

LC-MS – Liquid chromatography- mass spectrometry

MAPK- Mitogen-activated protein kinase

MHB – Mueller-Hinton Broth medium

MIC – Minimal inhibitory concentration

MM – Minimal media

MW – Molecular weight

NMR – Nuclear Magnetic Resonance

NP – Natural products

NRPS – Non-ribosomal peptide synthases

O.D – Optical density

OSMAC – One Strain-Many Compounds

PCR- Polymerase chain reaction

PDA – Photodiode array

PEG- Polyethylene glycol

PKA - Protein kinase A

PKS – Polyketide Synthase

RNA – Ribonucleic acid

qRT-PCR – Quantitative Real Time polymerase chain reaction

SMs – Secondary metabolites

TCs - Terpene cyclases

TF – Transcription factor

TFA – Trichloroacetic acid

TIC – Total Ion Chromatogram

TOF – Time of flight

TOR - Target of rapamycin

Chapter 1 - Introduction

1.1 Fungi

Fungi constitute a group of eukaryotic organisms widespread in nature (1). Nutritionally, they are heterotrophs, able to obtain nutrients from several organic compounds as a source of energy and carbon for cellular synthesis (1,2). Another important aspect is their ubiquity as they are present in almost every environment with high tolerance to pH, temperature and water availability, allowing these organisms to be easily spread and survive under a wide range of environmental conditions (3). Among the best described fungal species are saprophytes (obtain nutrients from dead organic matter) (*e.g*. *Aspergillus* spp. and *Neurospora* spp.(4)), pathogens, parasites or even symbionts (1,5).

Fungi play essential roles in ecosystems in the decay of organic matter, carbon recyclers or as plant and animals' pathogens (6), among others. Besides that, they also are important for human health and economy (3), beneficially as part of human nutrition (*e.g*. mushrooms) and health (*e.g*. antibiotic penicillin (7)) but also negatively acting as pathogens provoking serious health concerns such as invasive fungal infections (8) and causing severe plant pathologies that lead to economic losses in agriculture (9).

1.1.1 Fungal taxonomy

The classification of organisms has started with Linnaeus in 1735 with the division of life into two distinct kingdoms: animals and plants, the latter including fungi (10). Since then, the expansion of knowledge and increasing number of species described has required changes in the several division systems. The introduction of fungi as a kingdom by itself was only done in 1969 by Whittaker (11). In the most recent six kingdom system proposed by Cavalier-Smith in 1998, only "true fungi" (*i.e.* excluding slime moulds and oomycetes) were included in the kingdom *Fungi* (12,13). Typically four phyla were considered in this kingdom: *Chytridiomycota*, *Zygomycota*, *Ascomycota* and *Basidiomycota* (5). However, in 2007, a recent phylogenetic classification was proposed by Hibbett *et al.* (14) grouping the representative phyla *Basydiomycota* and *Ascomycota* in the subkingdom Dikarya. Overall, the classification considered one subkingdom (*Dikarya*), seven phyla and ten subphyla (14) .

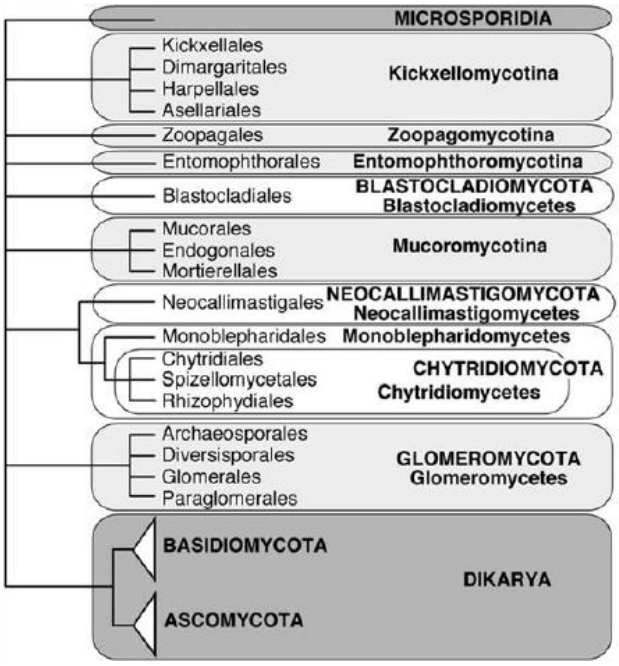


Figure 1.1. Phylogeny and classification of fungi (14) (the branch lengths do not aim to be proportional to genetic distances).

1.1.2 Ascomycota fungi

Approximately 65-75% of all known fungal species belong to the phylum *Ascomycota* (1,15). Three main subphyla are proposed in this classification: *Taphrinomycotina*, *Saccharomycotina*, *Pezizomycotina* (14). The subphylum *Pezizomycotina* contains the filamentous *Ascomycota* and comprises, among others, *Penicillium rubens (*from which the penicillin was discovered) (7), *Neurospora crassa* and *Aspergillus nidulans,* the last two considered model filamentous fungi in cell biology studies (16–18).

Filamentous fungi have the ability to form highly polarized hyphae. After a brief period of symmetric expansion, there is a switch to permanently apical extension. In this polar growth, a germ tube emerges and material is added to the apex, resulting in hypha (*i.e.* the basic unit of growth, an extending tubular cell) (19). The mycelium – a complex network of hyphal cells –results from the extension of hyphae units that branch sub-apically and also from hyphal anastomosis which consists of hyphal branches' fusion (and usually occurs in older mycelium) (20). This growth mechanism allow fungi to expand to wide areas in order to obtain nutrients (21). Hyphal growth might be suspended for many reasons (in situations of chemical stress or starvation and when entering sexual or asexual reproduction (22) and the fungus may remain under a dormant phase through the production of dormant resistant structures (*i.e.* spores) for propagation. Sexual spores, produced during meiosis, are known to be resistant and present higher genetic variability than the parental cells which provides a survival advantage in harsh conditions (5). When the environmental conditions become favorable the spores can germinate to initiate a new life cycle. Ascomycota fungi life cycle comprises both sexual and asexual reproduction (special detail about life cycle will be mentioned in relation to *Neurospora crassa)*.

1.1.3 Neurospora crassa

Neurospora crassa (from now on referred to as *N. crassa*) is a member of the large group of "higher" fungi from *Ascomycota* phylum. Like moulds in general, *N. crassa* presents a filamentous growth and it is one of the fastest-growing filamentous fungi, approximately 10 cm *per* day. This fungus is sometimes called the ‘orange bread mould’ because of its first appearance as an infestation of French bakeries in 1843 (18,23). It was also seen as the earliest colonizer of the remains of burnt vegetation(18,24). In 1941, the work of Beadle and Tatum using *N. crassa* led them to the famous ‘one gene-one enzyme’ hypothesis as well as the discipline of biochemical genetics as an experimental science (25). *Neurospora crassa* is being used as a model organism for biochemical, genetic and cellular research since the latter half of the 20th century because it is easy to grow, has a short life cycle and has haploid genetics (23,24,26).

Similarly to other filamentous fungi, *N. crassa* can undergo vegetative growth, asexual and sexual reproduction cycles. A general scheme of *N. crassa* life cycle is shown in Figure 1.2.

The sexual cycle is induced by nitrogen starvation or changes in temperature or light. Since *N. crassa* is a heterothallic fungus, the presence of two compatible partners to initiate sexual reproduction is required. In such case, the trichogyne (*i.e.* polarized structure from protoperithecium) of one mating-type fuses with the male conidium of the opposite mating-type. The conidium moves through the trichogyne into the ascogonial cell within the protoperithecium, which later becomes the perithecium (or ascocarp) (4,27).

The two haploid nuclei undergo repeated divisions within a developing dikaryotic ascogenous hyphal structure. Then, croziers (where nuclear fusion occurs) are formed and further differentiated into developing asci (27). At this stage, the perithecium contains ascus with eight sexual spores (ascospores) and each is dispersed until favorable conditions are set for the ascospore to germinate and restart the sexual cycle (4).

The production of asexual spores is usually triggered by circadian rhythms (28). In such conditions, the hyphae can differentiate into microconidia (*i.e.* uninucleated asexual spores scarce and with low viability) or conidiophores which originate chains of macroconidia (*i.e.* multinucleated asexual spores)(4).

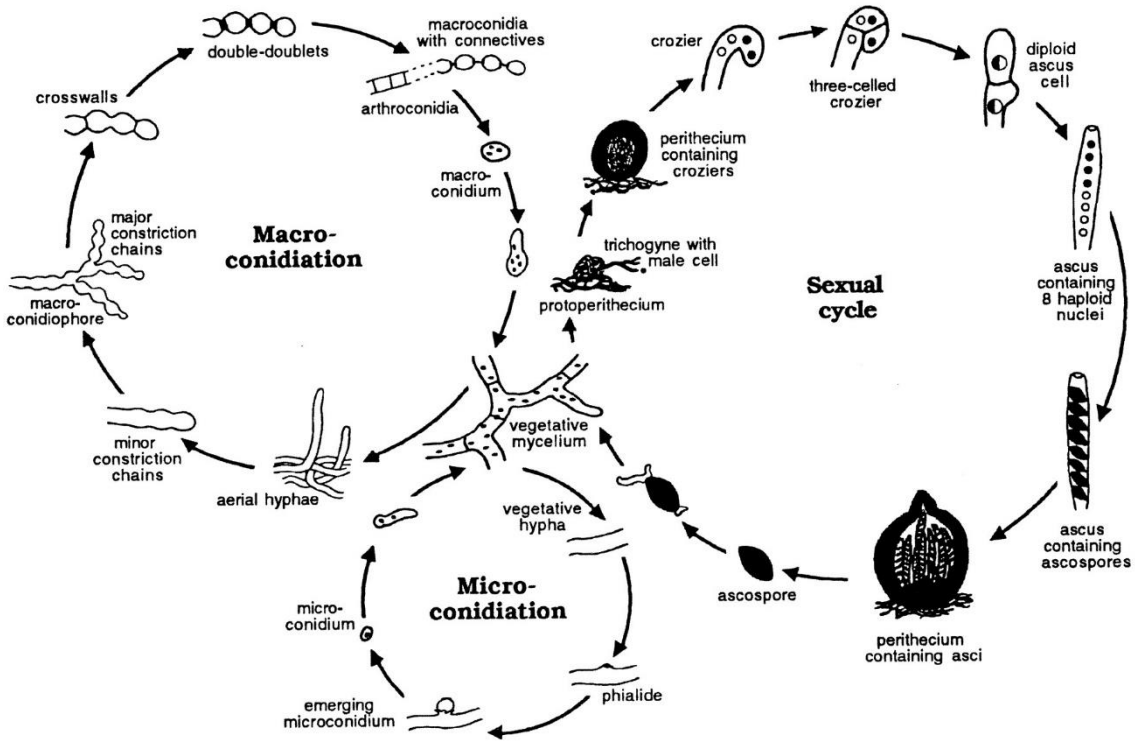


Figure 1.2. *Neurospora crassa* life cycle (4).

1.2 Fungal secondary metabolites

Filamentous fungi are abundant producers of secondary metabolites (SMs). These compounds, also termed natural products (NPs) are structurally diverse low-molecular mass molecules that are in general not necessary for the normal growth and development of the producer organism (29–31). These compounds are often synthesized from precursors derived from primary metabolism (32) when active growth has ceased or only under specific conditions (33).

The role that these compounds play for fungi is not clearly known. The most likely is that it helps fungi to survive in its ecological niche (30,34), using them for nutrient acquisition, interaction with other organisms, growth inhibition of competitors, as virulence factors or even to cope with environmental stress (29,35–37).

Since these natural products can have an effect on other microorganisms, fungi have a significant impact on human well-being which makes them an interesting study target. On the positive side, fungi are known to produce important compounds used as antibiotics (*e.g* beta-lactams), immunosuppressive (*e.g* taxol and camptothecin) and antitumor agents (*e.g*. Cyclosporin A), among others (38). On the other hand, fungi also produce mycotoxins that can be used as 'medically useful agents' at certain amounts (38). However, most mycotoxins appear as food contaminants which can affect both economy and health (32).

1.2.1 Classes and biosynthesis of fungal secondary metabolites

Fungal secondary metabolites are often located in clusters and their biosynthesis is complex (29). The complexity is due to a diversity of factors including the tight regulation of this process, the reactions required to convert primary building blocks into NPs, influence of external stimuli, the balance between primary and secondary metabolism and the developmental stage of the fungus (34,35).

The first step in the biosynthesis of a SM is catalyzed by one of the five “backbone” enzymes: polyketide synthases (PKSs), nonribosomal peptide synthases (NRPSs), hybrid NRPS–PKS enzymes, prenyltransferases (or dimethylallyl tryptophan synthases (DMATSs)), and terpene cyclases (TCs) (29,37,39). These multidomain enzymes are responsible for the production of the five different classes of fungal SMs: polyketides, nonribosomal peptides, NRPS-PKS hybrids, indole alkaloids and terpenes, respectively (30,40,41). The first two multidomain and multimodular enzymes, NRPSs and PKSs, are the responsible for the general structural scaffolds of most SMs (29,39).

Polyketides

Polyketides are the most abundant fungal secondary metabolites produced by the multidomain PKSs from the precursors acetyl-coA and malonyl-coA units (32). Biosynthesis is simply achieved by a mechanism that involves the iterative elongation of carboxylic acid building blocks. This enzyme system is organized into modules (Figure 1.3); each module is responsible for one chain elongation step and can be subdivided into domains (29). For PKSs, an elongation module can be divided into three domains: an acyltransferase (AT), acyl carrier protein (ACP) and ketoacyl synthase (KS). The polyketide scaffold can be further modified by tailoring enzymes (such as oxygenases, glycosyltransferase and other transferases) to confer additional structural functionalities to the end product (29). In addition, modular NRPSs and PKSs systems can cooperate and form the hybrid NRPSs-PKSs products (29).

This class of fungal SMs comprises the mycotoxins aflatoxin and fumonisin, the anticholesterolemic agents lovastatin (29) and compactin, and the pigments bikaverin and fusarubin (32), among others.

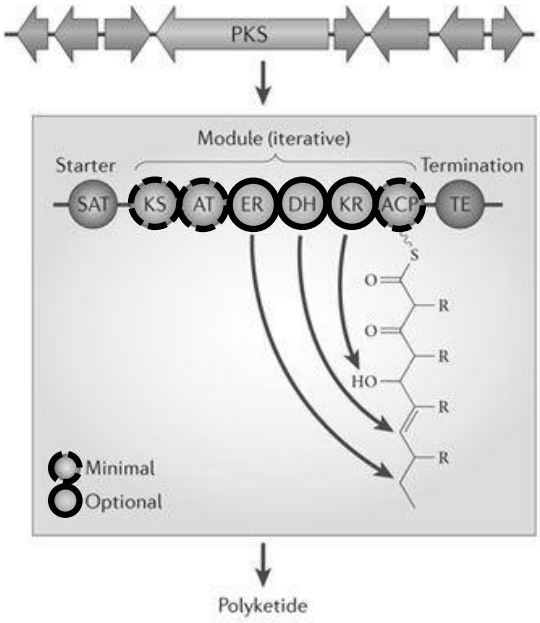


Figure 1.3. Gene clusters with a central polyketide synthase (PKS) gene. The domains of the encoded enzymes are indicated as spheres, with the minimal domains required to form a module of the enzyme in a dashed line. Adapted from (29)

Terpenes

Terpenes are biosynthesized by the enzymes terpene cyclases which are essential for the production of different terpenes. All terpenes are composed of several isoprene units and can be linear or cyclic, saturated or unsaturated and modified in various ways (40). Examples of natural products from this class are trichothecenes and gibberellins (32).

Indole alkaloids

Indole alkaloids comprise compounds biosynthesized from the aromatic amino acid tryptophan and dimethlyallyl pyrophosphate precursors (32). The first step in the biosynthesis of these compounds is the prenylation of tryptophan by the key enzyme prenyltransferase (or DMATS). Well-characterized metabolites that belong to this class are the ergot alkaloids produced by plant pathogenic *Claviceps* species, roquefortine C and related compounds such as meleagrin and glandicoline from *Penicillium* species (32).

Nonribosomal peptides

Nonribosomal peptides are derived from the multimodular enzymes NRPSs (40). As polyketides, the enzyme system can be divided into modules and each module contains several domains that allow the chain elongation of amino acid building blocks (Figure 1.4). The domains are usually the adenilation domain (A), a thiolation or peptidyl carrier protein domain (P) and a condensation/peptide-bond formation domain (C) (29,32,40).

These natural products are constituted from both proteinogenic and non-proteinogenic aminoacids and can be made of different lengths, can be linear or cyclized and may suffer variations

(40). Compounds within this class are the tripeptide beta-lactams, undadecapeptide immunosuppressive drug cyclosporine (29), siderophores and linear peptaibols (which belong to the class of peptaibiotics, explained below) (32).

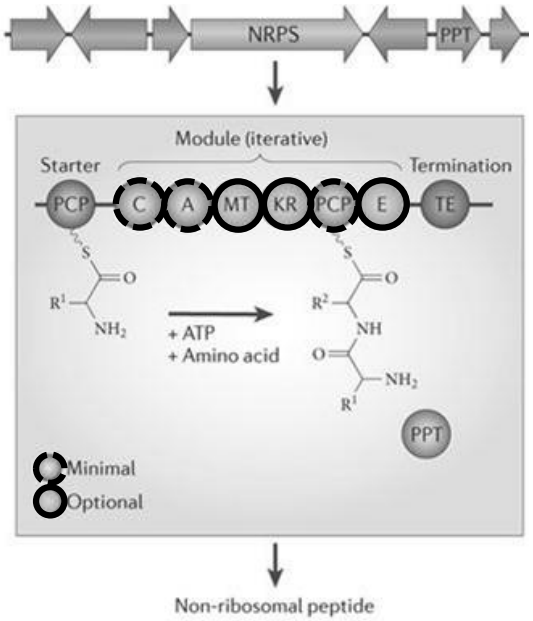


Figure 1.4. Gene clusters with a nonribosomal polyketide synthase (NRPS) gene. The domains of the encoded enzymes are indicated as spheres, with the minimal domains required to form a module of the enzyme in a dashed line.Adapted from (29).

Peptaibiotics

Among the group of non-ribosomal peptides, the family of antibiotic peptides termed 'peptaibiotics' is the largest and started to grow during the last two decades (42).

