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Fungal naphtho- γ -pyrones—secondary metabolites of industrial interest

Elodie Choque · Youssef El Rayess · José Raynal · Florence Mathieu

Abstract Naphtho- γ -pyrones (NGPs) are secondary metabolites mainly produced by filamentous fungi (*Fusarium* sp., *Aspergillus* sp.) that should be considered by industrials. Indeed, these natural biomolecules show various biological activities: anti-oxidant, anti-microbial, anti-cancer, anti-HIV, anti-hyperuricemic, anti-tubercular, or mammalian triacylglycerol synthesis inhibition which could be useful for pharmaceutical, cosmetic, and/or food industries. In this review, we draw an overview on the interest in studying fungal NGPs by presenting their biological activities and their potential values for industrials, their biochemical properties, and what is currently known on their biosynthetic pathway. Finally, we will present what remains to be discovered about NGPs.

Keywords Naphtho- γ -pyrones · Filamentous fungi · Aurasperone · Rubrofusarin · Anti-oxidant

Introduction

Because of their negative perception of chemicals, some consumers presently claimed and were ready to pay significant premiums for natural products (Young 1998; Sebranek and Bacus 2007; Karre et al. 2013; Aneja et al. 2014). In order to satisfy this demand, manufacturers are constantly seeking new natural molecules with helpful properties that could replace or

diminish synthetically produced chemicals in their products. In this search for new natural compounds, secondary metabolites produced by plant, fungi, or bacteria are an important potential resource due to their diversity and their various biological activities. For example, toxins such as aflatoxins, ochratoxins, or fumonisins have a detrimental effect on humans and animals (Nielsen et al. 2009; Kew 2013; Wu et al. 2014). On the other hand, other secondary metabolites are beneficial and can be used as food additives, pigments, antibiotics, anti-oxidant, or anti-cancer agents (Archer et al. 2008; Nielsen et al. 2009; Chang et al. 2011; Lee and Pan 2012).

Among the polyketide secondary metabolites prospected, naphtho- γ -pyrones (NGPs) are widespread in nature; hence, they are produced not only by a wide variety of filamentous fungi but also by lichen (Ernst-Russell et al. 2000), higher plants (Li et al. 2001; Graham et al. 2004; Lee et al. 2006), and echinoderms (Bokesch et al. 2010, Chovolou et al. 2011). These natural compounds present a broad range of biological activities such as anti-oxidant, anti-microbial, or anti-tumor (Koyama et al. 1988; Zhang et al. 2007; Barrow and McCulloch 2009; Nielsen et al. 2009; Lu et al. 2014). As an example of application of NGPs in industry, it was demonstrated that an *Aspergillus niger* extract containing NGPs protected lard from being oxidized (Zaika and Smith 1975). Besides, a patent was recently filed for the preparation of a herbal extract of *Cassia tora* leaves for treating anxiety disorders (WO 2010109318 A1). Relying on previous works describing hepatoprotective and anti-mutagenic properties of NGPs extracted from *Cassia tora*, authors of the patent suggest that NGPs extracted from the leaves of *Cassia tora* could be one of the active compounds for treatment against anxiety (Wong et al. 1989; Choi et al. 1997).

There is few data available in the literature regarding the biological role of NGPs in their producing organisms. YWA1, a monomer NGP, is described to be a precursor of the black

Elodie Choque and Youssef El Rayess contributed equally to this work.

E. Choque · J. Raynal · F. Mathieu (✉)
Laboratoire de Génie Chimique, UMR 5503 CNRS/INPT/UPS,
Université de Toulouse, INP-ENSAT, 1, avenue de l'Agrobiopôle,
31326 Castanet-Tolosan, France
e-mail: mathieu@ensat.fr

Y. El Rayess
Faculté des Sciences Agronomiques et Alimentaires, Université
Saint-Esprit de Kaslik, B.P. 446, Jounieh, Lebanon

pigment dihydroxynaphthalene (DHN)-melanin in *Aspergillus* sp. (Watanabe et al. 1998; Tsai et al. 1998) and of the red pigment aurofusarin in *Fusarium* sp. (Malz et al. 2005; Frandsen et al. 2006). Rubrofusarin, another monomer NGP, was also linked to aurofusarin synthesis in *Fusarium* sp. (Frandsen et al. 2006). Besides, recent results suggest that NGPs could be involved in virulence of producing fungal strains. Indeed, Slesiona and colleagues (2012) showed that YWA1 was sufficient to allow *Aspergillus fumigatus* escape from macrophages by inhibition of phagolysosomes acidification during the first step of infection in invasive bronchopulmonary aspergillosis (Langfelder et al. 1998; Tsai et al. 1998; Thywißen et al. 2011; Slesiona et al. 2012; reviewed in Heinekamp et al. 2012). Going further, Slesiona and colleagues (2012) conditionally expressed *wA* gene, required for YWA1 production, in *Aspergillus terreus*, a strain that does not produce this precursor. This conditional expression led to the production of YWA1 and was sufficient to prevent acidification of phagolysosomes (Slesiona et al. 2012). This result emphasizes the role of NGPs in the interaction of *Aspergillus* sp. with macrophages during the infection process. Some of the remaining questions are for example: are other NGPs linked to fungal pigmentation and/or virulence? Are they involved in other biological processes?

Unlike higher plants, filamentous fungi, such as *A. niger*, are frequently used in the fermentation industry for the production of primary and secondary metabolites like organic acids (Alvarez-Vasquez et al. 2000; Roukas 2000) and enzymes (Maldonado and Strasser de Saad 1998) because they are easy to handle and allow the massive production of interesting compounds. As NGPs represent the most abundant family of secondary metabolites in *A. niger* (Nielsen et al. 2009), it makes this filamentous fungus a good candidate for the production of these molecules. The aim of this review is to provide an overview of the production of NGPs by filamentous fungi. We will present their industrial potential, then their biochemical properties. Even though, several drawbacks, which will be presented in this review, now prevent their massive production. Describing what is known on the biosynthetic pathway of NGPs and what remains to be known will help us in improving the industrial process to produce/extract these potentially interesting molecules.

Biological activities of fungal NGPs and their potential industrial applications

The biological effects or activities of NGPs produced by different filamentous fungi have been partially studied. For example, the biological activities of some NGPs have already been reviewed showing anti-viral, anti-microbial, insecticidal, and anti-estrogenic activities (Barrow and McCulloch 2009; Lu et al. 2014). In the search of natural compounds with

attractive biological properties, NGPs produced by filamentous fungi were identified within two opposite strategies: (i) screening for a specific activity and elucidation of responsible compounds (Singh et al. 2003; Song et al. 2004; Shaaban et al. 2012; Kong et al. 2013) and (ii) biochemical discovery of new family members and testing of their putative anti-microbial properties (Ehrlich et al. 1984; Akiyama et al. 2003; Bouras et al. 2005). An exhaustive list of NGPs produced by filamentous fungi is presented in Table 1, including producer organisms and their described biological properties like anti-HIV, anti-tubercular, anti-hyperuricemia, anti-microbial, anti-tumor, and anti-oxidant, as many biological activities could be valued in pharmaceutical, cosmetic, and food industry.

