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REVIEW

Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group

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Abstract Filamentous fungi in the *Aspergillus* section *Nigri* (the black aspergilli) represent some of the most widespread food and feed contaminants known but they are also some of the most important workhorses used by the biotechnological industry. The *Nigri* section consists of six commonly found species (excluding *A. aculeatus* and its close relatives) from which currently 145 different secondary metabolites have been isolated and/or detected. From a human and animal safety point of view, the mycotoxins ochratoxin A (from *A. carbonarius* and less frequently *A. niger*) and fumonisin B_2 (from *A. niger*) are currently the most problematic compounds. Especially in foods and feeds such as coffee, nuts, dried fruits, and grape-based products where fumonisin-producing fusaria are not a problem, fumonisins pose a risk. Moreover, compounds such as malformins, naptho-γ-pyrones, and bicoumarins (kotanins) call for monitoring in food, feed, and biotechnology products as well as for a better toxicological evaluation, since they are often produced in large amounts by the black aspergilli. For chemical differentiation/identification of the less toxic species the diketopiperazine asperazine can be used as a positive marker since it is consistently produced by *A. tubingensis* (177 of 177 strains tested) and *A. acidus* (47 of 47 strains tested) but never by *A. niger* (140 strains tested). Naptho-γ-pyrones are the compounds produced in the highest quantities and are produced by all six common species in the group (*A. niger* 134 of 140; *A. tubingensis* 169 of 177; *A. acidus* 44 of 47; *A. carbonarius* 40 of 40, *A. brasiliensis* 18 of 18; and *A. ibericus* three of three).

Keywords Metabolomics · Fumonisin · Ochratoxin · Liquid chromatography–mass spectrometry · Polyketide synthase · Polyketide

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Introduction

The black aspergilli are some of the most important mycotoxigenic food and feed contaminants, especially in postharvest decay of fresh and dried fruits and certain vegetables, nuts, beans, and cereals [1, 2]. This is due to their fast growth, pH tolerance, and high abundance in many environments.

For the analytical chemist, issues such as fungal taxonomy and correct identification may seem of low relevance, but in fact biosystematics is a vital part of mycotoxin research and food safety. Since the profile of mycotoxins and other secondary metabolites is species-specific [3–5], correct identification at the species level provides the key for planning the analytical determination of all relevant compounds.

The *Aspergillus niger* group (the black aspergilli, *Aspergillus* subgenus *Circumdati* section *Nigri*) comprises 18 species, of which *A. niger, A. tubingensis, A. brasiliensis, A. acidus, A. carbonarius*, and *A. ibericus* are common, whereas the remaining species are rare and found mainly in tropical regions (Table 1). A cladification of *Aspergillus* section *Nigri* using the β-tubulin and calmodulin genes showed that three clades could be distinguished [5]: the *A. niger* clade, a clade consisting of the two rare species *A. homomorphus* and *A. ellipticus,* and the clade of uniseriate black aspergilli (*A. aculeatinus, A. aculeatus, A. japonicus*,

Table 1 Species in the *Aspergillus niger* group

Species	Rare	Species	Rare
A. acidus[a]		*A. ibericus*	
A. brasiliensis[a]		*A. niger*	
A. carbonarius		***A. piperis***	Yes
A. costaricaensis	Yes	***A. sclerotiicarbonarius***	Yes
A. ellipticus	Yes	***A. sclerotioniger***	Yes
A. heteromorphus	Yes	*A. tubingensis*[a]	
A. homomorphus	Yes	*A. vadensis*	Yes
Uniseriate black aspergilli			
A. aculeatinus		***A. japonicus***	
A. uvarum		***A. aculeatus***	

Species whose names are in **bold** have only been found in the tropics as yet. Important synonyms of *A. niger* are *A. awamori*, *A. phoenicis*, *A. kawachii*, *A. saitoi*, *A. usamii*, *A. foetidus*, *A. citricus*, and *A. ficuum*.

[a] Regularly confused with *A. niger*

and *A. uvarum*), the members of which differ significantly from the remaining black aspergilli regarding their morphology, physiological behavior, and secondary metabolite production (e.g., producers of neoxaline, asperparalines, secalonic acids, asperamide, and aculeasins) [5], and this third clade has therefore not been included in this review. The identity and metabolite production of the uniseriate black aspergilli is usually not confused with the identity and metabolite production of *A. niger* and other biseriate black aspergilli.