Peptaibiotics are biosynthesized exclusively by fungi (mainly soilborne and plant pathogenic) (43) and the designation comes from the fact that these compounds are peptides characterized by the presence of an atypical amino acid – α-aminoisobutyric acid (Aib) and remarkable antibiotic activities. These compounds are linear or cyclic non-ribosomal peptides which i) have a molecular weight of 500 to 2,200 Dalton, with 5 to 20 residues in length; ii) high content of Aib; iii) contain non-proteinogenic aminoacids and/or lipoamino acids (Figure 1.5) and iv) possess an acylated N-terminus and (v) in the case of linear peptides, have a C-terminal residue that can be a free or acylated amine-bonded 1,2-amino alcohol (subgroup referred as peptaibols) or an amine, amide, free amino acid, 2,5-dioxopiperazine, or sugar alcohol (43–45). Until today, more than one thousand of compounds have been identified and described in the literature and have been systematically compiled in the Peptaibiotics Database (46).

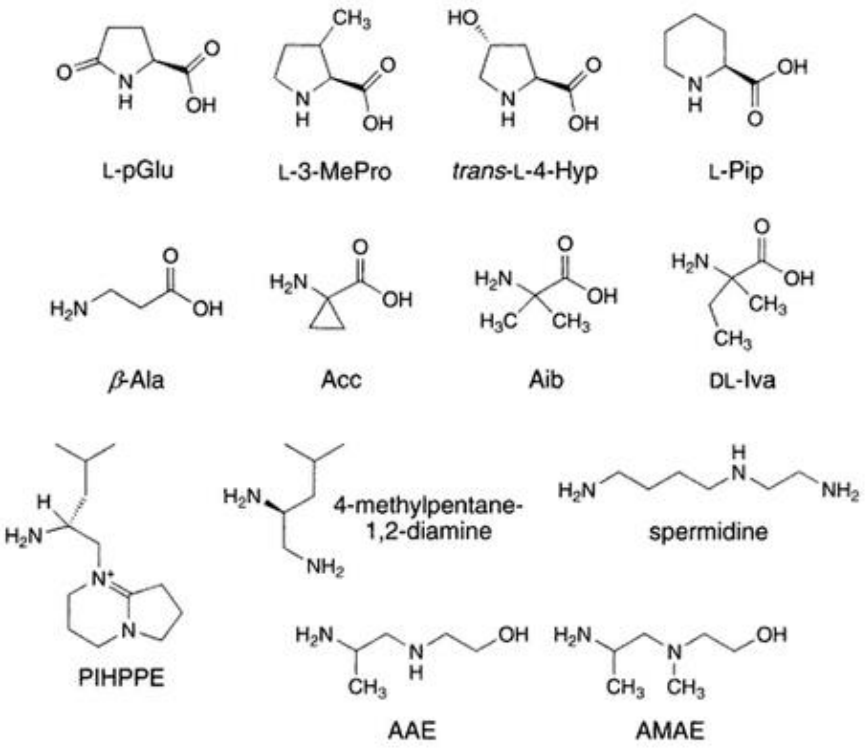


Figure 1.5. Uncommon constituents of peptaibiotics. Adapted from (45).

As unique and diversely structural compounds, these peptides may exhibit interesting bioactivities and can be used in a variety of applications as antifungal, antibacterial, anticancer and antiparasitc drugs (34,45). Part of the biological activities showed from these SMs is due to their linear and amphipathic nature which allows them to self-associate into oligomeric ion-channels and form pores in the lipid bilayer membranes, leading to cell death (42,44,47).

Neurospora crassa has its genome fully sequenced; surprisingly it has only ten predicted biosynthetic gene clusters (BGCs) related to secondary metabolism (37), compared to other filamentous fungi (with *ca.* 30 to 70 BGCs). There is one cluster associated with the production of a carotenoid (neurosporaxanthin) and a second one that can be associated with the production of the siderophore ferricrocin (by homology with *Aspergillus nidulans* genes). So, *N. crassa* is of interest because it has not been shown to produce any other secondary metabolites. Despite its limited SM biosynthetic potential, the BGCs have not been systematically solved. In addition, preliminary observations from the host laboratory showed evidence that supplementation of growth media with ionic liquids (both choline chloride (ChoCl) and 1-ethyl-3-methylimidazolium chloride ($[C_2mim]Cl$)) increased the expression levels of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (enzyme responsible for the production of the rare amino acid ACC) that in some fungi has been linked to neofrapeptins and acretocins (42,45), compounds belonging to the class of peptaibiotics. So, it seems possible that, in specific stress situations, *N. crassa* can produce peptaibotics containing ACC.

Several NRPS and related genes were identified in the *Neurospora* genome sequence (17). From whole-genome analysis of *Neurospora crassa* (strain OR74A) we can identify three NRPS genes (NCU04531, NCU07119 and NCU08441) and, three predicted NRPS-like genes (NCU05000, NCU08404 and NCU10423), coding for hypothetical proteins (37).

1.2.2 Regulation of secondary metabolites in fungi

Understanding the regulation of fungal secondary metabolism in response to diverse stimuli can provide information about the role of the compounds to the fungus and the ways to produce them (30).

The regulation of secondary metabolism in fungi is a very complex interconnecting network that responds to both developmental and environmental signals (48). The different environmental stimuli can be temperature, light, pH, carbon and nitrogen sources, redox status and also stimuli derived from interspecies communication (30,36).

In fungi, the genes involved in secondary metabolism are clustered, which are often found in sub-telomeric regions, at the end of a chromosome (49). In filamentous fungi, these clusters range from only a few to more than 20 genes (50). The reasons why the clustering occurs are not well understood but it has been suggested that it either results from horizontal transfer from prokaryotes (where clustering is common) or confers a selective advantage to fungi since it provides a more controlled and effective way of regulation (49,50).

The regulation of BGCs occurs at different levels, including global and pathway-specific regulators, signal transduction pathways and epigenetic modifications (36).

Global and cluster-specific regulators

Cluster-specific regulators are normally encoded within the cluster that they regulate, specifically modulating the genes of the cluster. On the contrary, global regulators are encoded by genes that do not belong to any BGC, regulating both genes related to secondary metabolism and others that generally respond to physiological and/or environmental processes (36).

In fungi, the response to environmental stimuli is mostly controlled by the Cys_2His_2 zinc-finger proteins (50). The most common of these global regulators are the AreA, PacC, CreA, CCAAT-binding complex (CBC) involved in the regulation of secondary metabolism in response to nitrogen, pH, carbon and iron, respectively (32,36). In addition, the global regulator LaeA is considered the major regulator of fungal secondary metabolism, discovered in *Aspergillus* species (51). LaeA participates in the light-sensing heterotrimeric velvet complex consisting of LaeA and the velvet proteins VeA and VelB, linking sexual development with SMs production in response to light (32).

The activation or repression of a given cluster is often achieved by the presence of cluster/pathway-specific regulators. The largest cluster-specific family unique to fungi is the $Zn(II)_2Cys_6$ family of transcription factors (TFs) (50). The best characterized TF is the aflatoxin/sterigmatocystin regulator AflR in *Aspergillus* (32). In particular, only two $Zn(II)_2Cys_6$ proteins (NIT-4 and QA1F) have been characterized in *N. crassa* (4).

Epigenetic regulation

Epigenetic modifications can derived from DNA, chromatin and RNA and include DNA methylation, RNA silencing systems, chromatin structure changes and centromere/telomere location (32).

The expression of secondary metabolism genes is also regulated by changes in chromatin structure and nucleosome packaging (32). The arrangement of SM genes into clusters suggests a regulation mechanism regarding the chromatin structure, with a restricted mode of action, yet able to regulate large genomic regions (29,36).

Chromatin is a highly condensed and wrapped complex of DNA and proteins in which the genetic material is packed. The repeating structural unit of chromatin is the nucleosome. The nucleosome consists of DNA wrapped around an octamer of four different core histone proteins (two copies each of H2A, H2B, H3 and H4) (52).

Histones are prone to modifications like methylation, acetylation, phosphorylation, sumoylation and ubiquitylation (29). These modifications can affect chromatin conformations and other recruited proteins that cause epigenetic changes (32). The main targets are usually the histones H3 and H4. In euchromatin – active state less condensed and with high transcription levels - lysines in the H3 and H4 tails are hyperacetylated and lysine 4 of H3 is trimethylated. On the contrary, in heterochromatin – silent state, highly compacted and with low transcription levels - lysines in H3 and H4 are hypoacetylated and lysine 9 of H3 is trimethylated (32,38).

Signal transduction pathways

The response to a stimulus is usually based on signaling pathways that regulates gene expression and consequently the secondary metabolism (40). The most studied pathways are cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), calcineurin/calmodulin, target of rapamycin (TOR), and mitogen-activated protein kinase (MAPK) (36).

MAPK signaling is conserved in all fungi and is usually divided into the signaling cascades of the cell wall integrity (cell wall biosynthesis and repair), high osmolarity glycerol (responds to osmostress) and pheromone (hyphal formation and sexual crossing) (36).

It was shown that the three major *N. crassa* MAPK signaling pathways cooperate during regulation of female sexual development and conidiation. In addition, MAK-1 MAPK cascade plays a role in tyrosinase expression required for secondary metabolite L-DOPA melanin biosynthesis (53). In some cases, these pathways strongly influence each other, demonstrating a cross-talk interaction (36). For example, it is suggested that tyrosinase expression is modulated by the action of cAMP and MAPK signaling pathways (53).

1.2.3 Ways to trigger the production of cryptic secondary metabolites

Most BGCs are cryptic (*i.e.* silent) or expressed at very low levels under standard laboratory conditions. This means that the identity, structure and function of most of the metabolites encoded by the cluster remains unknown (29,39,54). Genome mining has recently revealed that there is a larger number of secondary metabolism clusters than the ones that were already discovered (29). Furthermore, as more fungal genomes are sequenced and given the cryptic secondary metabolism gene clusters present, it is clear that there are a number of natural products yet to be discovered (29).

To gain access to these metabolites, their biosynthesis needs to be induced. With the genomics era, is now common to obtain a draft genome sequence of the microorganism of interest and use bioinformatics tools to try to identify potential BGCs which can then be manipulated by various approaches (39,55). This strategy emerged has a new field of genomics-driven natural product discovery (55).

Promising strategies to activate the BGCs were developed and have been recently reviewed (31,55). Often they are based on mimicking the natural environments by changing abiotic factors or by co-cultivation with other microorganisms. Other approaches target regulatory mechanisms at different levels, such as global and cluster-specific regulators, epigenetic mechanisms or signal transduction pathways (36).

Cultivation based approaches

It has been observed that different culture conditions are correlated with variations in SMs production (56). Cultivation based approaches are built on this relation and attempt to predict the natural conditions that lead to the production.

The simplest strategy used is the 'One Strain-Many Compounds (OSMAC)' approach, based on subjecting the fungus to changes in the growing environment, basically by altering parameters like pH, temperature, nutrient source and light, among others (36,54,55). By growing *Aspergillus nidulans* using this approach, more than 45 different metabolic profiles were obtained and led to the discovery of secondary metabolites aspoquinolones A–D (57) and aspernidines A and B (58).

Bacteria and fungi co-inhabit in soil, plants, mucosal membranes and the gut (39). Since microorganisms interact with each other in their natural environment and use secondary metabolism as a defense mechanism, co-cultivation of two or more strains together could constitute a driving force for the production of secondary metabolites (39). Co-cultivation came up as a relevant and efficient strategy after the discovery of the antibacterial polyketide enacyloxin by the bacteria *Gluconobacter* during a co-cultivation with *Neurospora crassa* or *Aspergillus oryzae* (31,59).

Molecular based approaches

Molecular based approaches target directly the framework of regulation mechanisms involved in the production of SMs. These approaches include genetic engineering and chemical manipulation of chromatin modifications.

Approaches for genetic engineering of fungi and other microorganisms include gene knock-out, gene silencing and gene overexpression (60) .

The most traditional method to identify new metabolites is accomplished by the inactivation/deletion of a gene of interest (*i.e.* possible candidate gene responsible for SM production) and subsequent analysis of the metabolic profile of the mutant strain, as compared to the wild-type (61). Using this approach, six NRPS from *Aspergillus nidulans* were deleted randomly leading to the production of an antibiotic emericellamide A (62).

Other strategies include the overexpression of a pathway-specific regulator and manipulation of global regulators. Bok and Keller found that the deletion of the global regulator LaeA decreased sterigmatocystin and penicillin formation in *Aspergillus nidulans* and gliotoxin production in *Aspergillus fumigatus* (39,51). On the contrary, its overexpression enhanced the production of these secondary metabolites (39,51). Bergmann *et al.* demonstrated that the overexpression of *apdR* (a Zn_2Cys_6 regulator of a hybrid PKS-NRPS gene cluster) allowed the identification of two new cytotoxic metabolites, aspyiridones A and B (54,63).

Another approach relies on the exchange of endogenous promoters of secondary metabolism BGCs for strong inducible promoters. For example, the exchange of *A. nidulans* promoter *acvA* for alchohol dehydrogenase 1 promoter, led to an increase of penicillin production (55,64).

Histone modifications have an important role in the activation of silent gene clusters once alterations in chromatin structure can trigger changes in gene expression on a genome-wide scale, including SM biosynthesis (29). Williams *et al. s*howed that fungal cultures treated with small-molecule epigenetic modifiers (such as inhibitors of histone acetyltransferases, histone deacetylase or DNA methyltransferases) could activate silent natural products pathways, leading to the production of new secondary metabolites (65). Deletion of genes connected to the chromatin modification machinery also has an impact. For example, deletion of *hdaA* (histone deacetylase) in *A. nidulans* resulted in transcriptional activation of sterigmatocystin and penicillin gene clusters (54,66). Moreover, the creation of an *A. nidulans* mutant with deletion of the enzyme CclA, a putative protein predicted to be required for H3 methylation, led to the activation of several BGCs and the production of several aromatic metabolites with a range of biological activities (55,67).

1.3 Ionic Liquids

Ionic liquids (IL) are, by definition, salts with a melting point below 100℃ (68–70). They usually comprise a bulky asymmetric cation and a weakly-coordinating anion which can be structurally modified, either on the anion, the cation or substituents on both (Figure 1.6). For this reason, there is a high structural diversity, and as a consequence different physical and chemical properties such as density, solubility and hydrophobicity (68,69). Besides that, ionic liquids have negligible vapor pressure, lack of flammability and high solvation potential making them interesting “green” chemicals (70).

For having such interesting characteristics, numerous applications of IL have been proposed in different areas such as organic synthesis, catalysis, separations and sustainable energies, among

others (71,72). At an industrial scale, some applications include the use of ILs in solar cells, batteries, paint additives and cellulose dissolution, etc (70). In addition, ILs are also suitable for chemistry processes connected with life-sciences, serving as solvents in enzymatic and whole-cell biocatalysis and as protein stabilization agents (70).

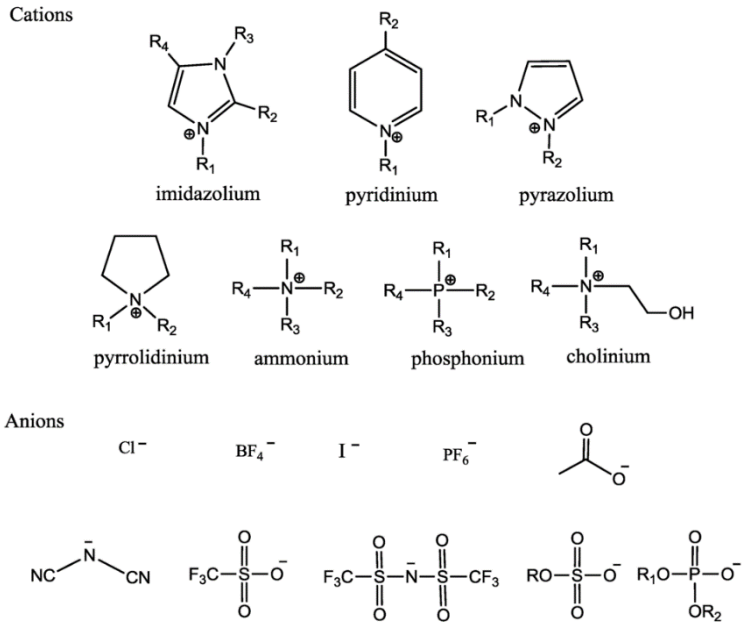


Figure 1.6. Examples of cations and anions commonly used in ionic liquid formulations. Adapted from (107).

However, ILs also carry concerns in terms of toxicity and biodegradability, affecting not only several organisms but also the environment as putative soil and water contaminants. These compounds are water soluble and also thermally and chemically stable, rising concerns in terms of environmental impact via wastewater effluents (69). A broad range of testing models – bacteria, mammalian cells, animals, fungi, algae and plants – have been used to evaluate the ecotoxicity of ILs since different organisms show very dissimilar behaviors when exposed to these chemicals (48).

Toxic effects are related to the intrinsic characteristics of the composing ions. It was shown that the increase of the chain length of side chains connected to the cationic head group leads to an inhibition enhancement of the ILs against numerous organisms (68,69,72). The effect of the anionic species seems to be less significant (70,72). This is likely because the lipophilic parts can be intercalated into the membrane and the ionic head group is partially solvated in aqueous solution (72). Taking this in mind, this hypothesis may be valid since it is observed that the mode of toxicity of ILs is related to necrosis, apoptosis and/or oxidative stress leading to loss of cell wall integrity and increase of membrane permeability (70,73).

Ionic liquids with certain molecular features enhance biodegradability (presence of esters, amides or linear alkyl chains, among others) (36,37). Specifically, on this study, the cholinium cation was reported to be readily biodegradable while imidazolium cation is non-biodegradable (70).

In addition, filamentous fungi show a high capacity to tolerate and even degrade fully or partially ionic liquids (48). In general, the imidazolium-based ILs were reported to be the most toxic, followed by the groups of pyridinium, pyrrolidinium, and piperidinium ionic liquids; cholinium salts were the less toxic (70). Additionally, when exposed to sub-lethal concentrations of ILs, *Ascomycota* fungal strains alter their metabolic footprint (74). Proteomic analyses of the two model fungi – *A. nidulans* and *N. crassa* – when exposed to both ChoCl and [C_2mim]Cl, showed that several biological processes and pathways were affected by either IL, provoking an accumulation of stress-responsive proteins and osmolytes (71). Within this study in particular, some results from the host laboratory showed that these chemicals can also induce metabolic alterations on fungal metabolism, boosting the diversity of low molecular-weight molecules synthesized by filamentous fungi (48,68,73,74).