It should be noticed that NGPs were first described as toxic compounds (Ghosal et al. 1979). We would like to discuss this point before going further into the description of beneficial biological activities of NGPs that could be valued by industrials. In late 1970s, Ghosal and collaborators showed that intraperitoneal injection of 50 mg/kg of mixture containing NGPs, extracted from *A. niger* van Teigh cultured on mango pulp, caused albino mice and rats death through central nervous system (CNS) depression (Ghosal et al. 1979). Conversely, another study showed that oral ingestion by rats of 0.1 % of their daily ration, of a methanol extract from *A. niger* ATCC 9029 containing NGPs, during 60 days did not seem to cause an acute toxicity for animals but show a hepatoprotective effect (Rabache and Adrian 1982). The differences between these studies resided in the fungal strain from which the NGPs were extracted, the organic solvents used for the extraction, the quantity, and the way it was administered. Besides, considering the way that NGPs were extracted before administration, it could not be excluded that mycotoxins should have been present in the mixture but biochemically undetectable. Indeed, other experimental studies show that NGP extracts could be toxic when intraperitoneally injected, even though, they do not exclude the putative presence of highly toxic bioactive malformin compounds in the extract (DeLucca et al. 1983; Ehrlich et al. 1984). Through bioactive fractionation, Zhan et al. (2007) show that cytotoxicity of an organic extract of *Aspergillus tubingensis* was due to malformin A1 and not to any of the ten NGPs present in the extract (asperpyrone D, TMC256A1, rubrofusarin B, fonsecin B, fonsecin, fonsecinone A, asperpyrone A, aurasperone A, dianhydro-aurasperone C, aurasperone E) (Zhan et al. 2007). None of these ten NGPs showed toxicity on different types of cancer cell line or normal human fibroblast when tested on cell culture to a concentration of 5 µg/ml (Zhan et al. 2007). It seems that NGPs are not toxic when a small dose is daily orally ingested (Rabache and Adrian 1982). Even though, chosen extracts will require toxicological assessments (dermatological, pharmaceutical, etc.) before their use in pharmaceutical, cosmetic, or food industry.

Table 1 Listing of fungal NGPs, their producing strains, and their described biological properties

NGPs	Strains	Activities	References
Asperpyrone	Asperpyrone A	<i>A. KJ-9; A. niger; A. tubingensis</i>	Akiyama et al. (2003); Zhang et al. (2007); Zhan et al. (2007); Li et al. (2013); Xiao et al. (2014)
	Asperpyrone B	<i>A. niger</i>	Anti-microbial Akiyama et al. (2003); Song et al. (2004)
	Asperpyrone C	<i>A. niger</i>	Anti-fungal Akiyama et al. (2003); Zhang et al. (2007)
	Asperpyrone D	<i>A. tubingensis; A. niger</i>	Zhan et al. (2007); Li et al. (2013)
	Asperpyrone E	<i>A. niger</i>	Li et al. (2013)
Aurasperone	Aurasperone A	<i>A. alternata; A. niger; A. tubingensis; A. fonsecaeus; Aspergillus</i> sp. FKJ-3451; <i>A. awamori; A. aculeatus</i>	Anti-microbial; xanthine oxidase inhibition Tanaka et al. (1966); Wang and Tanaka (1966); Ghosal et al. (1979); Priestap (1984); Ehrlich et al. (1984); Akiyama et al. (2003); Song et al. (2004); Campos et al. (2005); Zhang et al. (2007); Zhan et al. (2007); Sakai et al. (2008); Shaaban et al. (2012)
	Isoaurasperone A	<i>A. KJ-9; A. niger</i>	Ghosal et al. (1979); Ehrlich et al. (1984); Li et al. (2013); Xiao et al. (2014)
	Aurasperone B	<i>A. alternata; A. niger; A. fonsecaeus; A. awamori; A. vadensis</i>	Anti-fungal; anti-oxidant Tanaka et al. (1966); Tanaka et al. (1972); Priestap (1984); Ehrlich et al. (1984); Bouras et al. (2005); De Vries et al. (2005); Zhang et al. (2007); Shaaban et al. (2012)
	Aurasperone C	<i>A. alternata; A. niger; A. awamori</i>	Tanaka et al. (1966); Tanaka et al. (1972); Ehrlich et al. (1984); Bouras et al. (2005); Shaaban et al. (2012)
	Dianhydro-aurasperone C	<i>A. KJ-9; A. niger; A. M39; A. tubingensis</i>	Reverse multidrug resistance on human KB cells Ikeda et al. (1990); Zhang et al. (2007); Zhan et al. (2007); Li et al. (2013); Xiao et al. (2014);
	Aurasperone D	<i>A. niger</i>	Ghosal et al. (1979); Ehrlich et al. (1984); Bouras et al. (2005); Li et al. (2013)
	Aurasperone E	<i>A. niger; A. tubingensis</i>	Ehrlich et al. (1984); Bouras et al. (2005); Zhan et al. (2007)
	Aurasperone F	<i>A. alternata; A. niger</i>	Ghosal et al. (1979); Bouras et al. (2005); Shaaban et al. (2012)
	Isoaurasperone F	<i>A. niger</i>	Li et al. (2013)
	Aurasperone G	<i>A. niger</i>	Bouras et al. (2007)
Cephalochromin	Cephalochromin	<i>C. vilior; F508; Cephalosporium</i> sp.; <i>SCF-125; Nectria</i> sp.; <i>Pseudoanguillospora</i> sp.; <i>Verticillium</i> sp. K-113	Anti-proliferative effect cancer cell; anti-bacterial; nitric oxide production inhibition; calmodulin-sensitive phosphodiesterase inhibition; Haskins and Knapp (1969); Carey and Nair (1975); Matsumoto et al. (1975); Koyama et al. (1988); Hegde et al. (1993); Ishii et al. (2005); Zheng et al. (2007); Kock et al.

Table 1 (continued)