A. niger and *A. tubingensis* are probably the most common of the black aspergilli; however, in many studies describing secondary metabolites from these aspergilli, the producing organism has been identified as a black *Aspergillus* and then in many cases incorrectly named *A. niger*. A wrong identification may be further complicated by insufficient molecular identification based on sequencing of ribosomal DNA with low resolution [5, 6]. A polyphasic approach where many different types of characters (microscopy, metabolite profiling, molecular methods) are used is recommended for the identification of these aspergilli. Certain molecular methods have proven quite successful, including restriction fragment length polymorphism and β-tubulin, or calmodulin sequencing (reviewed in [5]).

Until recently, the main mycotoxin from the black aspergilli was considered to be ochratoxin A (OTA), produced in variable amounts within certain species of the group. *A. carbonarius* consistently produces large amounts of OTA, whereas only 6–10% of members of the *A. niger* group produce OTA and in 10–1000 lower amounts [7–9]. The third species producing OTA in section *Nigri* is *A. sclerotioniger*, but this species has only been found once in coffee. However, *A. niger* clearly has the widest distribution and has been reported to grow and damage a much larger number of crops and foods worldwide, including corn, peanuts, raisins, onions, mango, apples, and dried meat products [2]. This combined with the recent discovery that *A. niger* can also produce fumonisin B_2 (FB_2) and fumonisin B_4 (FB_4) [10, 11] (Fig. 1) necessitates the addition of fumonisins in a number of food and feed screening programs.

A. niger exhibits a remarkably versatile metabolism, which has made it one of the most important production organisms used for industrial fermentations [12, 13]. Since 1923, *A. niger* has been exploited commercially for its production of citric acid, mostly for use in food, cosmetics, and pharmaceutical preparations [14]. In addition, the fungus has been a rich source of industrial enzymes such as α-amylases, cellulases, and pectinases for use in the food industries since the 1960s [12]. *A. niger* possesses posttranslational mechanisms capable of correctly processing proteins that are difficult to express in traditional host organisms. As a result, it is widely used as a cell factory for heterologous expression of proteins [14].

A. niger has been considered to be nontoxic under industrial conditions [12], and thus to be considered a safe production organism. As a result, quite a number of *A. niger* fermentations have been granted the generally regarded as safe (GRAS) status by the US Food and Drug Administration [12]. However, the potential presence of both OTA and fumonisins in *A. niger* emphasizes the need to adjust and/or reconsider the screening procedures for simultaneous targeting of multiple classes of mycotoxins.

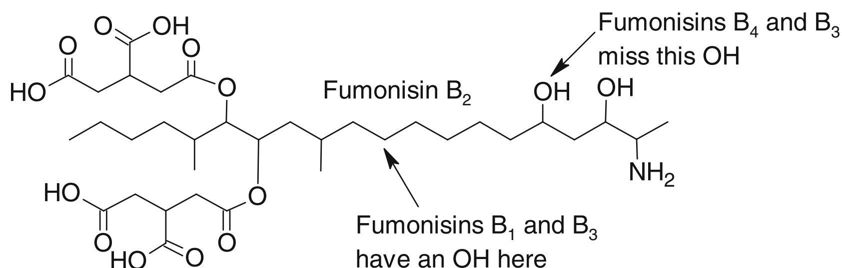

Fig. 1 Structure of fumonisin B_2 and the difference from the structures of fumonisin B_1, fumonisin B_3, and fumonisin B_4

In this review, we focus on the important secondary metabolites produced by members of the *A. niger* group relevant to the food, feed, and biotechnology industries. We have critically scrutinized the existing literature for reports of secondary metabolites claimed to be produced by *A. niger*, however often just found in a single strain or on a single occasion. In addition, we present analytical results from 25 years of metabolite profiling at the Center for Microbial Biotechnology of the black aspergilli. The strains investigated come from a large in-house collection (IBT collection, author address) and other fungal collections. The results are based on continuously obtained data from liquid chromatography (LC) with diode-array detection (DAD), 6 years of LC-DAD analyses combined with high-resolution time-of-flight (TOF) mass-spectrometric detection, and 1 year of screening with LC and tandem mass-spectrometric detection.