1.4 Importance of the study

Fungi are usually found in association with humans and other organisms, being the cause of numerous diseases. Most people typically suffer from superficial fungal infections (on epithelial surfaces as skin and mucosa) which are usually not lethal and relatively easy to treat. However, invasive fungal infections (colonization of bloodstream, organs and central nervous system) (75) have lower incidence but carry a bigger concern since they are harder to diagnose and treat and are associated with high mortality rates (over 50%) (8). Moreover, the incidence of life-threatening fungal infections is rising, in parallel with the increment of people with a compromised immune function (75).The global concern of fungal disease is significant but underappreciated relative to other diseases (75). Nearly two million people die every year with invasive infections, which may be a higher number than deaths from tuberculosis or malaria (8).

Infections with no reliable medical therapy are emerging since there is an increase of drug resistance (75). Fungi are also plant pathogens, causing devastating losses in crop yield worldwide (4). The evolution of resistance to antimicrobial agents used to control pathogens in medicine and agriculture is a rising problem and it seems clear that antifungal drug development is not moving at the same pace as the clinical needs (76).

Thus, there is an urgent need to discover new antifungal compounds for use in human and veterinary medicine and in agriculture and forestry. Given the remarkable success of microbial metabolites as starting points (*e.g*. penicillin discovery), there is a growing scientific interest on fungal SMs for the mining of microorganisms for new drug screening (55). Complete genome sequences of filamentous fungi began to reveal their potential to produce complex and specialized metabolites, giving rise to a new field of genomics-driven natural product discovery which consists of the production and identification of the fungal metabolic products by activating cryptic BGCs (36,55).

1.5 Objectives

This Master thesis aims to functionally characterize antimicrobial peptaibiotics produced by *Neurospora crassa* upon imposition of an unique chemical stimulus – an ionic liquid. The list of specific and sequential objectives is as follows:

1. To investigate the capacity of *N. crassa* to produce differential peptide metabolites when grown in media supplemented with the ionic liquid ChoCl. To address this objective we will resort on high performance liquid chromatography (HPLC), the central technique to isolate also the most promising candidates. The cultivation conditions will be optimized aiming to increase the diversity of differential peptides as well as their titers; this will include testing also different producing strains.

2. To chemically characterize the novel peptabiotics of *N. crassa*. This will be attempted relying on a suite of methods, including *inter alia* Nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) and amino acid analyses.

3. To initially characterize the inherent bioactivity of the differential peptides. Bioactivity assays (*ex vivo* anti-cancer and antibacterial activity assays) will be performed.

4. To unravel the genes involved in the biosynthesis of the peptide metabolites differential produced under the ionic liquid stimulus. We will essentially use quantitative gene expression analyses focusing on the response of several nonribosomal peptide synthases (NRPS), as well as genes involved in regulatory and related pathways.

Chapter 2 – Materials and Methods

2.1 Chemicals

The ionic liquids 1-ethyl-3-methylimidazolium chloride ([C2mim]Cl, >98%) and Cholinium Chloride (ChoCl, ≥98%), and the amino acid 1-Aminocyclopropane-1-carboxylic acid (ACC, ≥98%) as well as reagents to prepare growth media were purchased from Sigma-Aldrich.

Solvents used in metabolites extraction, namely ethyl acetate and methanol, were from VWR Chemicals and Merck, respectively. Chromatographic solvents were of High-performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific.

2.2 Fungal Strains

Neurospora crassa wild-type strain 2489 and mutant strains 16712, 12039, 13475, 12937 and 13480, all from Fungal Genetic Stock Center (FGSC), were cultivated in Erlenmeyers on Dichloran-Glycerol (DG18) (Oxoid) for 7 days at 30 ºC, in the dark without agitation. The spores were released by washing the culture with a NaCl (0.85% w/v) and Tween-20 (0.1% w/v) sterile solution while imposing a gentle scraping using sterile glass beads. The spores' suspension was filtered through a funnel with a miracloth. After collecting the spores, equal volumes of the suspension were centrifuged for 5 minutes, 12,857 *g* at 4 ºC (Eppendorf R5810R). The supernatant was discarded; the spores were washed in an equivalent volume of sterile NaCl solution (0.85% w/v) and centrifuged in the same conditions as before; these steps were repeated one more time. The pellet was homogenized in a sterile solution containing NaCl (0.85% w/v) and glycerol (30% v/v). After checking the O.D (600 nm; using the solution mentioned above as blank), the spore suspension was distributed in aliquots into cryovials that were kept for 1 h at 4 ºC, then overnight at - 20 ºC and at - 80 ºC for long term storage.

2.3 Determination of Minimal Inhibitory Concentrations (MIC)

The minimal Inhibitory Concentration (MIC) was determined by measuring the absorbance of the culture medium at 600 nm.

Each liquid medium (1 mL) was inoculated with a suspension of fungal spores in order to obtain the final concentration of 10^5 spores *per* mL and divided into four wells (250 µL each) of a 96 well-plate. The control samples (inoculated) and the blanks (non-inoculated) were produced in duplicate and incubated under the same conditions: negative control (ionic liquid free medium) and blank samples (media with addition of selected ionic liquids). Cultures were incubated in the dark at 27 ºC, without agitation. Daily measurements of the absorbance at 600 nm were performed, during 7 days, to measure the fungal growth (or lack thereof).

Water activity (a_w) of minimal media supplemented with the corresponding MIC of either ionic liquid was determined with a portable water activity indicator (HydroPalm AW1) following the manufacturer's instructions, at room temperature and in triplicate (resting periods of 10 minutes).

2.4 Conditions of fungal cultures

Minimal Media (50 mL) alone (control) or supplemented with 50% or 80% of the MIC for the respective tested IL ([C_2mim]Cl or ChoCl) were inoculated with 10^5 *N. crassa* spores *per* mL. Liquid cultures were incubated in dark under agitation (90 rpm) for defined temperature and incubation times. After the end of incubation, the fungal mycelia were separated from the extracellular extracts by filtration (Millipore glass fiber filter) and washed twice with 5 mL of a solution of NaCl (9% w/v) and Tween-20 (1% v/v). In some cases, complete mini protease inhibitors (Roche) were added to the filtrate accordingly to the manufacturer's instructions. Both fractions were immediately frozen in liquid nitrogen, stored at – 80 ºC and further lyophilized.

Minimal Media (MM) contained *per* liter of water, 1 g K_2PO_4, 1 g glucose; pH further adjusted to 7 with 10% phosphoric acid and then autoclaved at 115 ºC for 10 minutes; 10 mL of filter sterilized salts stock (100x) were added after autoclaving. 100x salts stock contained, *per* 100 mL, 30 g $NaNO_3$, 5 g $MgSO_4.7H_2O$, 5 g KCl, 100 mg $ZnSO_4.H_2O$, 50 mg $CuSO_4.5H_2O$, 10 µL HCl (37%) and 100 mg $FeSO_4.7H_2O$; stored at 4 ºC in the dark.

2.5 Secondary metabolites extraction and analysis

Lyophilized mycelium-free extracellular extracts were dissolved in 5 mL of MilliQ water and extracted with ethyl acetate (1:1, 3 x 1 min). The ethyl acetate fractions (hereafter referred as crude extracts) were recovered and dried under soft nitrogen flow. The respective methanolic fractions (hereafter referred to as peptide fractions) were obtained using the Sep-Pak plus C18 cartridge (Waters) as previously described (77). Briefly, the samples were re-dissolved in 10 mL of MeOH:water (1:2) and then passed with a syringe through a conditioned Sep-Pak C18 cartridge. The cartridge was washed with 10 mL of MilliQ water and 10 mL of methanol:water (1:2). The compounds (peptide fraction) were eluted with 10 mL of methanol 100% to a glass tube and dried in soft nitrogen flow. The conditioning of the cartridge was performed by passing through the cartridge 10 mL of methanol 100%, MilliQ water and methanol:water (1:2), successively. The samples were analyzed by High Performance Liquid Chromatography (HPLC) after making a sample solution with methanol (10% w/v).

For intracellular fraction analysis, frozen mycelium was ground in liquid nitrogen using a mortar and pestle and then extracted with ethyl acetate (5 mL, 3 x 1 min). Crude extracts were analysed by HPLC.

2.6 Chromatographic analysis

The samples were separated using an Acquity chromatographer with Photodiode Array (PDA) detector, cooling auto-sampler and column oven (Waters). A Symmetry C18 column (250 x 4.6 mm) packed with end-capped particles (5 µm, pore size 100 Å) (Waters) was used at 26 ºC and the samples were maintained at 4 ºC. Data were acquired using Empower 2 software, 2006 (Waters). Samples were injected using a 50 µL loop and the volume of injection was 50 µL. The mobile phase, at a flow rate of 0.9 mL/min, consisted of a solution of 0.1% trifluoroacetic acid (TFA) (Solvent A) and acetonitrile (ACN) (Solvent B), set as follows: the initial conditions were 99.5% A and 0.5% B, reaching

100% solvent B within 30 minutes and keeping those conditions for 10 minutes; 2 minutes to return to the initial conditions and 10 minutes to re-equilibrate de column. Samples running time was 52 minutes. The chromatographic profiles of the samples were obtained at the wavelength of 205 nm.

2.7 Collection of peptide fractions

Collection of the differential peaks from the peptide fraction was performed with a Fraction collector III (Waters) connected to the Acquity chromatographer (Waters). Semi-purified compounds (*i.e.* differential peaks displaying a single chromatographic peak) were collected using the same chromatographic method described above. The collected fractions were dried under nitrogen flow and kept at – 20 ºC until further analysis.

2.8 ACC supplementation assay

The protocol used was adapted from Petersen *et al.* (78). Culture media (*i.e.* MM with 0.1% glucose) was inoculated with 10^5 *N. crassa* spores *per* mL (50 mL cultures). The cultures were incubated at 30 ºC, in dark, under agitation (90 rpm). On the 6th day of incubation, the corresponding cultures were supplemented with ACC (at concentrations of 2, 5 and 10 mg/mL) and incubated again under the same conditions for an extra nine days. Controls included the negative control (media not supplemented with ACC) and the "IL control" (media supplemented with the corresponding IL). The cultures were made in duplicate. After the end of incubation time, the samples were treated the same way as mentioned above (see sections 2.4 and 2.5).

2.9 Nuclear Magnetic Resonance (NMR) analysis

^{1}H NMR and Diffusion Ordered Spectroscopy (DOSY) analyses of the peptide fraction were recorded on a Bruker 500 MHz spectrometer from CERMAX - Centro de Ressonância Magnética António Xavier at ITQB-NOVA.

2.10 Amino acid hydrolysis and analysis

Total hydrolysis of peptides present in the peptide fractions and in the semi-purified peptides (*ca.* six samples) as well as the standard amino acids (Aib and ACC) was performed using 6 N HCl for 1 hour at 150 ºC under inert atmosphere (nitrogen/vacuum cycles) in a Workstation Pico-Tag (Waters). Hydrolysed samples and the standard mixture of amino acid hydrolysates were derivatised following the manufacturer's instructions.

Further analysis was performed using the AccQ•Tag Ultra Amino Acid Analysis Method™ (derivatisation kit and standard mixture of amino acid hydrolysates, Waters). The obtained derivatives were separated on an AccQ•Tag Ultra column (150 mm x 3.9 mm, 4 µm) by reversed phase HPLC (Alliance, Waters), and detected by fluorescence (Waters 2475 fluorescence detector), according to the following details: the column heater was set at 39 °C, and the mobile phase flow rate was set to 1 mL/min. Eluents A, B, C and D were MiliQ water, ACN, sodium acetate 50 mM with 5 mM Triethylamine pH 5.5 and sodium acetate 100 mM with 5 mM Triethylamine pH 6.8, respectively. The

separation gradient was according to the Table 2.1. One microliter (1 µL) of sample was injected for analysis using a 250 µL loop. The FLR detector was set at 250 nm and 395 nm for excitation and emission wavelengths, respectively. Data were acquired using Empower Pro software (Waters).

Table 2.1. Separation gradient for amino acid analysis method. Eluents A, B, C and D were MilliQ water, ACN, sodium acetate 50 mM with 5 mM Triethylamine pH 5.5 and sodium acetate 100 mM with 5 mM Triethylamine pH 6.8, respectively.

Time (min)	% A	% B	% C	% D
0.01	3	2	75	20
5	3	2	75	20
23	3	7	70	20
43	5	21	15	59
44	0	30	0	70
48	0	30	0	70
50	3	2	75	20
55	3	2	75	20

2.11 Edman sequencing

Edman sequencing of semi-purified compounds (*ca.* five differential compounds) was performed. Edman sequencing was executed at ITQB NOVA Analytical service unit using Procise®, a Protein Sequencing System (model 491) of Applied Biosystems (ABI). Chemicals and sequencing protocols of ABI were used.

2.12 Total RNA extraction and cDNA synthesis

Mycelia from control or exposed to ionic liquid were recovered from the liquid cultures (see section 2.4). Approximately 100 mg of frozen mycelia were grounded with poly(vinylpolypirrolidone) (0.4 mg *per* mg of mycelia) using a TissueLyser LT (QIAGEN).The pulverized mycelia were then used in the extraction and purification of total RNA using RNeasy Plant Mini Kit (QIAGEN), according to the manufacturer's protocol.

Total RNA precipitation was performed. For this, 50 µL of 7.5 M NH_4OAc, 0.5 µL of RNase-free glycogen and 250 µL of absolute ethanol (stored at –20 ºC) were added to 100 µL of the sample (dissolved in RNase-free water) and mixed. Precipitation occurred at – 20 ºC overnight. Then, after centrifuging at ≥ 12,000 *g* at 4 ºC for 30 minutes to remove the supernatant, the pellet was washed twice with 500 µL of ethanol 80% (stored at –20 ºC); each washing step was followed by a centrifugation at ≥ 12,000 *g* at 4 ºC for 5 minutes. After complete drying of the pellet, the sample was resuspended in RNase-free water. Quality, integrity and quantity of total RNA were analyzed in a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Complementary DNA (cDNA) was synthesized from 100 ng of the total RNA using an iScript cDNASynthesis Kit (Bio-Rad) in an Applied Biosystems 2720 Thermal Cycler. The reaction protocol consisted of 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C (according to manufacturer's protocol).

2.13 *q*RT-PCR analysis

All real-time PCR (*q*RT-PCR) oligonucleotide pairs – based on *Neurospora crassa* (strain OR74A) genome (17) – were designed using NCBI/ Primer-BLAST web tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and produced by STAB VIDA (the primers are depicted in Table 2.2).

*q*RT-PCR analyses were performed in a Thermal Cycler (Bio-Rad) using EvaGreen Supermix (Bio-Rad), 250 nM of each oligonucleotide and cDNA template equivalent to 1 ng total RNA, in a final volume of 10 µL *per* well (three technical replicates). PCR conditions were: enzyme activation at 95 °C for 30 s; 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 59 °C for 30 s; and melting curve obtained from 65 °C to 95 °C, consisting of 0.5 °C increments every 5 s. Data analysis was performed using the CFX Manager Software v3.0 (Bio-Rad). Expression of each gene was calculated relative to control. Expression of all target genes was normalized to the expression of the coding gene for sed5 vesicle protein.

Table 2.2. List of designed *q*RT-PCR oligonucleotide pairs (forward and reverse) used in this study. Selected genes (NCU08441, NCU04657, NCU05000, NCU04531 and NCU07119) were the potential candidates for the production of the compounds of interest by *Neurospora crassa* as well as genes coding for LaeA, LaeA homolog and VeA (known global regulators) and the candidate housekeeping gene (NCU00778).

Gene code	Gene Description	Oligonucleotide (5′ to 3′)	
NCU08441	NRPS	F	ATGGGACTGGAAGAGATTGTCA
		R	CATTTCCATCCCCAGAAACAGA
NCU07119	NRPS 2	F	GAAGACCAACAGGAGTTTTCCA
		R	CGAGCTTATACCTCGACCCAA
NCU05000	Hypothetical protein, NRPS	F	AGCTCATTGACACGTACAGGA
		R	TTCTTCATCAACTCGGGAGTCA
NCU04531	Hypothetical protein, NRPS	F	GGACAAACGGGAACGGGAA
		R	CCATACTTCCCAGGACTGACA
NCU04657	ACC deaminase	F	GTGCTGGATGATCGGTACCA
		R	TCCATCATGCCCGCCAGA
NCU00646	Methyltransferase LaeA	F	CATTTCTTACGATGGGCCGAA
		R	ACCTTGATAACCTGCTCGTGA
NCU01731	VeA-like protein	F	CGTGCCTATTCCGTCAGCA
		R	TCATTTCAGGATCAGCCCTGA
NCU04904	Hypothetical protein; homolog to AN0807 (*laeA)*	F	GGCTGTGGTATAAGTTGGCAA
		R	CGTGAATCTTGGGATTGTTCCA
NCU00778	Sed5 vesicle protein	F	TCAGTGTTGGCTCCCATGTC
		R	GACGCGAGAAGTGCTTGAAC

2.14 Liquid chromatography-mass Spectrometry (LC-MS) analysis

Semi-purified peptides (*ca.* five samples) were analysed by microLC-MS using a Triple TOF 6600 MS system (Sciex) equipped with the DuoSprayTM ion source. Chromatographic separation was carried out in the Eksigent ekspert nanoLC425 in micro flow using an HALO C18 (50 × 0.5 mm,

2.7 µm particle size, 90Å) column from Eksigent at the flow rate of 10 µL/min. The mobile phase consisted of a solution of 0.1 % formic acid (solvent A) and a solution of acetonitrile containing 0.1 % formic acid (solvent B), set as follows: 5 % B in 2 min, followed by a linear gradient of 5–95 % B in 12 min, 2 min of 95 % B, 2 min to return to the initial conditions, and 5 min to re-equilibrate the column. MS was operated in positive ionization mode, with TOF MS scan with a m/z range 100-1000 for 250 ms. The top 20 m/z ions were selected for MS/MS analysis with 100 ms accumulation time for a total cycle time of 2.25 s. MS data were processed using the PeakView software using the extracted ion chromatogram for the compounds of interest. For compound identity a $\Delta(m/z) \leq 5$ ppm as well as a low noise/signal ratio, was considered.

2.15 Antibacterial assay

The antibacterial assays were executed following the standard methodology implemented by the Clinical and Laboratory Standards Institute (79). Dilutions from 3.35 to 0.026 mg/mL of each tested fraction were made in Mueller-Hinton Broth medium (MHB). The antibacterial activity was assessed against the gram-positive and gram-negative bacteria *Staphylococcus aureus* and *Escherichia coli*, respectively. All tests were done in triplicate; abiotic and biotic controls (medium alone and containing each bacteria, respectively) were also analyzed. The Inhibitory Concentration of 50% of the population (IC50) was established using a logistic model constructed in Microsoft Excel and then tested using XL-STAT (Addinsoft).