NGPs	Strains	Activities	References
		botulinum neurotoxin serotype A inhibitor	(2009); Cardellina et al. (2012); Hsiao et al. (2014)
	Cephalochromin 5-methyl ether	<i>Cephalosporium</i> sp.	Ishii et al. (2005)
	Cephalochromin A 5,5'-dimethyl ether	<i>Cephalosporium</i> sp.	Ishii et al. (2005)
Chaetochromin	Chaetochromin A	<i>Fusarium</i> sp.; <i>Chaetomium</i> sp.	Nitric oxide production inhibition; botulinum neurotoxin serotype A inhibitor; anti-diabetes
	Chaetochromin A 5,5'-dimethyl ether	<i>Chaetomium</i> sp.	Sekita et al. (1980); Koyama et al. (1988); Singh et al. (2003); Ishii et al. (2005); Paranagama et al. (2007); Cardellina et al. (2012); Xu et al. (2014); Qiang et al. (2014)
	Isochaetochromin A ₁	<i>Penicillium</i> sp. FKI-4942	Ishii et al. (2005)
	Isochaetochromin A ₂	<i>Chaetomium microcephalum</i>	Inhibition of triacylglycerol synthesis in mammalian cells
	Chaetochromin B	<i>Chaetomium</i> sp.; <i>Fusarium</i> sp.	Ugaki et al. (2012)
	Isochaetochromin B	<i>Fusarium</i> sp.	Xu et al. (2014)
	Isochaetochromin B ₁	<i>Penicillium</i> sp. FKI-4942; <i>Fusarium</i> sp.	HIV-1 integrase inhibition
	Isochaetochromin B ₂	<i>Penicillium</i> sp. FKI-4942; <i>M. anisopliae</i> ; <i>Fusarium</i> sp.	Inhibition of triacylglycerol synthesis in mammalian cells; anti-tubercular; HIV-1 integrase inhibition
	Chaetochromin C	<i>Fusarium</i> sp.	Singh et al. (2003); Ugaki et al. (2012); Kong et al. (2013)
	Chaetochromin D	<i>Fusarium</i> sp.	Koyama et al. (1988)
	Isochaetochromin D ₁	<i>Fusarium</i> sp.	Koyama et al. (1988); Singh et al. (2003)
Flavasperone	Flavasperone	<i>A. niger</i> sp.; <i>A. carbonarius</i> ; <i>A. fonsecaeus</i>	HIV-1 integrase inhibitory activity
Fonsecin	Fonsecin	<i>A. alternata</i> ; <i>A. carbonarius</i> ; <i>A. niger</i> ; <i>A. tubingensis</i> ; <i>A. fonsecaeus</i>	Anti-mycobacterial
	Fonsecin B	<i>A. alternata</i> ; <i>A. tubingensis</i>	Lund et al. (1953); Bycroft et al. (1962); Ghosal et al. (1979); Ehrlich et al. (1984); Zhang et al. (2008)
	10,10'-Bifonsecin B	<i>A. carbonarius</i>	Galmarini et al. (1962); Ehrlich et al. (1984); Bouras et al. (2005); Zhan et al. (2007); Zhang et al. (2008); Shaaban et al. (2012)
	Fonsecin monomethyl ether	<i>A. niger</i>	Zhan et al. (2007); Lee et al. (2010); Shaaban et al. (2012)
Fonsecinone	Fonsecinone A	<i>A. KJ-9</i> ; <i>A. niger</i> ; <i>A. tubingensis</i> ; <i>A. fonsecaeus</i> ; <i>Aspergillus aculeatus</i> ;	Zhang et al. (2008)
			Ehrlich et al. (1984)
			Priestap (1984); Akiyama et al. (2003); Song et al. (2004); Campos et al.

Table 1 (continued)

NGPs	Strains	Activities	References
		<i>Cladosporium herbarum</i>	(2005);Ye et al. (2005); Zhang et al. (2007); Zhan et al. (2007); Chiang et al. (2011);Xiao et al. (2014)
	Fonsecinone B	<i>A. niger</i> ; <i>A. fonsecaeus</i>	Anti-oxidant
	Fonsecinone C	<i>A. niger</i> ; <i>A. fonsecaeus</i>	Priestap (1984); Zhang et al. (2007)
	Fonsecinone D	<i>A. niger</i> ; <i>A. fonsecaeus</i>	Priestap (1984); Zhang et al. (2007)
Hypochromin	Hypochromin A	<i>H. vinosa</i>	Inhibitory effects on tyrosinase, proliferation, migration, and tubule formation Ohkawa et al. (2010)
	Hypochromin B	<i>H. vinosa</i>	Inhibitory effects on tyrosinase, proliferation, migration, and tubule formation Ohkawa et al. (2010)
	SC2051	<i>H. vinosa</i>	Inhibitory effects on tyrosinase, proliferation, migration, and tubule formation Ohkawa et al. (2010)
Indigotides	Indigotide B	<i>C. indigotica</i> ; <i>M. anisopliae</i>	Asai et al. (2012); Kong et al. (2013)
	Indigotide G	<i>M. anisopliae</i>	Kong et al. (2013)
	Indigotide H	<i>M. anisopliae</i>	Kong et al. (2013)
Nigerasperone	Nigerasperone A	<i>A. niger</i>	Zhang et al. (2007)
	Nigerasperone B	<i>A. niger</i>	Zhang et al. (2007)
	Nigerasperone C	<i>A. niger</i>	Anti-fungal; anti-oxidant Zhang et al. (2007)
Nigerone	Nigerone	<i>A. niger</i> ; <i>A. carbonarius</i>	Ehrlich et al. (1984); Divirgilio et al. (2007); Zhang et al. (2008)
	Nigerone methyl ether	<i>A. niger</i>	Ehrlich et al. (1984)
	Isonigerone	<i>A. carbonarius</i>	Zhang et al. (2008)
	6'-O-demethylnigerone	<i>A. carbonarius</i>	Zhang et al. (2008)
	8'-O-demethylisonigerone	<i>A. carbonarius</i>	Anti-microbial Zhang et al. (2008); Lee et al. (2013)
	8'-O-demethylnigerone	<i>A. carbonarius</i>	Anti-microbial Zhang et al. (2008); Lee et al. (2013)
Rubasperone	Rubasperone A	<i>A. tubingensis</i>	Huang et al. (2010)
	Rubasperone B	<i>A. tubingensis</i>	Huang et al. (2010)
	Rubasperone C	<i>A. tubingensis</i>	α -Glucosidase inhibition Huang et al. (2010)
	Rubasperone D	<i>A. tubingensis</i>	Huang et al. (2011)
	Rubasperone E	<i>A. tubingensis</i>	Huang et al. (2011)
	Rubasperone F	<i>A. tubingensis</i>	Huang et al. (2011)
Rubrofusarin	Rubrofusarin	<i>A. niger</i> ; <i>A. tubingensis</i> ; <i>Fusarium</i> sp.	Tyrosinase inhibition Ashley et al. (1937); Tanaka and Tamura (1961); Ghosal et al. (1979); Ehrlich

Table 1 (continued)