The overall aim is to provide an overview of the large numbers of compounds produced within this important group of filamentous fungi and to identify potential difficulties and pitfalls in the biochemical analysis of these compounds. Therefore, we discuss the individual compound classes, their biological significance, and natural abundance together with their spectroscopic and chromatographic properties relevant for their analytical determination.

Methodologies applied at the center for microbial biotechnology

Cultivation and extraction

Data obtained from analysis of *A. niger* group strains in our institution during the last 25 years were compiled. Cultures were identified on the basis of their morphology, metabolite profile, and partial sequencing (β-tubulin and calmodulin) as described elsewhere [15]. The strains were grown and extracted by one of the following three methods:

1. Combined chloroform–methanol (2:1, v/v) and acetone–ethyl acetate (1:1, v/v) extracts from cultures on yeast extract sucrose (YES) agar, Sigma YES agar, oatmeal agar, and potato sucrose agar (three plates each), as described by Frisvad and Thrane [16].These were made in the years 1983–1995, and comprise approximately 150 fungal isolates.
2. Extracts made by the microextraction procedure of Smedsgaard [17], where approximately 0.6-cm^2 plugs of culture from YES agar, Czapek yeast autolysate agar (CYA), CYA with 5% salt (CYAS), oatmeal agar, or malt extraxt agar [4] were extracted with methanol–dichloromethane–ethyl acetate (1:2:3 v/v) and 1% formic acid. These comprise some 200 fungal isolates and 200 extracts made in the years 1996–2009.
3. Extracts for fumonisin analysis made by the microextraction procedure mentioned above, but extracted using 75% methanol [10] in the years 2007–2009. Here mainly CYAS and partly YES agar cultures were extracted, which add up to a further 100 cultures and 200 extracts.

Metabolite analysis by LC-DAD

The large extracts from the years 1983–1995 were analyzed by LC [acidic, 15–100% acetonitrile (CH_3CN) gradient, 40 min, Nucleosil C_{18} column] with DAD (200–600 nm) as described by Frisvad and Thrane [16]. Data were available as printed reports with chromatographic traces (210 and 280 nm) and UV/vis spectra (200–600 nm).

Microextracts from the years 1995–2003 were analyzed by LC-DAD with parallel fluorescence detection (FLD) under similar chromatographic conditions using a Nucleosil BDB C_{18} column [17] with the FLD set at 230→333 and 230→450 nm.

Microextracts from the years 2003–2009 were analyzed by LC-DAD-FLD as mentioned above using a Luna C_{18} II column (15% CH_3CN to100% CH_3CN in 20 min) [18].

Metabolite analysis by LC-DAD–TOF mass spectrometry

These analyses were performed using a LC system coupled to an orthogonal TOF mass spectrometer (Micromass LCT oaTOF) equipped with an electrospray source [19]. Two different gradients were used: (1) Luna II C_{18} 15% CH_3CN to 100% CH_3CN in 20 min, 20 mM formic acid in both solvents [20]; (2) as described before but with the gradient starting at 30% CH_3CN and going to 60% CH_3CN in 5 min and then to 100% in 1 min.

About 300 extracts were analyzed by LC-DAD–TOF mass spectrometry (TOFMS) in the years 2003–2009; of these, most have also been analyzed by LC-DAD-FLD.

Metabolite analysis by LC–tandem mass spectrometry

This was performed using a Quattro Ultima triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source and operated in multiple reaction mode (MRM) [11]. Separation was done on a Gemini C_6-phenyl column (acidic, 20% CH_3CN to 55% CH_3CN in 6 min, then to 100% in 30 s). MRM transitions for OTA, fumonisin B_1 (FB_1), FB_2, fumonisin B_3 (FB_3), and FB_4 are described elsewhere [11, 21]. Malformin A_2 was detected using *m*/*z* 516→304 at 30 V and *m*/*z* 516→417 at 20 V; malformins C and A were detected using *m*/*z* 530→372 at 25 V and *m*/*z* 530→417 at 20 V; ochratoxin β was detected using *m*/*z* 223→103 at 36 V and *m*/*z* 223→159 at 36 V; ochratoxin α was detected using *m*/*z* 257→193 at 36 V and

m/*z* 257→221 at 33 V; and ochratoxin B was detected using *m*/*z* 370→205 at 36 V and *m*/*z* 370→324 at 36 V. About 350 extracts were analyzed by LC–tandem mass spectrometry (MS/MS) in the years 2008–2009; many of these have also been analyzed by LC-TOFMS and LC-DAD-FLD.