2.16 *Ex vivo* assay for anti-cancer activity

All tests were conducted as disclosed in published US patent application 20130136694 and as previously described (74). Briefly, egg chambers from female *Drosophila* (≤ 7 days old) were exposed to peptide fractions at standardized concentrations in Schneider's culture medium under controlled standard atmospheric conditions for up to 6 h. Egg chambers were observed using standard light and fluorescence microscopy and scored for presence/absence of polarity marker protein Par6. Each assay was done in triplicate. Controls for these assays include the compound *the*-103 (100% activity in *the*LiTE™) and negative control (solvent only).

Chapter 3 – Results

3.1 Determination of the MIC of ionic liquids in *Neurospora crassa*

It is well known that fungal processes such as growth and production of metabolites are affected by both abiotic and biotic factors. These include, among others, temperature, pH, presence of other organisms or foreign chemicals and water availability (36).

Neurospora crassa was subjected to a range of ionic liquid concentrations to test its susceptibility towards the presence of these chemicals. The minimal inhibitory concentrations (MIC) of [C_2mim]Cl and ChoCl against *N. crassa* are displayed in Table 3.1. MIC values were similar to those previously determined by Martins *et al* (73) and ChoCl and [C_2mim]Cl showed, respectively, low and moderate toxicity (68,70). The cholinium cation can be partially degraded by *N. crassa* (73) and can also be used as a carbon source (80) whereas the [C_2mim] cation did not appear to be degraded (73). Notwithstanding that cholinium cation is usually considered benign it showed to have an effect on growth inhibition. Previous reports on *A. nidulans* proteome grown in media supplemented with ChoCl suggested that the growth inhibition effect of the cation may be due to the accumulation of toxic by-products (*e.g.* cyanide) during its metabolisation (73). Importantly, it has to be taken into account that the MIC values should not be interpreted as absolute but rather as an indication of the upper inhibitory concentration limit.

Water availability is one of the major parameters affecting the growth of fungi. This factor can be measured by the water activity (a_w). Generally, germination of spores is optimal at a_w values higher than 0.7-0.8 and spores' formation frequently require higher a_w values than germination. In addition, the lowest a_w at which fungi growth has been observed was 0.61 (81). As depicted in Table 3.1, the growth media supplemented with either ionic liquid displayed a_w values of 1.0 which are considered to be optimal since growth inhibition only occurs at a_w values below 0.93 (5,82). Thus, in these media, growth inhibition of *N. crassa* did not appear to be related to water unavailability.

Table 3.1. Minimal inhibitory concentration (MIC) values of [C_2mim]Cl and ChoCl against *Neurospora crassa* and the corresponding water activity (a_w) of the growth media.

Ionic Liquid	MIC (M)	a_w
[C_2mim]Cl	0.4	1.0
ChoCl	1.2	1.0

The diversity of compounds produced by fungi depends on the composition of the growth media (56). Using this knowledge, a simple and effective technique with one single producing strain allowed the discovery of multiple secondary metabolites only by changing the growth environment (36,55). This technique is usually known as the OSMAC – One Strain Many Compounds approach. Taking the determined MIC values, sub-lethal concentrations of both

ionic liquids were used to impose a chemical stress to *N. crassa* cultures with the aim to activate the production of differential compounds.

3.2 Optimization of culture conditions

Growth media supplementation with different families of ionic liquids resulted in the altered metabolic footprint of different fungal strains (68). Moreover, supplementation of *Aspergillus nidulans* growth media with ionic liquids led to a dramatic alteration in the expression of several genes encoding for secondary metabolism. Concomitantly, the differential analyses (LC-MS) of the ensued fungal metabolome revealed 24 differential ion masses in cultures grown in a ChoCl (0.7 M) supplemented medium compared to control conditions (74). In addition, previous data from the team also demonstrated that the supplementation of the minimal growth media with either [C_2mim]Cl or ChoCl led to a higher metabolic diversity in *N. crassa* (data not shown). In addition, both ionic liquids also increased the expression of 1-aminocyclopropane-1-carboxyalate deaminase (NCU04657), which is the enzyme responsible for the formation of ACC (73). As previously described, ACC is a rare amino acid that has been linked to a specific group of non-ribosomal peptides namely peptaibiotics (42).

To investigate the hypothesis of peptaibiotics' production by *N. crassa*, preliminary studies have been performed with the crude extracellular fraction of cultures supplemented with either ionic liquid. NMR spectroscopy analyses corroborated the presence of peptides in these samples, and the corresponding mass spectrometry (MS) spectra showed a very weak signal with a *m/z* value of 1591, consistent with the identity of a peptaibiotic which usually display a molecular weight (MW) between 500 to 2,200 Da (data not shown).

However, in subsequent analyses *N. crassa* apparently lost the capacity to produce those compounds. Aiming to recover the capacity of the fungus to biosynthesize peptaibiotics when grown in medium supplemented with an ionic liquid, an extensive optimization of culture conditions was undertaken. The set of parameters analyzed during the course of optimization process are presented in Figure 3.1 and Table 3.2.

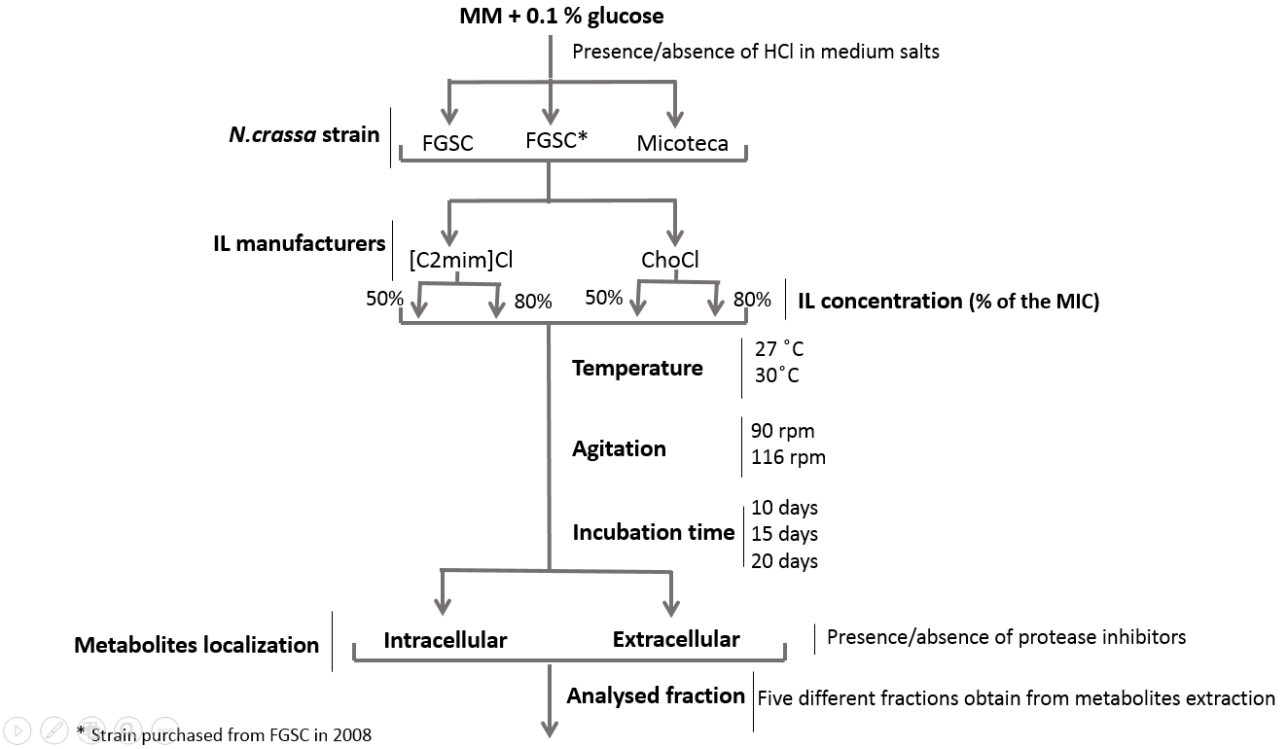


Figure 3.1. Set of parameters tunable during the optimization process. Scheme organization goes along the steps from fungal cultures until metabolites extraction and analysis. Strains were obtained from FGSC (Kansas State University) and Micoteca (University of Minho, Portugal).

Table 3.2. Set of experiments to optimize the differential production of peptaibiotics in *Neurospora crassa* cultures upon exposure to chemicals as ionic liquids. *FGSC stock was purchased in 2008.

Experiment number	Parameter	Experimental value	Final conditions
1	IL (concentration)	- ChoCl (50% and 80% of MIC) - [C_2mim]Cl (50% and 80% of MIC)	ChoCl 80%
2	IL manufacturers	Io-li-tec and Sigma-Aldrich	Sigma
3	Fraction analyzed	Sequential fractions attained during sample fractionation	Methanolic
4	Compounds localization	Intracellular and extracellular	Extracellular
5	Temperature	27 and 30 ºC	30 ºC
6	Incubation time	10, 15 and 20 days	15 days
7	Supplementation	Presence/absence of HCl in medium salts	Presence
8	Agitation	90 and 116 rpm	90 rpm
9	Protease inhibitors	Presence/absence	Presence
10	*N. crassa* strain	FGSC*, FGSC and Micoteca	FGSC

Media composition and cultivation parameters are known to have a great impact on microbial metabolite production (83). Not surprisingly, altering the agitation regime, the time of incubation and the ionic liquid concentration impacted differently the metabolic profile of *N. crassa* cultures; however, none of the profiles showed the differential accumulation of peptide metabolites. Parameters such as the incubation temperature and the supplier of the ionic liquid did not show major impact on the derived metabolic footprint (data not shown).

Only when using a new *N. crassa* strain from the FGSC did the supplementation of the growth media with the ionic liquid apparently lead to a differential metabolic profile (even though different from that observed in the preliminary assays). Analysis of the corresponding chromatographic profiles at 205 nm showed that only the peptide fraction presented significant differences between cultures grown in absence (control) or presence of the ionic liquid tested. ChoCl at a concentration of 80% of the MIC (*i.e.* 1 M) induced the higher production of diverse compounds; therefore, it was selected for subsequent studies. Final cultivation conditions used in the fungal cultures are depicted in Table 3.2.

3.3 Metabolic profiles of *Neurospora crassa* during exposure to Cholinium Chloride

The extracellular metabolites biosynthesized by *N. crassa* grown in medium supplemented with ChoCl at 80% of the MIC were extracted with ethyl acetate. The ensuing extracts were subjected to a further purification step using Sep-Pak C18 cartridges, a solid-phase extraction that relies on the capacity of the column to bind the peptides which can then be selectively eluted, hence purified and concentrated. The peptide fraction was resolved using HPLC. Representative chromatograms are depicted in Figure 3.2. It was possible to detect a set of 6 compounds (distinct retention times) that were only seen in the extracts derived from cultures grown in media supplemented with the ionic liquid. The differential compounds, which eluted at retention times of 7.7, 15.6, 17.3, 19.6, 29.7 and 33.6 minutes- hereafter referred as semi-purified peptides, were collected using a microcollector coupled to the HPLC.

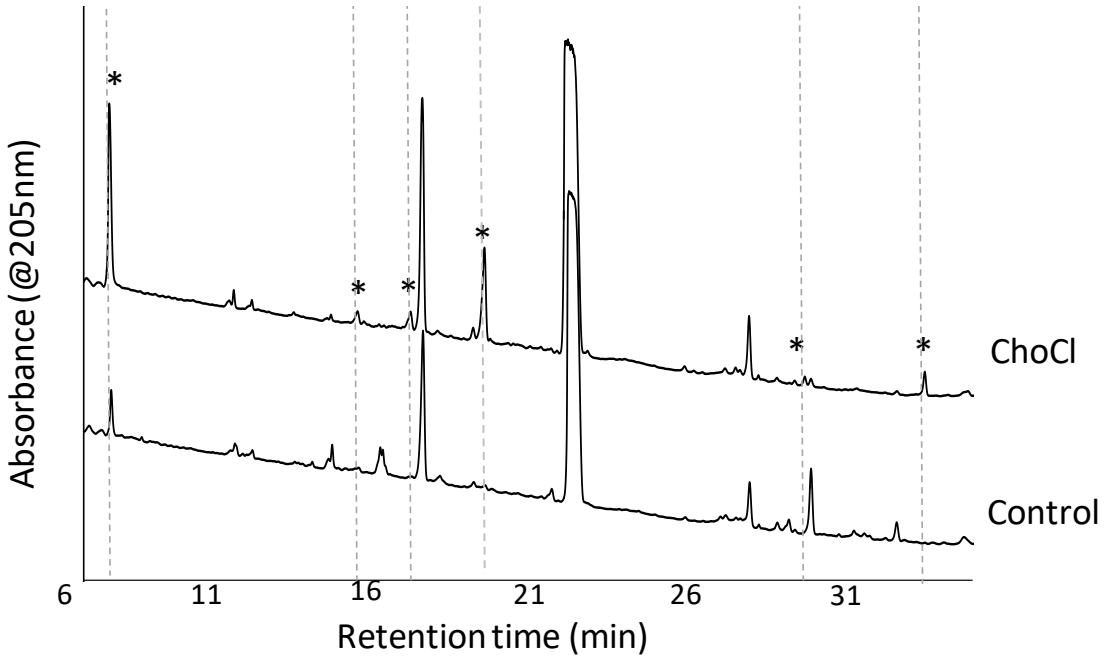


Figure 3.2. Aligned chromatographic profiles (205 nm) of the peptide fraction of *Neurospora crassa* wild-type cultures grown during 15 days in control media or media supplemented with choline chloride (1 M). Asterisks highlight the differential peaks at retention times of 7.7, 15.6, 17.3, 19.6, 29.7 and 33.6 minutes. All biological replicates were reproducible (data not shown). Protease inhibitors are known to elute at retention time of 22.2 minutes. 3 technical replicates were performed.

3.4 Characterization of the isolated compounds produced by *Neurospora crassa* after exposure to Cholinium chloride

NMR analysis

To confirm the hypothesis of peptaibiotics production by *N. crassa* cultures in media containing an ionic liquid supplement, the total peptide fraction was first analyzed by NMR spectroscopy to confirm the peptidic nature and the range of sizes of the extant compounds. The proton spectrum shows signals between 6 and 8 ppm (Figure 3.3) which are associated with peptide bonds (amide).

In addition, DOSY analysis was also performed. This technique aims to separate NMR signals of different species according to their diffusion coefficient; rate of diffusion is inversely related to molecular weight/size. The DOSY spectrum shows that the compounds mixture have a size higher than N-acetylglucosamine (molecular weight of 221 Da), here presented as a standard compound with a known molecular weight (Figure 3.4).

These results together show the presence of peptides with a molecular weight higher than 221 Da that can match the peptaibiotics size range (500-2,200 Da) (45). The NMR analyses were also attempted with the semi-purified peptides but the amounts collected so far were below the sensitivity level of the methods, hence higher amounts of the compounds are required to proceed further (ongoing work).

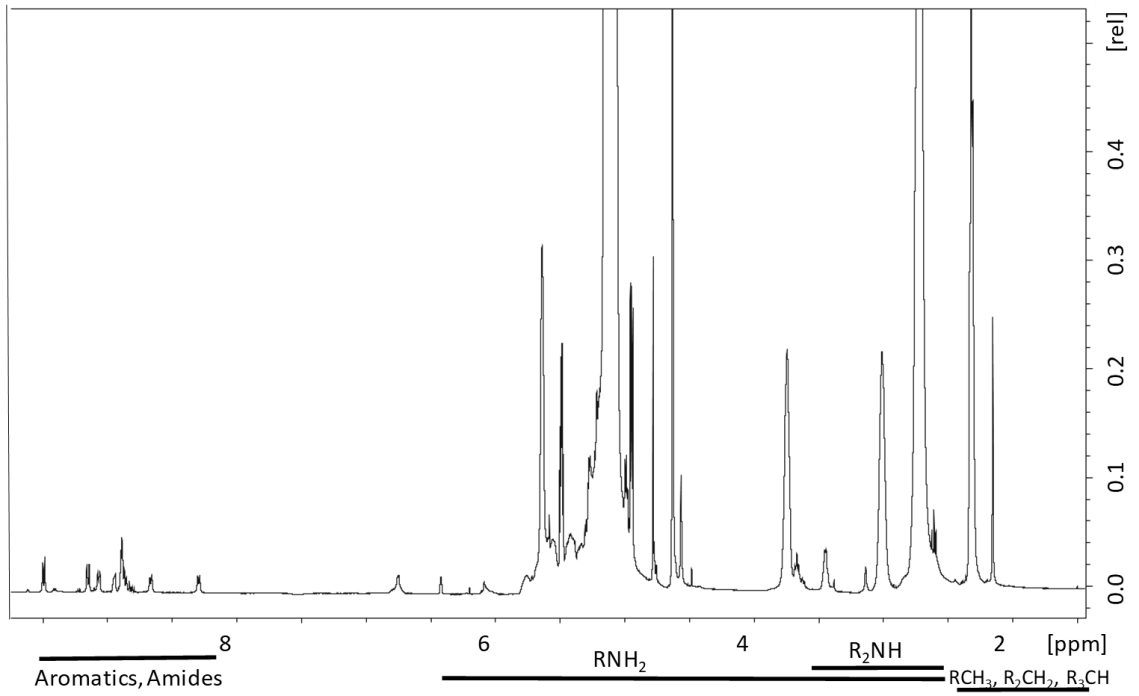


Figure 3.3. ^{1}H NMR spectra of total peptide fraction recovered from *Neurospora crassa* cultures grown 15 days with cholinium chloride (1M).