NGPs	Strains	Activities	References
	Rubrofusarin B	<i>A. KJ-9</i> ; <i>A. alternata</i> ; <i>A. M39</i> ; <i>A. niger</i> ; <i>A. tubingensis</i> ; <i>A. carbonarius</i> ; <i>Cladosporium</i> <i>herbarum</i>	et al. (1984); Huang et al. (2010) Ikeda et al. (1990); Song et al. (2004); Ye et al. (2005); Zhan et al. (2007); Zhang et al. (2008); Huang et al. (2010); Shaaban et al. (2012); Xiao et al. (2014)
	Rubrofusarin-6- <i>O</i> - α -D-ribofuranoside	<i>A. niger</i>	Li et al. (2013)
	Rubrofusarin-6- <i>O</i> - β -(4- <i>O</i> -methylglucopyranoside)	<i>T. lutearostata</i>	Isaka et al. (2007)
TMC 256	TMC-256A1	<i>A. carbonarius</i> ; <i>A. tubingensis</i> ; <i>A. niger</i>	IL-4 signal transduction inhibition Sakurai et al. (2002); Zhan et al. (2007); Zhang et al. (2008); Li et al. (2013)
	TMC-256B1	<i>A. niger</i>	IL-4 signal transduction inhibition Sakurai et al. (2002)
	TMC-256C1	<i>A. niger</i>	IL-4 signal transduction inhibition Sakurai et al. (2002)
Ustilaginoidin	Ustilaginoidin A	<i>Villosiclava virens</i>	Shibata et al. (1963); Koyama et al. (1988); Koyama and Natori (1988)
	Isoustilaginoidin A	<i>Verticillium</i> sp. <i>K-113</i>	Matsumoto et al. (1975)
	Dihydroisoustilaginoidin A	<i>A. butyri</i> ; <i>Verticillium</i> sp. <i>K-113</i>	Nitric oxide production inhibitory activity Matsumoto et al. (1975); Ishii et al. (2005)
	Ustilaginoidin B	<i>Villosiclava virens</i>	Shibata and Ogihara (1963)
	Ustilaginoidin C	<i>Villosiclava virens</i>	Shibata and Ogihara (1963)
	Ustilaginoidin D	<i>M. anisopliae</i> ; <i>Fusarium</i> sp.	Anti-tubercular Koyama et al. (1988); Singh et al. (2003); Kong et al. (2013)
	Ustilaginoidin E	<i>Fusarium</i> sp.	Koyama et al. (1988)
	Ustilaginoidin F	<i>Villosiclava virens</i>	Koyama et al. (1988)
	Ustilaginoidin G	<i>Villosiclava virens</i>	Koyama et al. (1988)
	Ustilaginoidin H	<i>Villosiclava virens</i>	Koyama et al. (1988)
	Ustilaginoidin I	<i>Villosiclava virens</i>	Koyama et al. (1988)
	Ustilaginoidin J	<i>Villosiclava virens</i>	Koyama et al. (1988)
YWA1	YWA1	<i>Aspergillus</i> sp.; <i>Fusarium</i> sp.	Fujii et al. (2001); Frandsen et al. (2011)

Going through medical consideration, some NGPs have been described for their anti-tubercular, anti-tumor, anti-HIV, and anti-hyperuricosuric effects (Singh et al. 2003; Song et al. 2004; Shaaban et al. 2012) (Table 1). For example, aurasperone A and rubrofusarin B, extracted from *A. niger* IFB-E003, show a xanthine oxidase inhibitory effect equivalent to the one of allopurinol, a commonly used anti-hyperuricosuric. These NGPs also show anti-tumor properties against a colon cancer cell line (Song et al. 2004). In the search of new and more powerful drug against tuberculosis, Shaaban et al. (2012) show that rubrofusarin B and aurasperone A, extracted from *Alternaria alternata* D2006, have high antimicrobial properties and show really weak toxicity at a 10 µg/ml concentration (Shaaban et al. 2012). Anti-tumor and anti-microbial activities of NGPs were recently detailed by Lu and collaborators (2014). Finally, considering anti-HIV drugs, four NGPs of the chaetochromin family, extracted from *Fusarium* sp., show good inhibitory properties against HIV-1 integrase and should be deeply studied for their therapeutic use (Singh et al. 2003).

Thanks to literature and personal unpublished data, it is possible to think that one of the most attractive properties of NGPs was their anti-oxidant capacity. First described by Rabache and colleagues (1982), the anti-oxidant activity of NGPs seems to have both a beneficial effect on rats growing and a hepatoprotective activity, probably due to a better intestinal absorption of retinol and tocopherol (Rabache et al. 1982). Xie et al. (2012) have recently shown that a previous animal treatment with Cassia seed ethanol extract (CSE) protected mice against CCl₄-induced liver injury via an enhancement of the anti-oxidant capacity (Xie et al. 2012). It was previously demonstrated that CSE contained NGPs (Kitanaka et al. 1998; Li et al. 2001). Anti-oxidant properties of NGPs were also described for nigerasperone C, aurasperone B, fonsecinone B, and fonsecinone D, extracted from *A. niger* EN-13, which show an anti-oxidant capacity two times lower than the one of BHT (Zhang et al. 2007). Besides, experiments using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) test on *A. niger* C-433, which did not produce ochratoxin A (Bouras et al. 2005; Bouras et al. 2007), show that the anti-oxidant power of an ethanol extract containing NGPs is equivalent to vitamin C (unpublished personal data). Unlike natural anti-oxidants currently used (vit. C, vit. D, vit. E, etc.) (Scotter and Castle 2004; Burke 2007; Stamford 2012), the anti-oxidant capacity of this ethanol extract is also stable over the time and resists to temperatures up to 60 °C (unpublished personal data). Study of the anti-oxidant properties of NGPs will allow having significant impacts in a variety of areas. As described above, the anti-oxidant activity of NGPs prevents lard oxidation (Zaika and Smith 1975), so NGPs could be a good substitute to actual conservators such as sulfur or synthetic anti-oxidants. In health and cosmetics industry, NGPs could be an ideal dietary

supplement or formulate in cream as anti-skin-aging compounds (Ye et al. 2014).

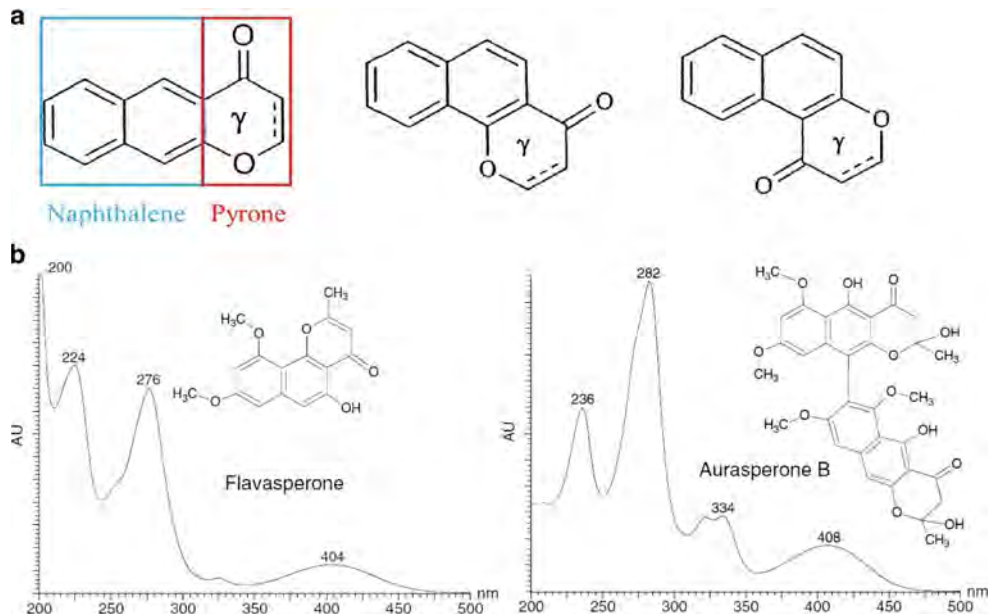
Chemistry of fungal NGPs

Naphtho- γ -pyrones (NGPs) belong to the family of naphthopyrones. They are C13 (C6-C4-C3) basic skeleton molecules consisting of a naphthalene core and a pyrone core. Greek letters (α , β , γ) indicate the position of the oxygen atom relative to the carbonyl group on the pyrone core (Barrow and McCulloch 2009) (Fig. 1a). The naphthopyrone group comprises 18 isomeric forms. Only three can exist as NGPs. As shown in Fig. 1a, one isomeric form has the basic skeleton organized in a linear form where the remaining two forms have angular organization. Up to now, the NGP family (Table 1) is composed of various monomers, fonsecin, flavasperone, rubrofusarin, etc. (Fig. 2a), and dimers, asperpyrones, aurasperones, fonsecinones, nigerones, etc, whose diversity is presented in Fig. 2b. Dimers result from the association of two monomers, depending on their structure (linear and angular), the position of the chemical bond between monomers, and the decoration present on both naphthalene and pyrone cores (Ehrlich et al. 1984; Chiang et al. 2011). As mentioned in Fig. 2, some NGPs share the same structure, but they are conformational isomers. Conformation of these NGPs was recently reviewed by Lu et al. (2014).