Reference standards

For the LC-DAD-FLD analyses, OTA, ochratoxin B (Sigma-Aldrich), ochratoxin α, kotanin, nigragillin, and malformins A, A_2, and C (gifts) were coanalyzed. For LC–mass spectrometry (MS), FB_1, FB_2, and FB_3 (Biopure, Tuln, Austria) and AAL toxins TA_1 and TB_1 (Sigma-Aldrich) were also included. Tensidols A and B and aurasperones A, B, and E were purified and validated by NMR [22].

Data analysis

LC-DAD and LC-DAD-FLD data were analyzed manually by comparing retention times, and UV spectra for the detected peaks as well as the retention times of the fluorescence peaks. These data were matched with LC-DAD-TOFMS data for tentative identification of the peaks by searching Antibase (Wiley) for compounds with the same accurate mass (±0.01-Da tolerance) and similar UV properties and estimated retention time (based on theoretical log*D* values) [19]. Compounds that we were able to tentatively identify in extracts from black aspergilli were aurasperones C–G, pyrophen, funalenone, fonsecinones B and C, fonsecin (TMC-256B1B), TMC-256A2, rubrofusarin B, fonsecin B, tensyuic acid A, asperazine, pyranonigrins A–D, and FB_4. The identity of FB_4 was further validated by showing that its accurate mass, retention time and tandem spectra from 25–50 eV were identical to those of to the presumed FB_4 in culture extracts from *Fusarium verticillioides*.

Analysis of biological significant metabolites

The black aspergilli can produce a diverse range of mainly polyketide-derived secondary metabolites along with nonribosomal peptides and a number of compounds of mixed biosynthetic origin. Annotation of the fully sequenced genome of *A. niger* showed the presence of an impressive 34 polyketide synthase (PKS)-encoding genes as well as 17 nonribosomal peptide synthase (NRPS) genes and seven PKS-NRPS hybrids, accentuating the biosynthetic potential and versatility of this species [13]. So far, a total of 145 metabolites (Table 2) have been isolated and their structures elucidated from the biseriate black aspergilli.

Ochratoxins

Historically, OTA and its precursors (ochratoxins B, β, and α) have been the most significant mycotoxins produced within the *A. niger* complex. In extracts from approximately 400 agar cultures, OTA was always detected in amounts at least 10 times higher than the precursors using LC-FLD, LC-TOF analysis, and LC-MS/MS, and in many cases only OTA was detected.

It has been claimed that *A. tubingensis* can produce OTA [23, 24], presumably owing to unspecific chemical analyses. It was not possible to confirm this from the same cultures in our laboratory by LC-FLD, LC-TOF analysis, nor LC-MS/MS in culture extracts from up to ten different media. Other researchers have also doubted OTA production by *A. tubingensis* [3, 25].

Owing to the array of adverse toxicological effects on animals and humans, OTA is one of the most studied mycotoxins. The very low regulatory levels in food and feed mean that numerous highly sensitive analytical methods have been developed [26–28] and these have been reviewed several times [29, 30]. Thus, OTA will not be in the major scope of this paper since many of the commodities where *A. niger* is a problem have already been covered [29, 31–35]. However, it must be emphasized that we have found that direct analysis of OTA from *A. niger* using LC-DAD is not possible in crude extracts owing to coeluting malformins and naptho-γ-pyrones (NGPs) (see later), of which the NGPs are present in more than 100 times higher quantities than OTA. This is illustrated in Fig. 2 showing LC-DAD-TOFMS chromatogram with the OTA peak hidden underneath the peaks of rubrofusarin B and malformins. Furthermore, with use of our LC-DAD-FLD method, four to five peaks of closely eluted substances are detected from many black *Aspergillus* extracts (230→333 nm, acidic conditions). This indicates a risk that some of these compounds may be coeluted with OTA even under other slightly different conditions. Thus, careful control of chromatographic conditions is necessary. Careful control of potential carryover between samples in the autosampler should be done if high-level OTA-producing *A. carbonarius* strains are analyzed in sequence.