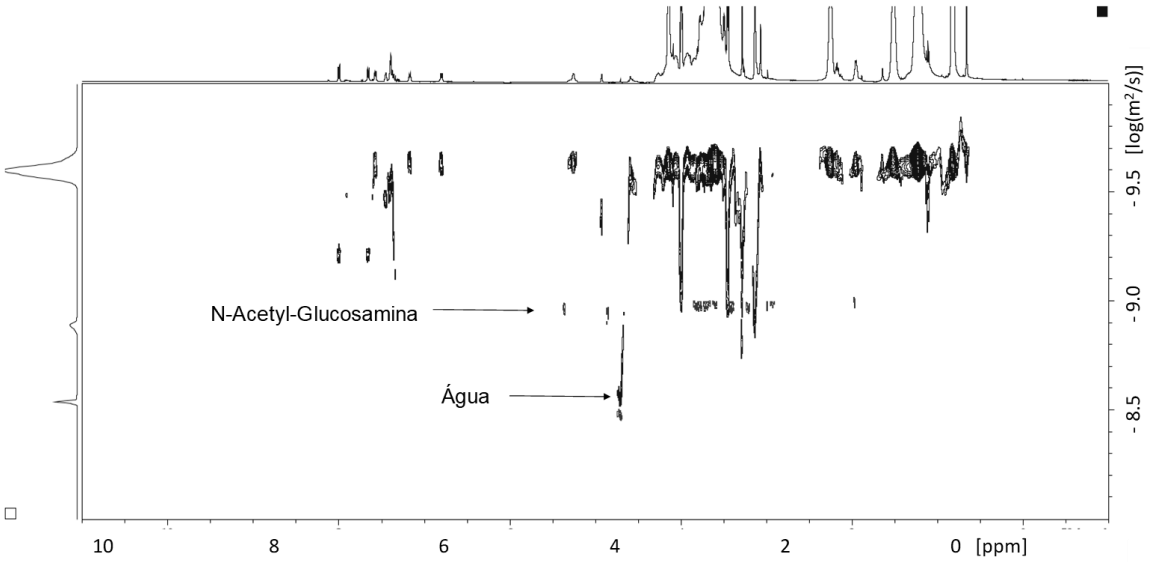


Figure 3.4 DOSY spectrum of total peptide fraction recovered from *Neurospora crassa* cultures of grown 15 days with cholinium chloride (1M).

Amino acid analysis

One of the main characteristics of peptaibiotics are the high content of the amino acid Aib (43). Therefore, evaluation of the presence of Aib in the peptides produced by *N. crassa* grown in media containing an ionic liquid supplement, would allow their characterization as peptaibiotics or not. Moreover, since previous studies showed that supplementation of this ionic liquid could lead to increased levels of ACC production (73), the presence of ACC was also evaluated.

Hydrolysates of the peptide fraction collected from cultures grown in media supplemented with cholinium chloride as well as from control cultures (standard growth media) were analyzed using the AccQ•Tag Ultra Amino Acid Analysis Method™. The presence of Aib could be only detected in the hydrolysates of the peptide fraction derived from cultures grown with the ionic liquid supplementation; ACC was also detected even though in low quantity (Figure 3.5). Overall, these observations support the hypothesis that cholinium chloride has the capacity to trigger the production of peptaibiotics in *N. crassa*.

Semi-purified peptides collected from *N. crassa* extracts cultures grown in media containing the ionic liquid supplement at retention times of 7.7, 15.6, 17.3, 19.6, 29.7 and 33.6 minutes were also analyzed using the AccQ•Tag Ultra Amino Acid Analysis Method™ (Figure 3.6). The chromatogram for the standard mixture of amino acid hydrolysates is depicted in Figure A2 (see Annex 2).

Aib and/or ACC were detected only in two of the peptides, however due to the low amounts herein analyzed one cannot disregard the possibility that for some peptides the rare amino acids amounts were below the detection limits of the method. Only the peptide eluted at 17.3 minutes contains the amino acid Aib as well as ACC (Figure 3.6 B), whereas the peptide eluted at 15.6 minutes contains ACC but apparently does not contain Aib (or is below its detection limit) (Figure 3.6 A).

To determine the complete amino acid sequence of the semi-purified peptides, Edman sequencing was also performed but failed. Peptaibiotics usually have the N-terminal acylated (*i.e.* blocked) (45), hampering sequencing by Edman degradation.

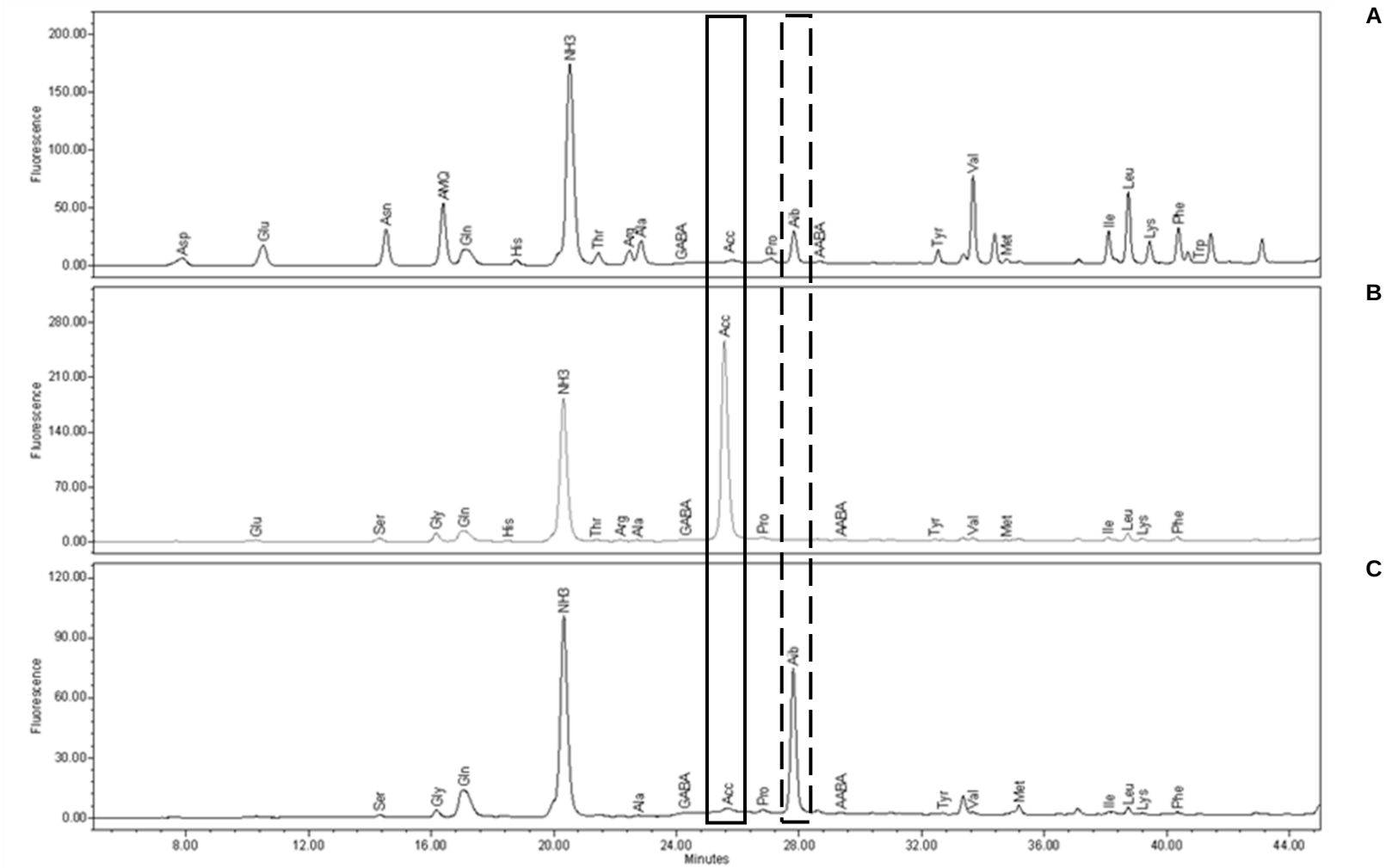


Figure 3.5. Chromatographic analyses of hydrolyzed samples of A) total peptide fraction recovered from *Neurospora crassa* cultures grown in media containing the cholinium chloride supplement; B) standard amino acid ACC (100 mM) and C) standard amino acid Aib (100 mM). Hydrolysate of the control peptide fraction was not included for a matter of simplicity.

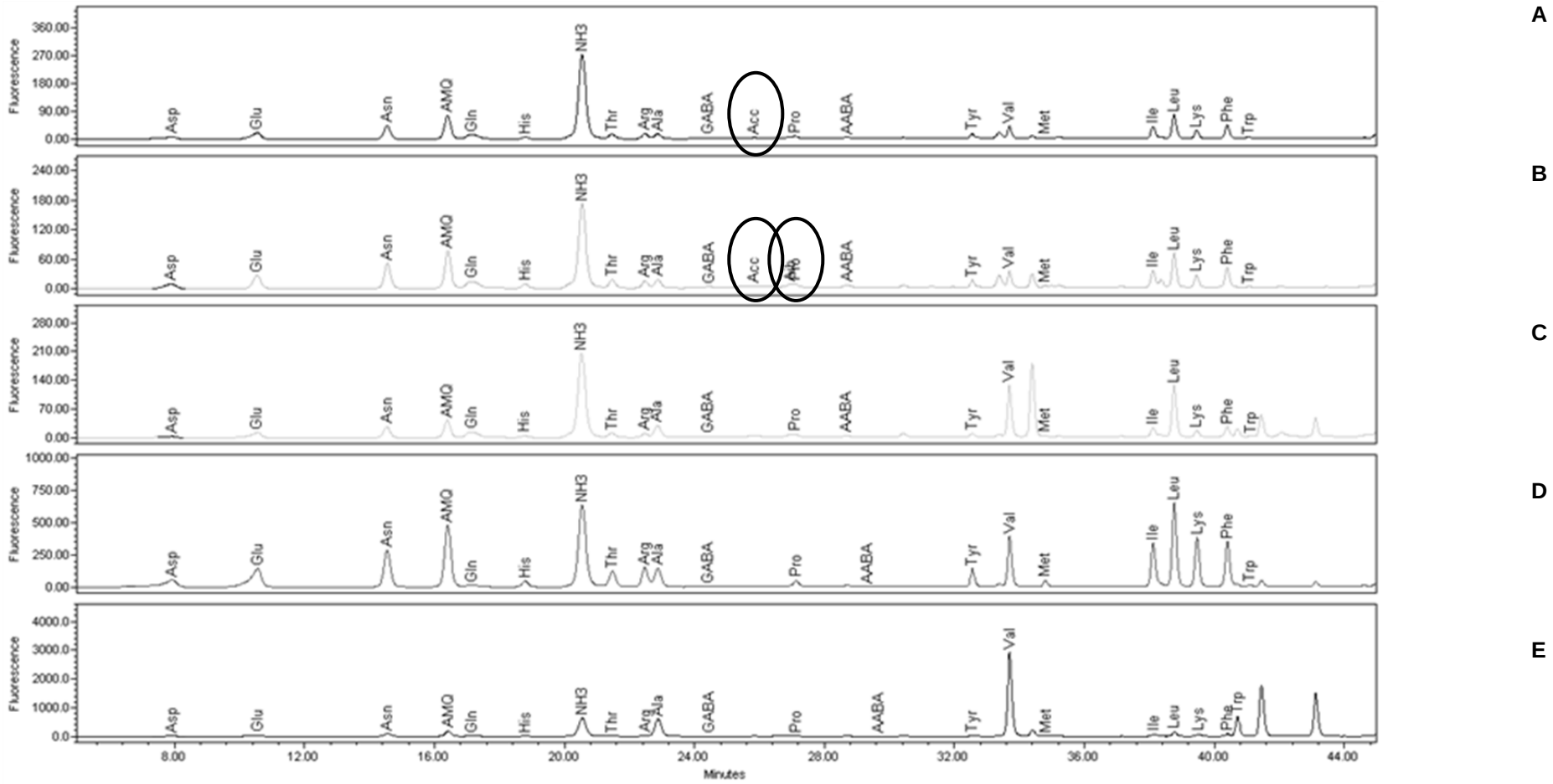


Figure 3.6. Chromatographic analyses of hydrolysates of the semi-purified peptides recovered from *Neurospora crassa* cultures grown in media containing the cholinium chloride supplement, and collected at retention times of A) 15.6, B) 17.3, C) 19.6, D) 29.7 and E) 33.6 minutes.

Chromatographic and NMR analyses showed that the supplementation of the growth media with an ionic liquid increased the diversity of peptide metabolites biosynthesized by *N. crassa*. The amino acid analyses confirmed that at least one of the biosynthesized peptides contains Aib, therefore classified as a peptaibiotic – the first discovery of *N. crassa* capacity to biosynthesize this class of bioactive metabolites. Even though some of the differential compounds herein analyzed may not be peptaibiotics, they may hide interesting biological activity. In addition, peptides produced by fungi and bacteria that contain ACC but lack Aib have already been reported to display interesting biological activities (insecticide activity, anti-cancer and antibacterial) (84).

ACC supplementation

The supplementation of fungal cultures with an amino acid was shown to influenced the production of metabolites of interest (78). Since compounds with ACC could be detected through the amino acid hydrolysis analysis, herein we have also tested the supplementation of *N. crassa* cultures with this rare amino acid aiming to observe an increased peptaibiotics' production.

The chromatographic profiles of the extracellular peptidic fraction are shown in Figure 3.7. Out of the six compounds that were putatively differentially induced by the ionic liquid supplementation, four were also apparently altered by the presence of ACC. Moreover, compounds eluting at 17.3 and 19.6 minutes apparently accumulated in the media in a concentration dependent manner. The amino acid hydrolysis of compounds eluting at 19.6 and 29.7 min that were induced by the ionic liquid did not show the presence of ACC (Figure 3.6); this may be explained by the low amount of these compounds in the growth cultures, hampering the analysis. In contrast, the compound eluting at 15.66 minutes that was induced by the ionic liquid contains ACC but was not detected by chromatographic analyses after supplementation with ACC.

Further analyses will aim to verify the presence or not of ACC and Aib in the compounds produced in growth medium containing ACC as supplement. Combined analyses of the peptides produced in media supplemented with either ionic liquid or ACC provide an unexpected technical means to investigate the biosynthesis of peptidic metabolites in *N. crassa*. One interesting observation to explore further is that the accumulation of the peptides is apparently positively regulated by the ACC concentration, opening the possibility to explore also a concentration-dependent effect in the ionic liquid supplementation.

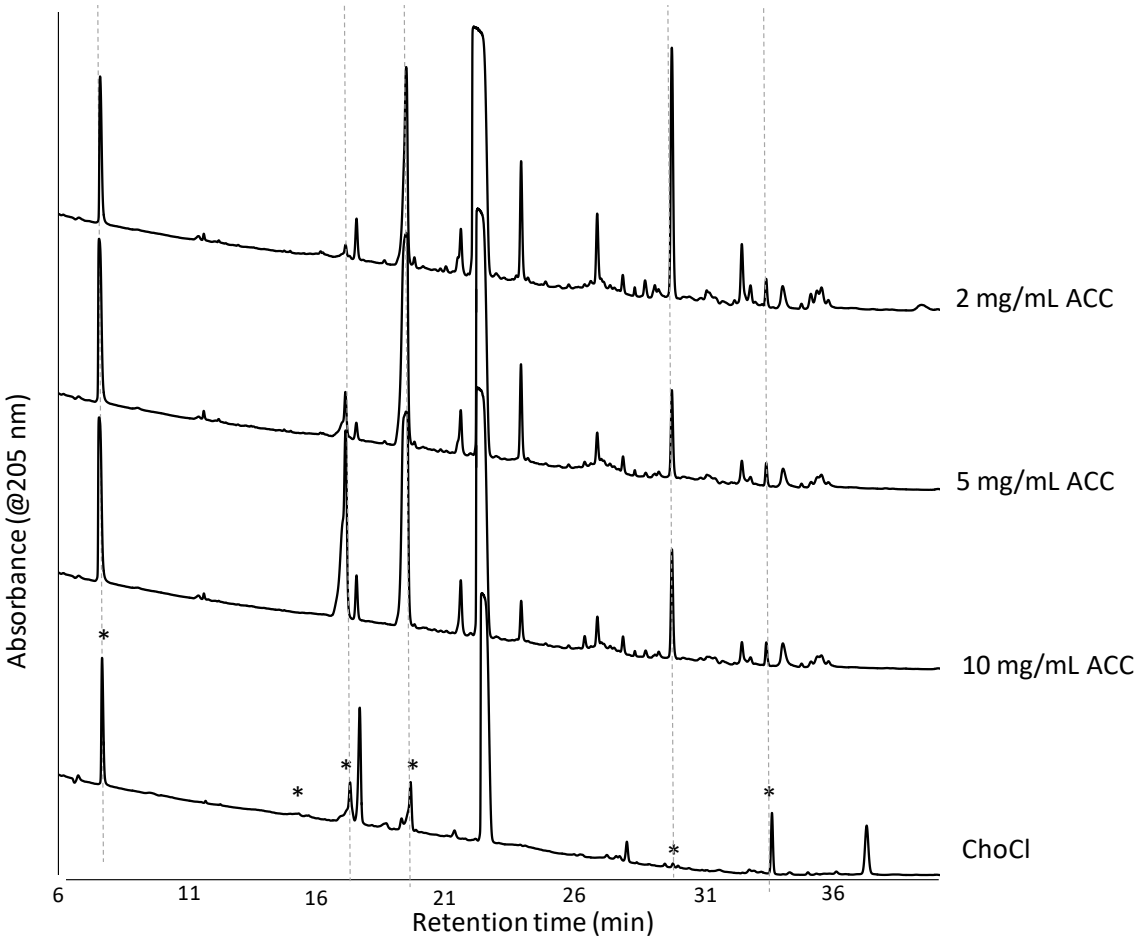


Figure 3.7. Aligned chromatographic profiles (205 nm) of the peptide fraction of *Neurospora crassa* wild-type cultures grown in media supplemented with cholinium chloride (1 M) or ACC (2, 5 and 10 mg/mL). Asterisks highlight the differential peaks at retention times of 7.7, 15.6, 17.3, 19.6, 29.7 and 33.6 minutes, only produced in the ChoCl supplemented medium and not in control conditions. Protease inhibitors are known to elute at retention time of 22.2 minutes. Cultures were done in triplicate and all replicates were reproducible (representative chromatograms depicted).

LC-MS analysis

To further analyze the semi-purified peptides derived from *N. crassa* cultures grown in media containing the ionic liquid supplement, all the samples were subjected to LC-ESI-MS analyses (untargeted). The corresponding total ion chromatograms, which are depicted in Figure 3.8, clearly demonstrate that the samples comprise more than a single compound. To further investigate the presence of peptaibiotics, we focused the analysis on the most intense peaks of the total ion chromatograms (TICs) (+/- 0.1 min) that display masses with signal intensity above 10^5. Several series were detected with mass increments of 44 Da, consistent with a polymeric interference, *i.e.* loss of a [C_2H_4O] group from *e.g.* polyethylene glycol (PEG), Triton or Tween (85,86). Ion masses of 317.0745 and 723.4113 were indeed observed in most samples, which may be assigned to dibutylphthalate and PEG, respectively (86). Optimization of a method to clean the peptide samples and/or avoid their contamination during fractionation and analysis is therefore necessary (*e.g.* TiO_2 was described to eliminate PEG from samples (87)).