The relationship between the structure of NGPs and their biological activities is poorly described in the literature. Concerning the anti-oxidant activity, it is possible to make a comparison with what it is described on naphtho- α -pyrones by Leitão et al. (2002). They suggest that the anti-oxidant capacity of naphthopyrones produced by *Paepalanthus* sp. is due to presence and number of free hydroxyl groups (Leitão et al. 2002). Unpublished personal data show that aurasperone C has a higher anti-oxidant capacity than aurasperone B and F (unpublished personal data). As shown in Fig. 2b, aurasperone C has five free hydroxyl groups while aurasperone B and F have only four hydroxyl groups. Besides, aurasperone F has a higher anti-oxidant activity than aurasperone B, suggesting that the position of the hydroxyl groups is important too.

Elucidation of NGP structures so far was mostly done using semi-preparative reverse-phase HPLC purification and mass spectrometry analysis (Campos et al. 2005; Bouras et al. 2005). It was shown that NGPs contain a fully conjugated system, giving rise to very characteristic UV/vis spectra, strongly absorbing between 200 and 450 nm (Fig. 1b). The absorption spectra mainly depend on the isomeric form and the polymerization degree (Nielsen et al. 2009) (Fig. 2b). As example, flavasperone (monomeric, angular) presents two peaks with equal maximum

Fig. 1 Biochemical properties of NGPs. **a** Linear and angular forms of NGPs. Both present a naphthalene core and a pyrone core. **b** UV/vis spectra of flavasperone (monomer) and aurasperone B (dimer) adapted from Nielsen et al. (2009)



absorbance (224 nm, 276 nm) whereas aurasperone B (dimeric, linear) shows a maximum absorbance peak (282 nm) and three little peaks (236, 334, 408 nm) (Nielsen et al. 2009; for UV/vis spectra references, please refer to Table 1) (Fig. 1b). Unfortunately, despite of the possible discrimination of different NGPs using their specific absorption spectra, reliable standards are missing to precisely estimate the NGP concentration of purified extracts. Indeed, the only standard presently available is rubrofusarin, a NGP monomer linear, isolated from *Fusarium graminearum* (Adipogen, CAS 3567-00-8).

Their specific structure confers NGP hydrophobic characters allowing their extraction by several organic solvents, such as ethyl acetate (Xiao et al. 2014; Li et al. 2013; Zhang et al. 2007), acetone (Zhang et al. 2007), methanol (Akiyama et al. 2003; Bouras et al. 2005; Bouras et al. 2007), chloroform/methanol mix (Song et al. 2004), or methylene chloride (Ehrlich et al. 1984). Thanks to this hydrophobic property, it is also possible to consider their extraction by a nontoxic organic solvent as ethanol, as it is already done in higher plants (Kitanaka et al. 1998; Li et al. 2001; Xie et al. 2012). Even though, the issue remains the purity of the extract because one-step extraction with any organic solvent leads to a crude extract containing all hydrophobic compounds present in matrices. Right now, purification process for isolation and structure elucidation of NGPs is composed of numerous steps of drying, elution, and chromatography (Akiyama et al. 2003; Li et al. 2013; Xiao et al. 2014). However, purity of these molecules is not a prerequisite for their potential industrialization, as ethanol extracts are already used (Xie et al. 2012, WO 2010109318 A1). Considering their attractive biological properties, it is interesting to better understand their biosynthetic pathway in order to optimize their production.

The poorly known biosynthetic pathway of NGPs

In the purpose of improving NGP production/extraction process, a strong knowledge of their biosynthetic pathway is a relevant fact. Unfortunately, only few data are available in the literature. The first step of NGP biosynthetic pathway elucidation was done by Watanabe and colleagues (1998) (Watanabe et al. 1998; Watanabe et al. 1999). Studying conidial pigmentation in *Aspergillus nidulans* (*A. nidulans*), they showed that *wA* gene encodes a polyketide synthase responsible for the formation of the linear monomer NGP, YWA1, required for the formation of the dark green conidial pigment (Watanabe et al. 1998). Indeed, *A. nidulans* strain lacking *wA* gene produces white conidia. It was lately shown that the C-terminal part of *wA* was required for the naphthopyrone synthesis (Fujii et al. 2001). Indeed, *A. nidulans* strain expressing a C-term-modified *wA* gene produces a heptaketide isocoumarin instead of YWA1. Authors assume that the C-term part of *wA*, and particularly a Claisen cyclase domain, is involved in the cyclization of the second aromatic ring of YWA1 (Fujii et al. 2001).

At the same time, Tsai and colleagues (1998), working on conidial morphology and virulence of *A. fumigatus*, characterize *alb1* gene, *A. nidulans* *wA* ortholog, putatively encoding the pentaketide tetrahydroxynaphthalene (THN) (Tsai et al. 1998). THN is a precursor in the DHN-melanin, a well-known virulence factor of both plant and human (Langfelder et al. 2003; Heinekamp et al. 2012). *alb1* was then overexpressed in *Aspergillus oryzae*, which does not possess an orthologous gene (Watanabe et al. 2000). This overexpression leads to the production of the heptaketide naphthopyrone YWA1 instead of THN (Watanabe et al. 2000). These results suggest that *alb1* encodes a naphthopyrone synthase instead

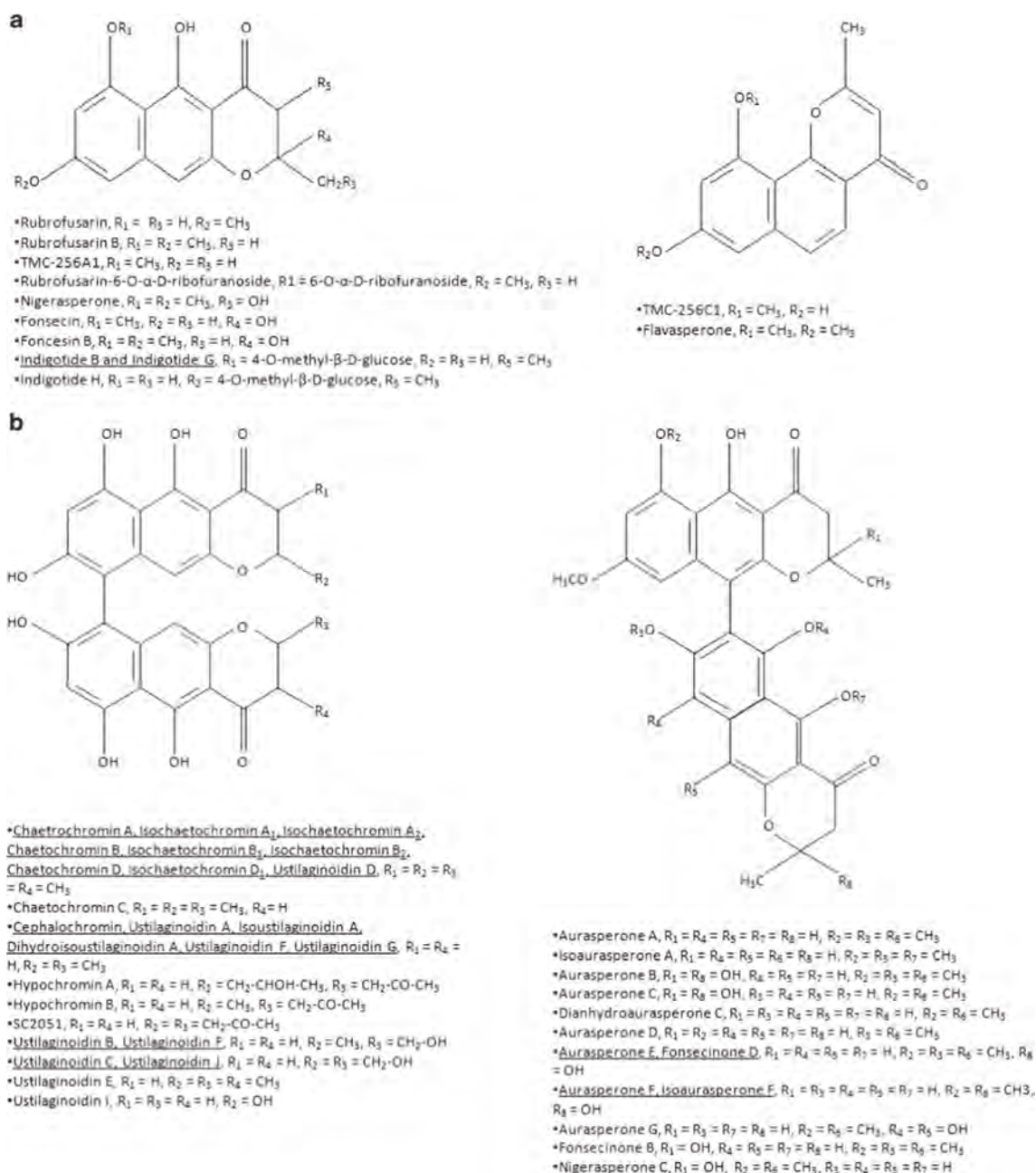


Fig. 2 Structural formula of the presented naphtho- γ -pyrones. **a** NGP monomers. **b** NGP dimers. Underlined NGPs names with the same structural formula represent stereoisomers

of a THN synthase. Besides, it was then described that the esterase-like enzyme Ayg1 allows the formation of THN from YWA1, in *A. fumigatus* (Fujii et al. 2004). Interestingly, in *Colletotrichum lagenarium*, it was shown that synthesis of the THN pentaketide only required the presence of the polyketide synthase PKS1 (Watanabe and Ebizuka 2004). Unlike *Aspergillus* sp., NGP production is not described in *Colletotrichum* sp. So, in *Aspergillus* sp., the presence of this additional first step, leading to the formation of YWA1, suggests that this monomer NGP could be a common precursor for both DHN-melanin and NGPs.

Considering NGP biosynthesis in *Aspergillus* sp., two simultaneous studies show that *alba*, *alb1* ortholog in *A. niger*, was responsible of YWA1 synthesis which seems to be a precursor of both DHN-melanin and NGPs produced by this filamentous fungus (Jørgensen et al. 2011b; Chiang et al. 2011) (Fig. 3). Jørgensen et al. (2011b) tried to elucidate black pigmentation biosynthetic pathway in *A. niger* N402, using UV mutagenesis to select spore color mutants. Thus, they described three different spore color mutants: fawn, olive, and brown. Using a complementation method, they showed that a specific gene required for DHN-melanin biosynthesis