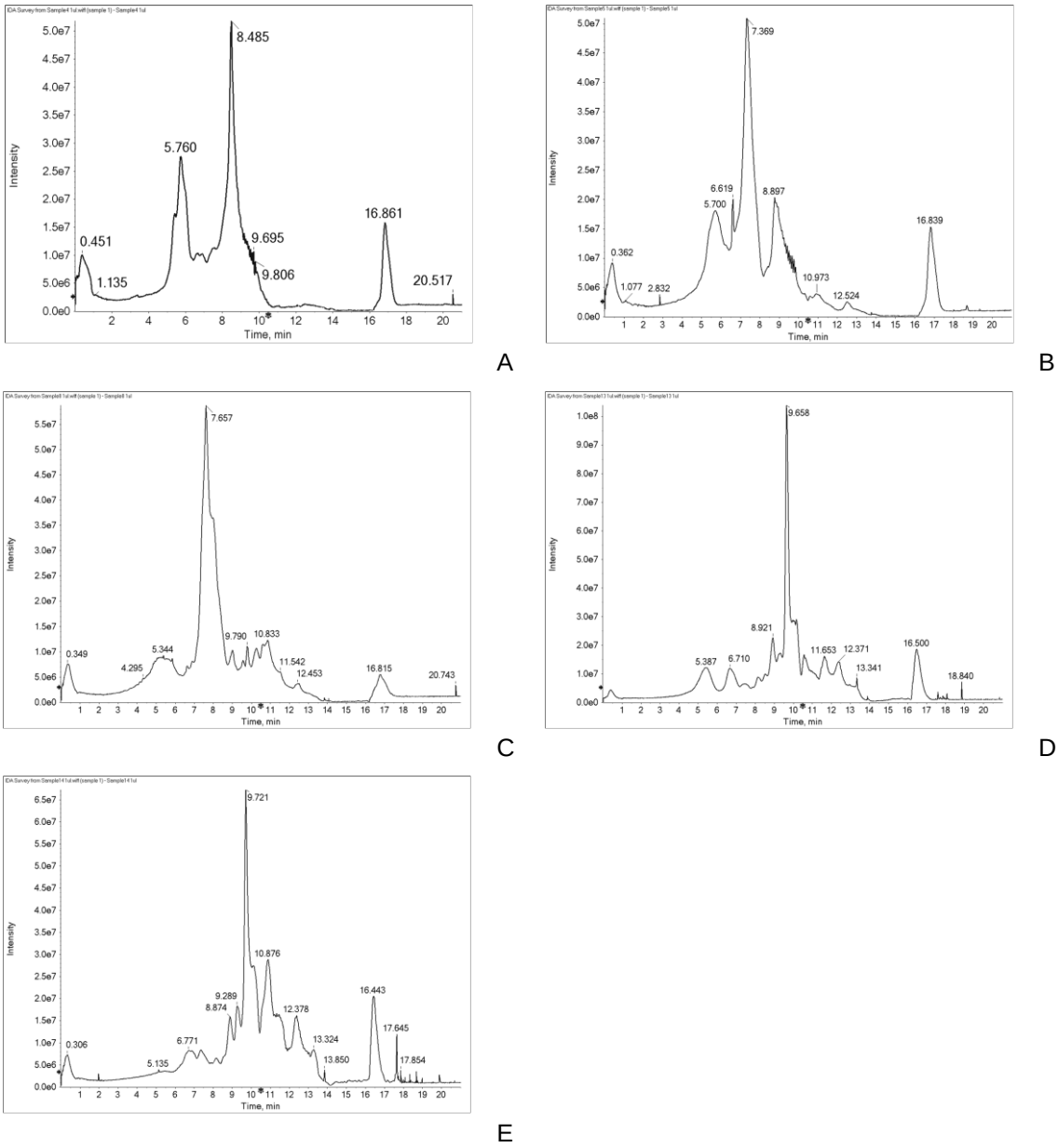


Figure 3.8. Total ion chromatograms obtained for the semi-purified peptide fractions eluting at 15.6 (A), 17.3 (B), 19.6 (C), 29.7 (D) and 33.4 (E) min.

Despite this obvious pitfall, in three out of the five analyzed semi-purified peptides, ion masses not fitting the series of masses from the polymeric contamination could be extracted (Table 3.3). Their exact mass values fit in the range described for the peptaibiotics (*i.e.* 500 Da to 2,200 Da) (45), therefore we have searched the peptaibiotics database (1390 accessions; (46)). Three masses display no match; possibly constituting new peptaibiotics. Only the mass of 1359.8669 (extracted from the semi-purified peptide eluted at 33.4 min) matches that of the known peptaibiotic Trichobrachin IIa A. The full sequence of amino acids (fifteen in total) of this peptaibiotic, originally isolated from *Trichoderma parceramosum* Bissett *(Trichoderma longibrachiatum* Rifai*)*, has been disclosed (77) (Table 3.4).

Table 3.3. Putative peptaibiotics masses present in the semi-purified peptides.

Semi-purified peptides elution time	Most intense peak in TIC (retention time, minutes)	Ion mass (m/z)
17.3 min	6.619	685.4630
29.7 min	9.658	1447.9218; 1491.9478
33.6 min	9.721	1359.8669

Interestingly, among the amino-acids comprised in the semi-purified peptide eluted at 33.4 min, Ala and Val are the most abundant (Figure 3.6 E), notwithstanding that Aib could not be identified. The total ion chromatogram of this sample (Figure 3.8) shows the presence of several compounds which may interfere with the amino acid analysis. This result as well as those of the other putative peptaibiotics (Table 3.3), deserves further analysis and validation *e.g.* MS/MS analyses.

Table 3.4.Amino acid sequence of Trichobrachin IIa A

Mod N terminal	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15
Acetic acid	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val

3.5 Understanding the biosynthesis of peptide metabolites produced by *Neurospora crassa*

To further validate the hypothesis that the compounds produced belong in fact to the class of peptaibiotics, the identification of the enzymes and/or cluster responsible for their production was attempted. Peptaibiotics belong to the class of non-ribosomal peptides which are produced by a backbone enzyme NRPS. In *N. crassa* genome, we could identify 4 candidate NRPS genes, namely NCU04531, NCU05000, NCU07119 and NCU08441, using the SMURF tool (37). NCU04657, the gene coding for ACC deaminase (*i.e.* ACC producing enzyme) was also subject of study with the aim to understand how ACC influences the biosynthesis of peptaibiotics.

Mutants' metabolic profile during exposure to cholinium chloride

The preferential method to elucidate the biosynthetic pathways of secondary metabolites relies on methodical gene deletion targeting one cluster at a time *i.e.* genes putatively coding for biosynthetic enzymes (61).The differential analysis of the metabolic profiles

of the knock-out mutants compared to the parental strain can reveal the association of peptaibiotics to specific genes. Chromatographic profiles of *N. crassa* knock-out mutants grown in media supplemented with ChoCl (1 M) are represented in Figure 3.9.

In the metabolic profiles of the mutants compared to the wild-type the compound eluting at 7.7 minutes did not show any alteration, hence it is most probably produced by another biosynthetic pathway not related to peptaibiotics production (Figure 3.9). In fact, since a similar compound may be present at lower levels in cultures grown in control conditions (Figure 3.2), this compound has been ignored in subsequent analyses.

Compounds eluting at 15.6 and 29.7 minutes were not detected in any of the chromatographic profiles derived from cultures of the mutants. Therefore, the analysis will be focused on the compounds eluting at 17.3, 19.6 and 33.6 minutes. When NCU04531 is deleted, the strain loses the capacity to produce the compound(s) eluting at 33.6 minutes under the stimuli of the ionic liquid, suggesting a major role of this NRPS in the biosynthesis. The mutant deleted of NCU04531 apparently produced in medium supplemented with ChoCl higher amounts of the compound(s) eluting at 17.3 minutes compared to the wild type. In addition, all mutants herein tested have apparently increased the production of the compound(s) eluting at 19.6 minutes when grown in media supplemented with the ionic liquid. Regarding the NCU04657 mutant grown in media containing the ChoCl supplement, the metabolic profile showed an increase in the compounds eluting at 17.3, 19.6 and 33.6 minutes, hence an alternative source of ACC for the biosynthesis of the peptaibiotics may exist. While this remains highly speculative for compounds eluting at 19.6 and 33.6 min, the compound(s) eluting at 17.3 minutes contain both ACC and Aib (Figure 3.6).

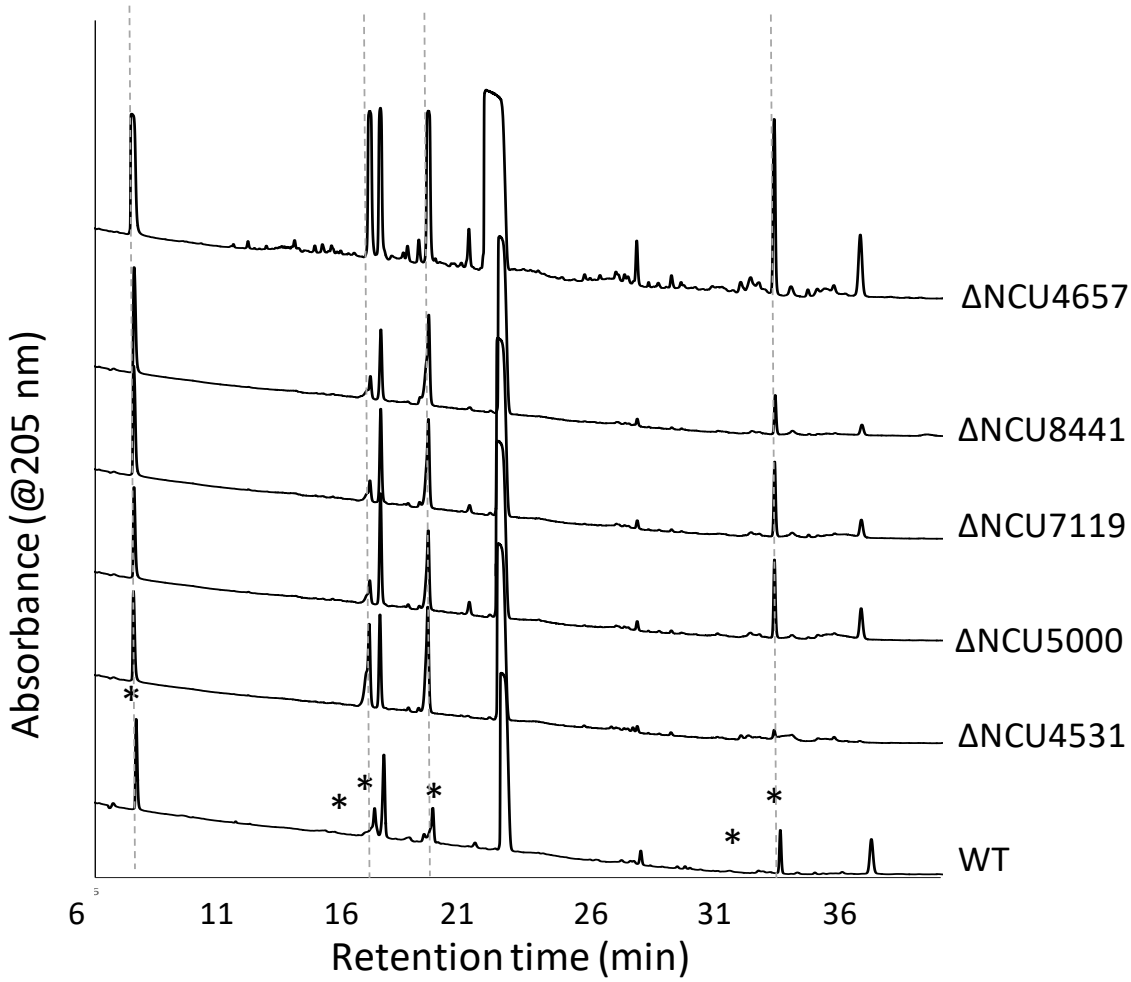


Figure 3.9. Chromatographic profiles of peptide fractions (crude) derived from fungal cultures grown in medium supplemented with ChoCl: Neurospora crassa wild type (WT) and strains carrying deletion in specific NRPS genes (ΔNCU04531, ΔNCU05000, ΔNCU07119 and ΔNCU08441) and for ACC deaminase (ΔNCU04657). Asterisks highlight the differential peaks at retention times of 7.7, 15.6, 17.3, 19.6, 29.7 and 33.6 minutes, only produced in the ChoCl supplemented medium and not in control conditions in the wild-type strain. Protease inhibitors are known to elute at retention times of 22.2 minutes. Cultures were done in triplicate and all replicates were reproducible (representative chromatograms depicted).

q*RT-PCR analysis*

qRT-PCR analyses were performed to follow the transcription of specific NRPS encoding genes in media supplemented with an ionic liquid (Figure 3.10) or ACC (Figure 3.11) compared to control conditions, aiming to identify the NRPS mediating the biosynthesis of the formed peptaibiotics.

In the wild-type strain (Figure 3.10 A) only the NCU08441 gene underwent up-regulation during growth in media supplemented with ChoCl compared to control conditions, with the remaining NRPS genes being down-regulated. Moreover, the NCU04657 knock-out mutant showed a down-regulation of the NCU04531 gene in the ChoCl medium compared to control conditions, suggesting that this NRPS enzyme may use ACC (Figure 3.10 B).

When the NCU04531 knock-out mutant was grown in media supplemented with ChoCl, the expression levels of NCU08441 were greatly increased (Figure 3.10 C). These genes may be interdependent and may even share precursors. However, the expression of NCU04531 gene in the NCU08441 knock-out mutant in media supplemented with ChoCl (Figure 3.10 D) was not significantly altered, suggesting that their interdependence is uneven and possibly involves unknown regulators. Deletion of NCU07119 gene led to the up-regulation of NCU04531 gene in the ChoCl medium (Figure 3.10 E) suggesting that it is in a downstream biosynthetic pathway, possibly using the product of the NCU04531 encoding gene as precursor. Finally, the NCU05000 knock-out mutant led to the up-regulation of NCU07119 gene in the ChoCl medium (Figure 3.10 F) which means that probably uses precursors formed through the activity of NCU07119.

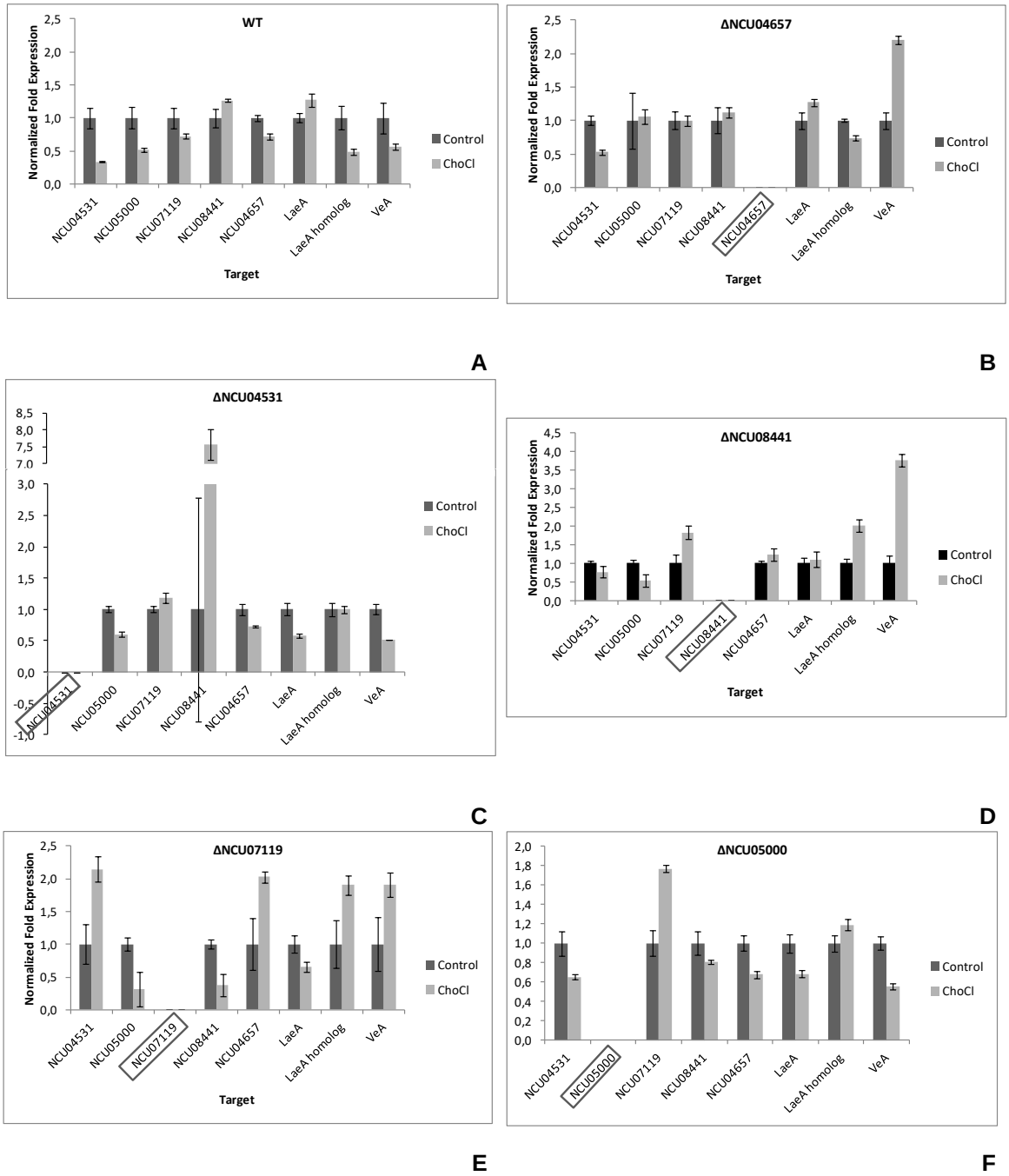


Figure 3.10. Analysis of relative expression of four NRPS genes (NCU04531, NCU05000, NCU08441, NCU07119) and ACC deaminase gene (NCU04657) by RT-PCR. *Neurospora crassa* wild-type (A) and the deleted mutants for NCU04657 (B), NCU04531 (C), NCU08441 (D), NCU07119 (E) and NCU05000 (F) were grown in media supplemented with ChoCl (1M). Values represent relative gene expression in pair-wise comparisons with the control (*i.e.* media not supplemented). sed5 vesicle protein gene was used as internal control.