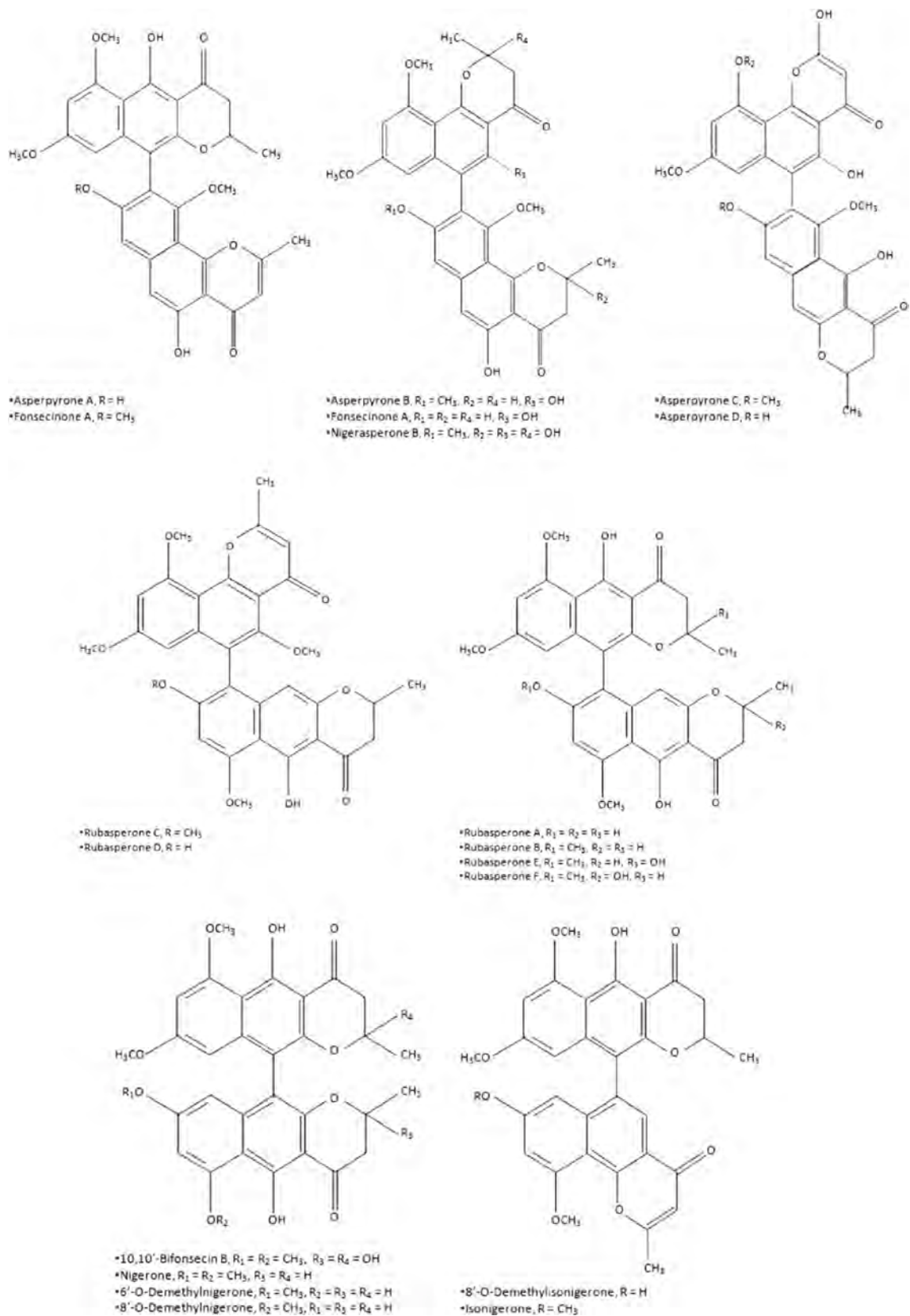


Fig. 2 (continued)

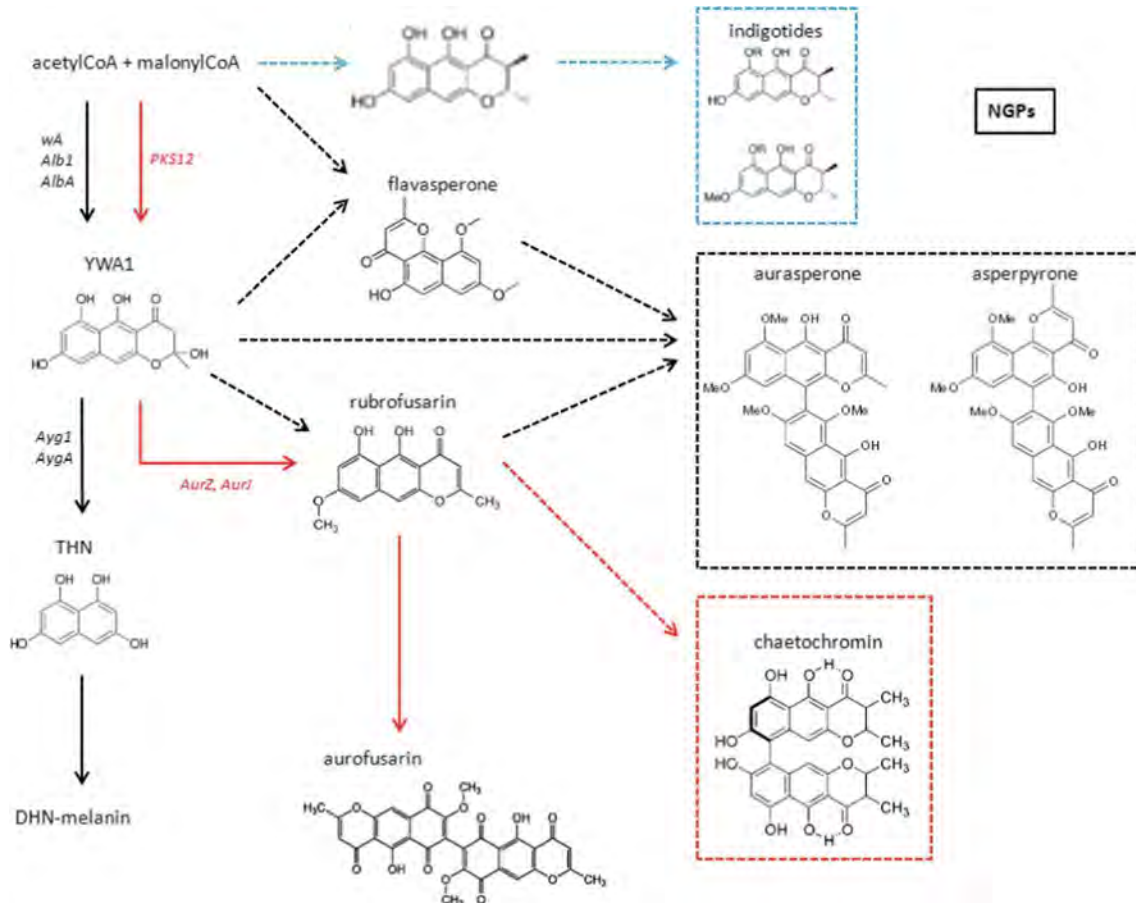


Fig. 3 Biosynthesis pathways of NGPs. *Black arrows* represent common biosynthesis pathways of DHN-melanin and NGPs in *Aspergillus* sp., *red arrows* represent putative common biosynthesis pathways of aurofusarin and NGPs in *Fusarium* sp., and *blue arrows* represent NGP biosynthetic

pathway in *Cordyceps indigotica*. When *arrows are represented with a full line*, the biosynthetic pathway is known whereas when *arrows are represented with dotted line*, the biosynthetic pathway has to be discovered

was mutated in each color phenotype. Mutation on *albA* was responsible for the “fawn” phenotype while mutations on *aygA* and *abrA* (ortholog of *A. fumigatus* *ayg1* and *abr1*) gave olive and brown phenotypes, respectively. Interestingly, they also showed that in addition to a light color indicating a defect in pigment synthesis, fawn mutants were not able to produce NGPs, suggesting that *AlbA* is also involved in the biosynthetic pathway of NGPs (Jørgensen et al. 2011b). Olive and brown mutants were still able to produce some NGPs, suggesting that *AygA* and *AbrA* are not involved in NGP biosynthetic pathway (Jørgensen et al. 2011b). Those results were confirmed by Chiang and collaborators (2011) who deleted *albA* and *aygA* in *A. niger* ATCC1015 in order to determinate cryptic secondary metabolites by suppressing NGP production (Chiang et al. 2011). However, compared to the work done by Jørgensen et al. (2011a, b), fungal phenotypes of mutants are different. Various hypotheses can be made to explain those differences: genetic background of each strain, culture medium and growth conditions, hazardous mutation versus deletion, or the major point of DNA repair mechanism disruption used by Chiang et al. (2011) to facilitate *A. niger*

transformation and which can lead to huge genetic variation. Indeed, it may be seriously considered that disruption of DNA repair machinery could let appeared spontaneous mutations emphasizing the colorless phenotype and the lack of NGPs.

The precursor YWA1 was also described in the biosynthetic pathway of the homodimeric naphthoquinone aurofusarin, a red color pigment produced by *Fusarium* sp. (Fig. 3) (Malz et al. 2005; Frandsen et al. 2006; Frandsen et al. 2011). Identification of the gene cluster responsible for the synthesis of aurofusarin indicates that nor-rubrofusarin and rubrofusarin, two NGP monomers, were precursors of this red pigment (Frandsen et al. 2006). It was first admitted that *F. graminearum* PKS12 was the polyketide synthase responsible of nor-rubrofusarin production as an intermediate to aurofusarin (Malz et al. 2005). Determination of *AurZ* and *AurJ* functions allows demonstrating that rubrofusarin synthesis passes through the production of YWA1 by PKS12 (Frandsen et al. 2011) (Fig. 3). As described above for *wA* gene in *A. nidulans*, it was shown that a Claisen cyclase domain in the C-terminal part of PKS12 was required for the formation of YWA1 (Sørensen et al. 2012). Disruption of this

PKS12 domain leads to the formation of a lactone, citreoisocoumarin, instead of YWA1. This result suggests the presence of a conserved regulatory mechanism in the formation of YWA1. Then, AurZ is responsible for the production of nor-rubrofusarin from YWA1, and AurJ was required next for the production of rubrofusarin (Frandsen et al. 2011). Rugbjerg et al. (2013) showed that PKS12, AurZ, and AurJ were necessary and sufficient for the production of rubrofusarin. Indeed, heterologous expression of these three proteins in *Saccharomyces cerevisiae* allows weak production of rubrofusarin by the yeast (Rugbjerg et al. 2013). Interestingly, some NGP dimers, such as chaetochromin or ustilaginoidin, are also produced by *Fusarium* sp. (Singh et al. 2003). However, any study describes a link between aurofusarin biosynthetic pathway and the one of chaetochromin or ustilaginoidin. Questions remaining are does YWA1 and/or rubrofusarin are precursors for NGP biosynthesis in *Fusarium* sp. and is rubrofusarin the second key step, after YWA1, in the general biosynthetic pathway of NGPs (Fig. 3). Besides, it has to be noticed that AurZ is the first representative of a novel class of dehydratases that act on hydroxylated γ -pyrones (Frandsen et al. 2011). This information will be significant for bioinformatic selection of putative candidates involved in the biosynthetic pathways of NGPs in fungal organisms.

Obviously, NGPs and fungal pigments are clearly biosynthetically linked in both *Aspergillus* sp. (DHN-melanin or *A. nidulans* dark green pigment) and *Fusarium* sp. (aurofusarin) (Malz et al. 2005; Chiang et al. 2011; Jørgensen et al. 2011b). It was even proposed that a second type of polymeric subunit made upstream from YWA1 could be incorporated into the final melanin polymer in *A. fumigatus* (Tsai et al. 1999; Fujii et al. 2004; Wheeler et al. 2008). The role of this particular biosynthetic link between NGPs and pigment is not yet elucidated and introduces important questions on the fungal role of these molecules.

Does NGP biosynthesis could depend of more than one pathway? Some studies suggest that it could be the case. Indeed, still working on conidia coloration of *A. niger* N402, Jørgensen et al. (2011a, b) show that flavasperone and aurasperone B, two NGPs, were associated to conidia formation (Jørgensen et al. 2011a). Besides, they showed that flavasperone production did not show the same dependence on AlbA depending on the culture conditions, submerged against subaerial (Jørgensen et al. 2011a, b). This difference could be explained by induction of a complementary PKS or an efficient metabolism of flavasperone in subaerial settings. In the same study, they determined that low level of aurasperone B was found in a mutant derived from $\Delta albA$ background suggesting partial redundancy to synthesize NGPs (Fig. 3) (Jørgensen et al. 2011a). The question here is does *A. niger* genome contain a candidate polyketide synthase for this complementary biosynthetic pathway hypothesis? It

was shown that more than 70 % of fungal PKS-encoding gene clusters were transcriptionally suppressed under various standard laboratory culture conditions (Scherlach and Hertweck 2009; Zerikly and Challis 2009; Fisch et al. 2009). That is why, treatment of *A. niger* ATCC 1015 with suberoylanilide hydroxamic acid (SAHA), an inhibitor of histone deacetylase (HDAC) used for epigenetic studies, led to transcription up-regulation of many secondary metabolites encoding biosynthetic gene clusters (Fisch et al. 2009). Comparing the conserved domain composition of the polyketide synthases present in *A. niger*, they showed that only one PKS, An03g05440, possesses the same conserved domains as AlbA (Fisch et al. 2009). Besides, genes encoding those PKS show a strong homology. Interestingly, An03g05440 was strongly upregulated by SAHA whereas *albA* was not. This specific property of An03g05440 can lead to the use of SAHA for a better understanding of NGP biosynthetic pathway in *A. niger*. Supporting those results, the use of an HDAC inhibitor on *Cordyceps indigotica* (*C. indigotica*) culture allows the purification of indigotides, characterized as NGPs (Asai et al. 2012) (Fig. 3). Trying to understand the way that indigotides are synthesized by *C. indigotica*, Asai and collaborators (2012) established the profile of a NGP precursor synthesized from malonyl CoA, different from YWA1. This putative precursor supports the hypothesis of multiple biosynthetic pathways for the production of NGPs.

Further perspectives

Manufacturers are always seeking for new molecules with beneficial biological properties. Numerous natural extracts are studied for their putative biological properties at a laboratory scale (Akiyama et al. 2003; Shaaban et al. 2012; Xie et al. 2012; Kong et al. 2013). Fungal NGPs show biological properties such as anti-oxidant, anti-tumoral, and anti-microbial activities that could find potential applications in food, health, and cosmetics areas. They are natural molecules produced in large amount by industrial fungi, such as *A. niger* (Nielsen et al. 2009). Besides, they can be co-extracted, with other hydrophobic compounds, in a single step with ethanol allowing accreditation for “non-organic” label, attractive for industry (Kitanaka et al. 1998; Li et al. 2001; Xie et al. 2012). Personal experimental data suggest that ethanol extracts containing NGPs highly resist to temperature up to 60 °C conserving their biological properties, particularly their anti-oxidant activity, and seem to be strongly stable over the time. The dark color of some extracts issued from *A. niger* may be incompatible for their industrial use from consumer’s perceptions. However, these crude extracts could still be commercialized as dietary supplements. Besides, the black color of the extracts could still be removed by addition of melanin

inhibitors in culture media or using genetically modified strains unable to produce melanin as described by Jørgensen et al. (2011a, b) and Chiang et al. (2011).

Optimization of the production/extraction process of fungal NGPs has to be done. The first step will be the improvement of culture conditions such as culture media, temperature, pH, incubation time, and the fungal strain chosen. Then, a particular attention should be made at the purification process. As hydrophobic molecules, NGPs are currently extracted with different organic solvents resulting to the simultaneous extraction of other hydrophobic compounds and thus requiring various purification steps, predominantly chromatography (Bouras et al. 2005; Campos et al. 2005). Depending on the potential industrial applications, purification should not be always necessary; the use of a fungal bioactive extract containing NGPs could be possible, as it is already the case for ethanol crude extracts produced by plants (WO 2010109318 A1; Xie et al. 2012).

In addition, determination of NGP biosynthetic pathway is absolutely required for the improvement of the production/extraction of fungal NGPs. This knowledge will help guide the production of a specific NGP directly in the culture according to the chosen biological property. Besides, a complete knowledge of this pathway should allow the heterologous synthesis of targeted NGPs as it was done by Rugbjerg and colleagues (2013) for rubrofusarin (Rugbjerg et al. 2013). This specific production will greatly facilitate the current extraction process.

Conflict of interest The authors declare that they have no conflict of interest.

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