The analysis of gene expression in *N. crassa* wild-type strain was also performed after ACC supplementation (Figure 3.11). The addition of ACC to the growth medium showed no alteration in the expression of the gene coding for NCU05000. The results also showed that ACC led to the up-regulation of genes encoding for NCU04531, NCU07119 and NCU08441. This may imply that although alternatives sources of ACC may exist, the pathways involving the later genes are positively regulated by ACC exogenous supplementation. This observation also

partially reinforces the possibility of a cross talk mechanism between the corresponding biosynthetic gene clusters.

The expression levels of genes encoding for regulators of the secondary metabolism - LaeA and its homolog, and VeA (88) - in both wild-type and the knock-out strains grown in media supplemented with the ionic liquid compared to control conditions, were not strongly correlated with the transcription levels of the NRPS genes (Figure 3.10). The most interesting observation is that ACC supplementation positively impacts the expression of the NRPS genes, yet inversely correlated with LaeA expression and directly corrected with LaeA homolog expression. A link between these regulatory pathways may exist.

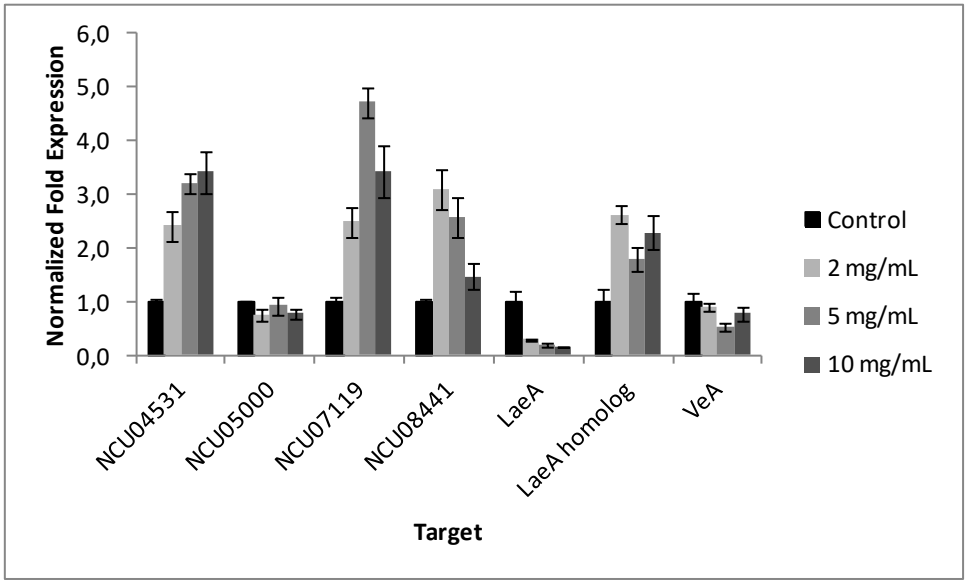


Figure 3.11. Analysis of relative expression of four NRPS genes (NCU04531, NCU05000, NCU08441, NCU07119) by RT-PCR. *Neurospora crassa* wild-type was grown in media supplemented with 2, 5 and 10 mg/mL of ACC. Values represent relative gene expression in pair-wise comparisons with the control (*i.e.* media not supplemented). sed5 vesicle protein gene was used as internal control.

3.6 Biological activity assessment of *Neurospora crassa* extracts after exposure to Cholinium chloride

The most interesting and appellative application of peptaibiotics is their use as anti-bacterial, -viral, -cancer, -fungal drugs, among others (45). For that purpose, anti-cancer activity tests were performed using the peptide fraction. Antibacterial activity assay against the gram-positive and gram-negative bacteria *Staphylococcus aureus* and *Escherichia coli*, respectively, was also performed; however, the assay did not provide consistent results and needs to be carried out again.

Disruption of cell polarity is a hallmark of cancer (89). *theLiTE*™, a validated *ex vivo* assay is based on exposing *Drosophila* epithelial tissues to test candidate compounds measuring the polarity modulating activity. The focus goes on target Par6, a polarity complex

protein responsible for construction and maintenance of apical junctions, essential for epithelial polarity (90).

TheLiTE™ was used to screen the presence of compounds with anti-carcinoma potential in the peptide fractions of *N. crassa* culture extracts. The cut off for a positive result is 50% which means that the testing compound is capable to impair the fluorescent ring of polarity marker protein Par6 in more than 50% of the analyzed eggs. The positive control (*i.e.* 30 µM of candidate compound *the*-103) affects 100% of the *Drosophila* eggs while the blank (*i.e.* solvent only which is 0.6 % (v/v) DMSO) presents no significant activity. Only the peptide fraction without protease inhibitors showed activity higher than 50% (Figure 3.12); this activity results from the differential metabolites production since the respective negative control did not show significant activity. In addition, the peptide fraction with protease inhibitors can also be marked as a positive result since the assessment of this potential in mixtures of compounds can be more complex and the cut off is usually lower. Moreover, an explanation for its lower activity may have to do with the fact that the presence of protease inhibitors contributes to a dilution effect.

To further analyze the potency of the compounds present in both peptide fractions, dosage dilutions were performed and tested. Surprisingly, the peptide fraction with protease inhibitors showed a reproducible cell polarity modulation effects at all doses tested (Figure 3.13), suggesting it to be more potent than other compound mixtures tested; the same was not true to the peptide fraction without protease inhibitors (data not shown).

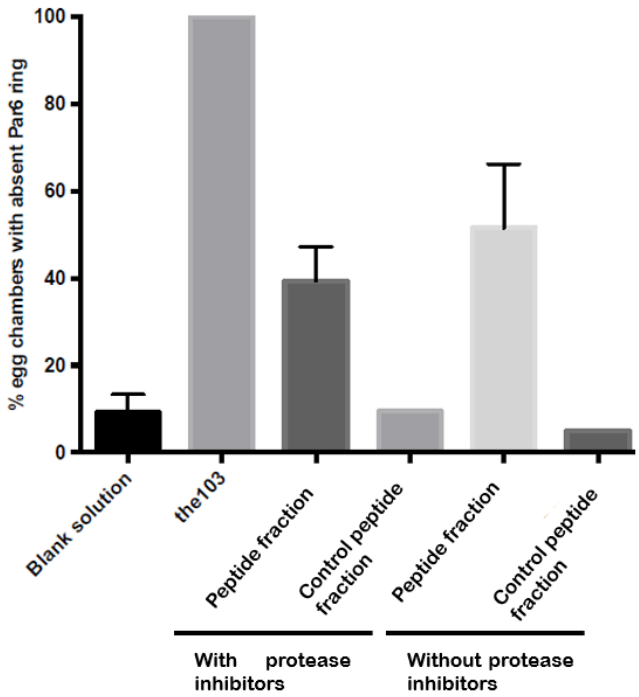


Figure 3.12. Ex vivo theLiTE™ assay of peptide fractions recovered from *Neurospora crassa* cultures alone (control) or supplemented with cholinium chloride and in the presence or absence of protease inhibitors. The figures represent the % of egg chambers showing Par6 ring impairment in the presence of blank solution and of *the*-103 compound; or in presence of the peptide fractions (48 µg/mL) recovered from control cultures and cultures exposed to cholinium chloride with and without protease inhibitors.

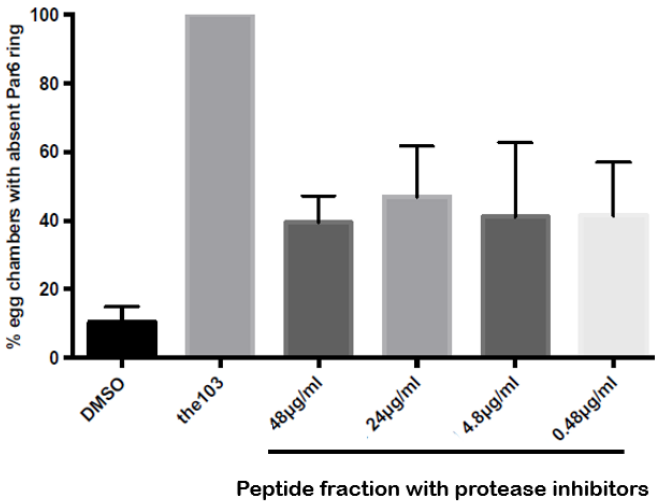


Figure 3.13. Ex vivo theLiTE™ assay of peptide fraction recovered from *Neurospora crassa* cultures supplemented with cholinium chloride in the presence of protease inhibitors. The figure represents the % of egg chambers showing Par6 ring impairment in the presence of blank solution and of *the*-103 compound; or in presence of dosage dilutions (48, 24, 4.8 and 0.48 µg/mL) of the peptide fraction obtained after exposure with the ionic liquid cholinium chloride, in the presence of protease inhibitors.

Chapter 4 - Discussion

Previous observations showed that the supplementation of ionic liquids to the growth media of *N. crassa* induced the production of differential compounds possibly belonging to the class of peptaibiotics; a particular class of peptide metabolites that have been demonstrated to display interesting biological activities (34). During a previous study, *N. crassa* lost the capacity of production of differential peptides in media supplemented with an ionic liquid. To attempt the recovery of such unique biosynthetic capacity, we have herein undertaken an extensive optimization of the growth conditions (Schematically represented in Figure 3.1 and Table 3.2). Final conditions leading to a differential metabolic profile were set: growth medium containing as supplement 1M of ChoCl (cultivation for 15 days, 30 ℃ with agitation). The differentially accumulated peptides apparently differ from those preliminary observed most likely because the cultivation conditions set here are also different (*i.e.* [C_2mim]Cl at 50% of the MIC was replaced by ChoCl at 80% of MIC).

A set of six differential compounds (eluting at retention times 7.7, 15.6, 17.3, 19.6, 29.7 and 33.6 minutes) were produced by *N. crassa* cultures grown in media containing a sub-lethal concentration of ChoCl (*i.e.* 1 M) (Figure 3.2). Only the compounds eluting at 15.6 and 17.3 minutes were demonstrated to contain ACC and ACC plus Aib, respectively (Figure 3.6). This corroborates *N. crassa* capacity to produce peptaibiotics (Aib containing compounds) as well as peptides containing ACC; constituting an unexpected first discovery in the model fungus *N. crassa*. The other differential peptides may also contain one or both rare amino acids; a hypothesis that deserves careful analysis and validation since their detection may have been hindered by the limited amounts of peptides tested. Some amino acids may be below the detection limits of the used techniques and their hydrolysis may also result in their degradation (91). Additional tests will therefore consider higher amounts of the semi-purified peptides. In addition, the preliminary LC-ESI-MS data is also consistent with the identity of peptaibiotics, notwithstanding that the samples showed the presence of contaminants which may suppress the mass signals of the peptides. Further purification and cleaning of the semi-purified peptides is therefore required before completion of the mass spectrometry analyses. Despite this experimental drawback, at least four masses could be extracted (from three semi-purified peptides) (Table 3.3), all of which fall in the mass range of peptaibiotics with one matching the known peptaibiotic Trichobrachin IIa A. This observation is inspirational and efforts to solve their chemical identify will be done, comprising not only further MS-based assays but also NMR and amino acid analyses, following the methods already tested. The presence of the differential peptides in an additional set of cultures grown in media containing the ionic liquid supplement has been validated, and the peptides already fractionated as herein established. These samples will be first used for NMR and biological activity assays, as well as for the required optimization before new MS-based assays are attempted.

Peptaibiotics constitute one of the most interesting classes of fungal secondary metabolites since they display broad-spectrum antibiotic and membrane disrupting activities (43). Further analyses of the semi-purified peptides are necessary to demonstrate their potential antibacterial and antifungal activity. Nonetheless our preliminary results showed that the peptide fraction differentially produced when *N. crassa* growth medium is supplemented with ChoCl, displays promising anti-cancer activity (Figure 3.12 and Figure 3.13). The anticancer activity levels herein observed are extremely high, inspiring the search for the most active compound within the tested extracts. A new set of samples – semi-purified peptides - is currently under evaluation.

Inspired by the potential biological activity of the differential peptides, we have analyzed also their biosynthesis especially as this knowledge could help in the development of improved strategies for the production of higher titers of targeted peptides. For that purpose, knock-out strains carrying single NRPS gene deletions (as well as the deletion of the ACC deaminase gene) were functionally analyzed. The assays included analysis of the differential chromatographic profiles derived from cultures grown in media supplemented with the ionic liquid (Figure 3.2) or ACC (Figure 3.7), as well as the analysis of the expression levels of the target genes (which included the exploratory analysis of some regulatory genes) (Figure 3.10 and Figure 3.11). To facilitate an integrated analysis, the obtained data is represented qualitatively in Table 4.1,Table 4.2 and Table 4.3). Although the amino acids composing the differential peptides derived from cultures of mutants grown in the presence of ChoCl or the wild type strain grown in medium supplemented with ACC are not yet known, we will assume herein their identifies based solely on their chromatographic retention times.

The chromatographic analyses of the peptide fractions from *N. crassa* NRPS deletion mutants (Table 4.1) clearly show that the NCU04531 gene is linked to the production of a compound eluting at 33.6 minutes (*i.e.* its production is decreased/lost in the mutant). The compounds eluting at 15.6 and 29.7 minutes remained unseen in all the peptide fractions from the mutated strains most probably due to their low amounts in these extracts. For the remaining peptides, no direct relation between their production and the deletion of a specific NRPS gene was observed. One possible explanation for the formation of the peptides is the existence of cross chemistry between the NRPS genes, even though they are physically separated in different clusters, some of which may even be located at distinct chromosomes – a hypothesis elegantly described by Mortensen and co-workers (92).

Table 4.1 Summary of results of chromatographic analyses of *Neurospora crassa* knock-out mutants cultures supplemented with ChoCl. Symbols mean the changes compared to the profile observed in wild-type culture supplemented with ChoCl (increase ↑, decrease ↓, not observed × and not altered ~).

CHROMATOGRAPHIC ANALYSIS						
Strain	Differential peaks (retention time, minutes)					
	7.7	**15.6** ACC	**17.3** ACC&Aib	**19.6**	**29.7**	**33.6**
ΔNCU04531	~	×	↑	↑	×	↓
ΔNCU08441	~	×	~	↑	×	~
ΔNCU07119	~	×	~	↑	×	↑
ΔNCU05000	~	×	~	↑	×	↑
ΔNCU04657	~	×	↑	↑	×	↑

Exposure to ChoCl increased the expression levels of NCU08441 gene in the wild-type strain compared to the control, whereas the remaining NRPS genes were down-regulated (Table 4.2). One hypothesis would be that the NCU08441 gene encodes for a NRPS that plays a “central role” in the production of differential peptides accumulating under the ionic liquid stimuli. Contrary to this idea, under the ionic liquid stimuli, the NCU08441 deleted mutant showed a similar profile of peptides compared to the wild-type strain (Table 4.1), except for the compound eluting at 19.6 minutes that increased as in all the remaining mutants. The differential compounds accumulating at retention time 19.6 may compete for the same pool of precursors as the remaining compounds. The expression levels of the NRPS genes in the mutant strains under the ionic liquid supplementation reinforce the idea of a cross-chemistry between the different NRPS gene clusters. The deletion of the NCU08441 gene impacts positively the gene NCU07119. NCU04531, NCU07119 and NCU05000 genes are also apparently linked, as the deletion of one impacts the expression of the other. A hierarchical interdependence between these genes could be suggested as follows (Figure 4.1):

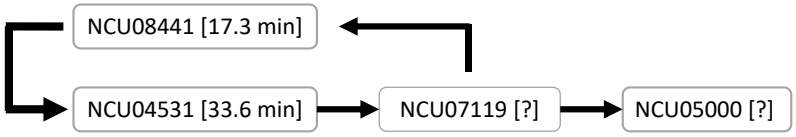


Figure 4.1. Representative scheme of the cross-talk of NRPS backbone enzymes. In brackets, the possible association between the peptide observed and the NRPS involved in the production.

The gene NCU04531 was correlated with a compound eluted at 33.6 min; its deletion led to increased levels of a peptide eluting at 17.3 min when grown in the ionic liquid media. One possibility is that this compound is linked to the activity of the NRPS gene NCU08441. This hypothesis requires careful validation since a compound eluting at the same retention time is still detected in the chromatograms of the knock out NCU08441 strain. The remaining peptides cannot be preliminary linked to the producing genes since they remained unseen in the chromatograms, with the exception of compounds eluting at 19.6 min that systematically

increased. One explanation is that the latter compound is associated with the expression of an NRPS-like gene; *N. crassa* has two, namely NCU08404 and NCU10423 genes (obtained through SMURF tool (37)), which have not been yet analyzed – opening up a new set of experiments to be undertaken in a near future. The interpretations done require validation and testing and may differ if the pool of differential peptides is expanded; although unlikely one cannot disregard the intrinsic limitations of the extraction and analytical methods herein used. One of the first steps in subsequent assays is the identification of the peptides produced by the mutants during growth in media containing an ionic liquid supplement, ultimately to verify whether they are or not similar to the peptides produced by the wild-type strain grown in the same conditions.

Table 4.2 Summary of results of RT-PCR analyses of *Neurospora crassa* knock-out mutant cultures supplemented with ChoCl for the target genes for NRPS (NCU05000, NCU08441, NCU07119 and NCU04531) and ACC deaminase (NCU04657). Symbols mean the changes of gene expression compared to the profile observed to the control (media not supplemented) (increase ↑, decrease ↓, not observed × and not altered ~).

RT-PCR ANALYSIS					
Strain	Expression levels of target genes				
	NCU05000	NCU08441	NCU07119	NCU04531	NCU04657
WT	↓	↑	↓	↓	↓
ΔNCU04531	↓	↑	~	×	↓
ΔNCU08441	↓	×	↑	↓	↑
ΔNCU07119	↓	↓	×	↑	↑
ΔNCU05000	×	↓	↑	↓	↓
ΔNCU04657	↑~	↑~	~	↓	×

ACC supplementation of the growth media provoked an effect comparable to that of the ionic liquid supplementation; ACC apparently induced production of similar peptides lacking only the peptide(s) eluted at 15.6 min (Table 4.3). Interestingly, the production of most of the differential peptides (except that eluted at 33.3 min) was dependent on the concentration of ACC, increasing proportionally for higher concentrations. Consistently, ACC exogenous supplementation positively influenced the NRPS genes, except NCU05000 (Figure 3.7 and Table 4.3). The hypothesis of interdependence between the clusters is reinforced by these data. Cultivation of the NCU04657 deletion mutant (*i.e.* the non-producing ACC strain) in media supplemented with the ionic liquid led to increased levels of the differential peptides eluting at 17.3, 19.6 and 33.6 minutes (Table 4.1). This observation suggests that these compounds can use another source of ACC rather than its formation through the ACC deaminase; nonetheless, their chemical identities and ACC contents requires careful validation. This hypothesis is strengthened by the analysis of RT-PCR data (Table 4.2) since the expression of the NRPS genes was not significantly affected in the mutant carrying a single deletion of NCU04657 gene.

We have also preliminary analyzed the expression of genes known as global regulators of secondary metabolism in filamentous fungi, namely LaeA and VeA as well as a LaeA

homolog. Our aim was to identify a master regulator, if any, that could boost the production of peptaibiotics in *N. crassa*. The obtained data raise the hypothesis that in *N. crassa* the production of peptaibiotics is not tightly regulated by these global regulators. No obvious relation between their expression levels and that of the NRPS genes could be established consistently across all conditions and phenotypes tested. Therefore, especially as these global regulators are also not well characterized in *N. crassa*, better understanding of their regulatory impact in the formation of peptaibiotics will not be an immediately important focus in future assays.

Table 4.3 Summary of results of chromatographic analyses of *Neurospora crassa* wild-type cultures supplemented with ACC. Symbols means the changes compared to the profile observed to growth media supplemented with ChoCl (increase ↑, decrease ↓, not observed × and not altered ~).

ACC supplementation						
ACC concentration (mg/mL)	Differential peaks (retention time, minutes)					
	7.7	**15.6** ACC	**17.3** ACC&Aib	**19.6**	**29.7**	**33.6**
2	↑~	×	↑	↑	↑	×
5	↑~	×	↑	↑	↑	×
10	↑~	×	↑	↑	↑	×

As higher amounts of the compounds are required to complete their chemical characterization, one possibility is to optimize growth conditions that increase the production titers in the wild-type strain, exploring as supplements of the growth medium either the ionic liquid or the ACC. Another possibility - assuming that the peptides biosynthesized under the ionic liquid stimuli are conserved in the mutant strains – is to take advantage of the metabolic profile of specific mutants seeking augmented production of a target peptide.

The answer to most of the above mentioned questions relies on the use of experimental methods which have been already established. Nonetheless, two important biological questions which have not yet been considered deserve special attention:

i) what is the functional role of the peptaibiotics biosynthesized by *N. crassa*; *i.e.* are these compounds related with the capacity of *N. crassa* to thrive in drastic environments such as burnt vegetation?;

ii) why has a peptaibiotic producing *N. crassa* phenotype evolved to a non-producing one? The two *N. crassa* strains that show distinct phenotypes were both purchased from FGSC; in fact the non-producing strain has been used in the initial studies, generating the first evidence of peptaibiotics biosynthesis. These observations raise exciting questions about these phenotypic features, which may be linked or not to epigenetic modifications. Other reasons may be behind the differential behavior of the strains, namely the biosynthesis of the peptaibiotics may be associated either with the presence of an endosymbiont (93) or with mobile genetic elements, such as conjugative plasmids, that are lost through sub-culturing of the fungus (94). To address these challenges the best strategy would be to perform RNAseq and compare the producing and non-producing strains; these methods are now in use in the host laboratory.

Chapter 5 - Conclusions

The obtained data are consistent with the initial hypothesis that certain ionic liquids can trigger the production of structurally diverse low-molecular mass compounds containing Aib and/or ACC in *N. crassa.* Supplementation of the growth media with ChoCl stimulates the biosynthesis of diverse peptides, comprising compounds that contain Aib. The presence of Aib in some of the peptides is consistent with the existence of peptaibiotics in the fungal cultures herein investigated. Furthermore, the presence of peptaibiotics containing also the rare amino acid ACC supports the idea that *N. crassa* can biosynthesize diverse unusual peptides. Data are extremely relevant because compounds belonging to the class of peptaibiotics, usually display potent biological activity, as herein preliminary demonstrated when considering their potential anticancer activity. Therefore the data set included in this Master Thesis opens the possibility to use *N. crassa* cultures grown under an ionic liquid stimulus (or maybe an ACC stimulus) to produce and identify novel compounds with broad pharmacological activity. From the biotechnological and industrial point of view, disclosing the regulatory mechanisms behind the production of these compounds in the model fungus could have valuable outcomes. Only some initial hints on the NRPS genes responsible for the production of the peptaibiotics have been generated, most remaining at this stage speculative and requiring re-assessment at the chemical and genetic level. Nonetheless, two NRPS constitute our initial future targets. NCU04531 is possibly associated with the production of a compound eluting at 33.3 min (further assays required to confirm whether it contains ACC and/or Aib) and NCU08441 may be linked to a compound eluting at 17.3 (which contains ACC and Aib; and one of its extracted ion masses fits peptaibiotic features).

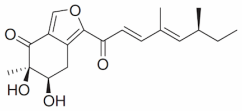
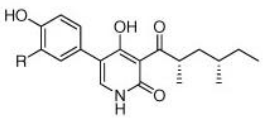
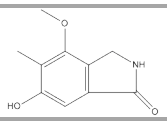
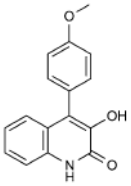
This work generated valuable knowledge on *N. crassa*. Results were largely unexpected because this model fungus is famous for its low secondary metabolite producing capacity, having only ten predicted gene clusters and hence not interesting from this point of view. Surprisingly, *N. crassa* was shown herein to produce unusual peptide-based secondary metabolites with remarkable biological activity. Surely, after validation and reassessment of these major findings, the secondary metabolism of *N. crassa* will not be seen as weak.

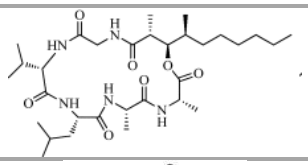
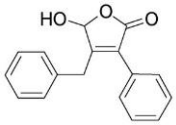
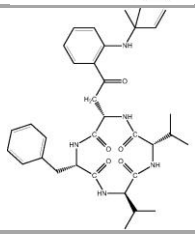
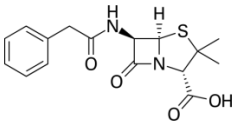
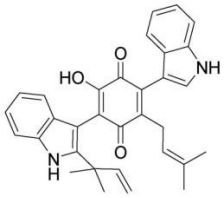
Annex 1- Experimental exploratory work in *Aspergillus nidulans* biosynthesis of peptide metabolites

During the course of the optimization of cultivation conditions that led to peptaibiotics production in *N. crassa* exposed to ChoCl, initial steps were taken to expand these findings to another model fungus, namely *Aspergillus nidulans*. Contrary to *N. crassa* this fungus is a great producer of secondary metabolites, comprising several putative NRPS genes. Our previous studies demonstrated the stimulatory effect of ChoCl in the production of ACC in *N. crassa* - seen at the proteome level through the up-accumulation of ACC deaminase in the mycelial proteome grown in ChoCl medium (73). A probable similar effect was noticed in *A. nidulans* cultures exposed to ChoCl. However, the effect was only visible due to the up-regulation of the gene encoding the ACC deaminase yet not translated into the differential proteome (73). With the goal to understand if ChoCl (or other ionic liquids) could also act as stimuli for the production of peptide-based metabolites, especially peptaibiotics in *A. nidulans*, we have run a series of exploratory work which will be described in the next paragraphs in the style of a short communication.

Nearly 70 backbone enzymes for secondary metabolism have been defined in the *A. nidulans* genome (92,95); however, so far only 25 of those has been characterized their respective secondary metabolite(s). *Aspergillus nidulans* encodes in its genome 12 NRPS, one hybrid PKS/NRPS and 14 NRPS-like genes (96) (Table A1) yet not all have been characterized.

Table A1. Examples of NRPS-derived secondary metabolites in *Aspergillus nidulans*.

Secondary metabolite	Encoding gene	Structure	REF
Asperfuranone	AN3494 (NRPS-like) and AN3495 (NRPS)		(97)
Aspyridone	AN8412 (Hybrid PKS-NRPS)		(63)
Cichorine*	AN6444 (NRPS-Like; not backbone gene)		(98)
Cyclopeptins (*e.g*. 4'-Methoxyviridicatin)	AN9226 (NRPS)		(99)

Emericellamide	AN2545 (NRPS)		(96)
Microperfuranone	AN3396 (NRPS-like)		(100)
Nidulanin A	AN1242 (NRPS)		(92)
Penicillin	AN2621 (NRPS)		(101)
Siderophores	AN0607 (sidC; NRPS), AN7884 (NRPS) and AN6236 (NRPS)		(102,103)
Terrequinone	AN8513 (NRPS-Like)		(104)

Previous studies by the host laboratory have provided evidence of the impact of ionic liquids in the ability of filamentous fungi to produce cryptic SMs (68,70). These studies included analyses of how ChoCl supplements induced alterations in the transcriptome and the metabolome of *Aspergillus nidulans* (74). The supplementation of *A. nidulans* growth media with this ionic liquid led to dramatic alterations in the expression levels of genes coding in secondary metabolism. In particular, 5 predicted uncharacterized NRPS underwent major up-regulation when the fungus was grown in media supplemented with 0.7 M ChoCl (*i.e.* 50% of the MIC).

In the present study, 2 genes coding for putative NRPS, namely AN5318 and AN2064, and for ACC deaminase - AN8899, were targeted for the production of single deletion mutants. This was accomplished using a well-established gene deletion and transformation method. The parent strain was FGSC A1149, which is a uracil auxotrophic and *nkuA* deletion (non-homologous recombination deficient) strain commonly used for transformation and efficient construction of gene knockouts. The strategy for gene replacement method used a fusion PCR method that relies on *Aspergillus fumigatus* pyrG as selectable marker (AfpyrG) (105). In brief, gene replacement constructs included 1 kb upstream of the relevant genomic sequence, the selectable marker pyrG and 1 kb downstream. Constructs were then transformed into A1149 mycelia protoplasts (produced using an optimal enzymatic-based protocol) re-suspended in buffer by adding the fusion DNA along with a PEG solution. Homologous recombination during

transformation leads to replacement of the target gene with AfpyrG. The transformants were selected by their ability to grow in YAS medium supplemented with pyridoxine; and the mutations were confirmed by double digestion using restriction enzymes that cut specifically in the relevant genomic coding sequence or in AfpyrG. Until now, ΔAN5318 and ΔAN2064 deletion mutants were successfully established yet the deletion of AN8899 gene remains to be confirmed.

The biosynthetic capacity of the mutants and wild type strain (parental strain) were done in media supplemented with ChoCl at 50% or 80% of the MIC. The MIC values for *A. nidulans* wild-type strain, which were obtained as described for *N. crassa* (see Materials and Methods, section 2.3), were 1.2 M and 1.8 M for 50 % and 80 % of the MIC, respectively. The MIC obtained for the wild-type strain was used as gold standard for assessing the sensitivity of each mutant; this allows the comparison of the effect of ionic liquid stimuli using the same conditions.

Aspergillus nidulans cultures (5 mL) were initiated from conidia (10^6 conidia *per* mL) in a 1 % glucose mineral growth media (106) without (control) or with the ChoCl supplements and incubated (dark, 30°C at 90 rpm) for 10 or 15 days. At the end of incubation, the mycelia were recovered by filtration (glass fiber pre-filters) and both mycelia and filtrate were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. The lyophilized filtrates were extracted and processed as described for *N. crassa* (see Materials and Methods, section 2.5). The crude metabolic extracts were analyzed by liquid chromatography (Materials and Methods, section 2.6). A representative zoom of a chromatogram is depicted in Figure A1, highlighting the differential accumulation of compounds in cultures grown in the presence of the ionic liquid supplement compared to the control conditions. Moreover, despite that some of the differential peaks are lacking in the profiles of ΔAN5318 and ΔAN2064 strains grown under the ChoCl stimulus, the complexity of their profiles hinders a more targeted analysis. Taken as an example, in Figure A1 the chromatographic profile of the ΔAN5318 strain in the ChoCl media lacks some of the peaks that were induced by the ionic liquid in the wild-type strain (bold dashed line, see arrows). The crude extracts will be fractionated to collect only the peptide fractions (Sep-pak extraction, Materials and Methods, section 2.6) and a differential analysis will be undertaken, aiming to link one specific peptide to its producing NRPS gene. The fractionation of the differential peptides will then proceed as optimized for *N. crassa*.

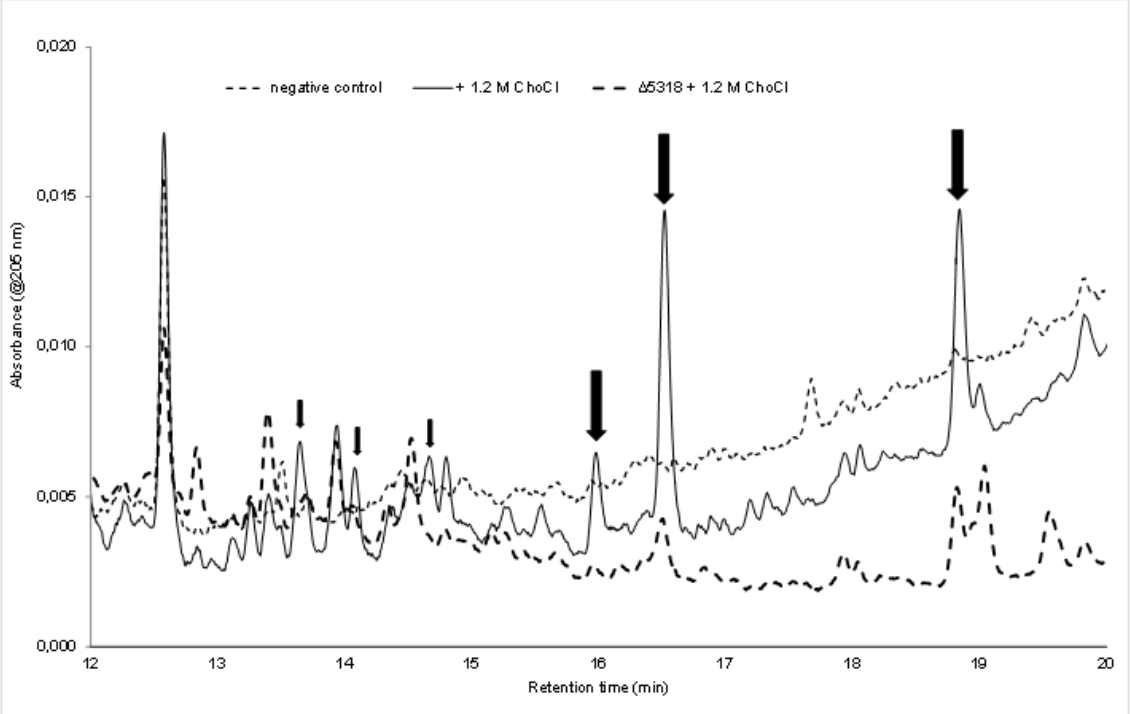


Figure A1. Aligned chromatographic profiles (205 nm) of the crude fraction of *Aspergillus nidulans* wild-type and ΔAN5318 cultures grown during 15 days in control media or media supplement with choline chloride (1.2 M). Arrows highlight the differential peaks. All replicates were reproducible (data not shown).

Annex 2- Standard mixture of amino acid hydrolysates

Amino acid standard used in the HPLC method for amino acid analyses (see Materials and Methods, section 2.10).

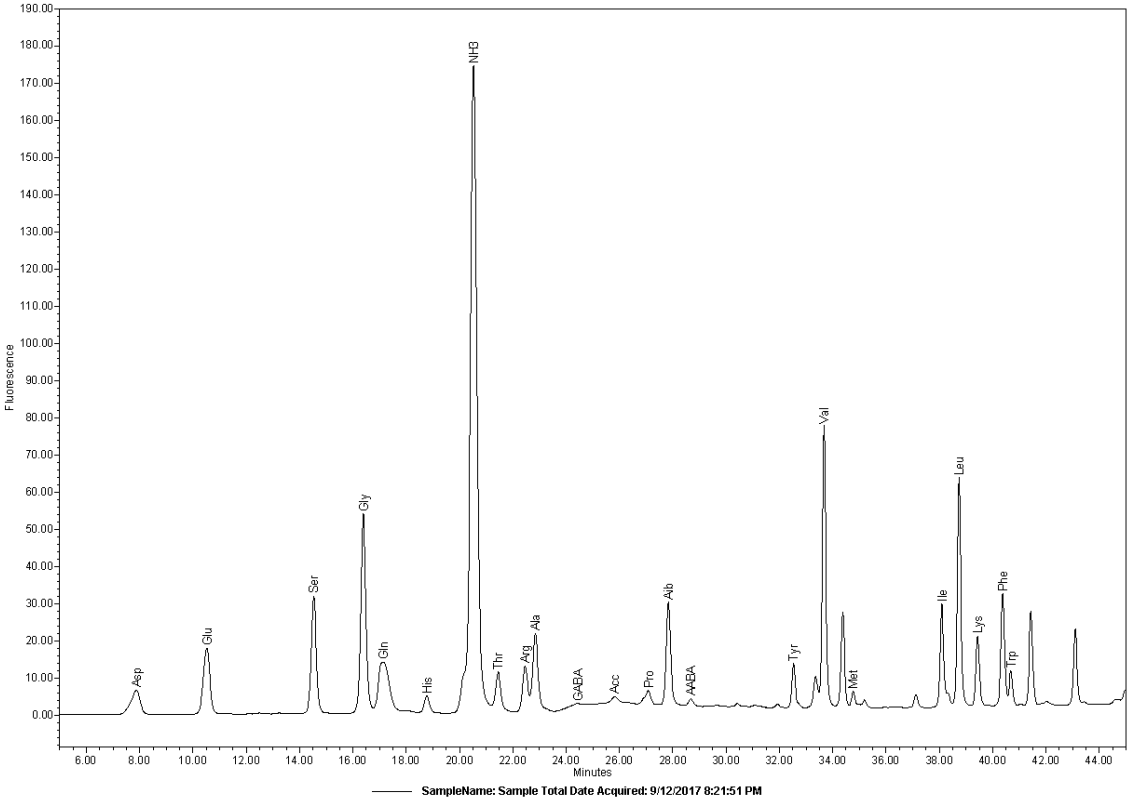


Figure A2. Standard mixture of amino acid hydrolysates. The standard is a part of the AccQ•Tag Ultra Amino Acid Analysis Method™ analyzed in the HPLC using the same method as for the remaining samples.

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