For OTA analysis, our LC-TOFMS was approximately twofold less sensitive than FLD. Here sensitivity could be enhanced tenfold by shifting to alkaline conditions [32]; however, other compounds would suffer from a pH change (e.g., by postcolumn addition of NH_4OH solution) and comparison with old data will not be possible. Our LC-MS/MS instrument is about 20–50 times more sensitive than acidic FLD and has until now not yielded any interfering peaks on any of the two MRM transitions. It should be noted that the latest LC-TOFMS and Orbitrap instruments will, according to the manufactures, provide 10 times

Table 2 Secondary metabolites and mycotoxins from *A. niger* and related species

Component	Species	Elementary composition	UV/VIS	Monoisotopic mass (Da)
Glyoxylic acid [86, 87]		$C_2H_2O_3$		74.0004
Glycolic acid [86]		C_2H_4O		76.0155
Oxalic acid [88]		$C_2H_2O_4$		89.9947
Hydroxypyruvic acid [89]		$C_3H_4O_4$		104.0104
(+)-Parasorbic acid (Antibase2008)		$C_6H_8O_2$		112.0519
Sorbic acid (Antibase2008)		$C_6H_8O_2$		112.0519
Fumaric acid [90]		$C_4H_4O_4$		116.0104
2-Phenylethanol [91]		$C_8H_{10}O$		122.0726
Glutaric acid [86]		$C_5H_8O_4$		132.0423
Asperyellone [92]		$C_{20}H_{22}O$		278.1671
Phenylacetic acid [91]		$C_8H_8O_3$		136.0519
Phenoxyacetic acid [91]		$C_8H_8O_3$		152.0468
p-Methoxyphenylacetic acid [91]		$C_9H_{10}O_3$		166.0624
4-Hydroxymandelic acid [88]		$C_8H_8O_4$		168.0417
Dehydrocarolic acid [93]	*A. brasiliensis*	$C_9H_8O_4$		180.0417
Cyclo-L-Ala-L-Leu [94]		$C_9H_{16}N_2O_2$		184.1206
Citric acid [95]	*Nigri*[a]	$C_6H_8O_7$		192.0265
D-Galactonic acid		$C_6H_{12}O_7$		196.0583
7-Hydroxy-4-methoxy-5-methylcoumarin [96, 97]	*A. niger* (reported as *Cladosporium herbarum*)	$C_{11}H_{10}O_4$		206.0574
Hexylitaconic acid [98, 99]	*A. niger*	$C_{11}H_{18}O_4$		214.1200
3-Methyl-8-hydroxy-4-decanoate [91]	*A. niger*	$C_{12}H_{22}O_3$		214.1564
Ochratoxin β	*A. niger, A carbonarius, A. sclerotioniger*	$C_{11}H_{10}O_5$		222.0523
Nigragillin [93]	*A. niger, A. tubingensis*	$C_{13}H_{22}ON_2$	262 (26,200)	222.1739
Pyranonigrin A (pyranopyrrol A) [57, 75]	*Nigri*	$C_{10}H_9NO_5$,	210, 252, 316	223.0481
Aspernigrin A [57, 96]	*A. niger*	$C_{13}H_{12}N_2O_2$		228.0893
Carbonarone A [83]	*A. carbonarius*	$C_{13}H_{11}NO_3$		229.0733
Carbonarone B [83]	*A. carbonarius*	$C_{13}H_{11}NO_3$		229.0733
Carbonarone A [83]	*A. carbonarius*	$C_{13}H_{11}NO_3$	279 (100%), 317 (29%), 401 (25%)	229.0739
Carbonarone B [83]	*A. carbonarius*	$C_{13}H_{11}NO_3$	340 (100%)	229.0739
Tensidol A [100]	*A. niger, A. tubingensis*	$C_{13}H_{11}NO_3$	206 (100%), 250 (30%)	229.0739
2-Methylene-3-hexyl-butanedioic acid [98]	*A. niger*	$C_{11}H_{18}O_5$		230.1149
Pyranonigrin D (pyranopyrrol D) [57]	*Nigri*	$C_{11}H_9NO_5$		235.0481
2-Carboxymethyl-3-hexylmaleic acid anhydride [98, 101]	*A. niger*	$C_{12}H_{16}O_5$	253	240.0992
Tensyuic acid [102]	*A. niger*	$C_{11}H_{16}O_6$	203 (6,000), 205 (5,600)	244.0947
Tensyuic acid F [102]	*A. niger*	$C_{11}H_{16}O_6$	199 (11,000)	244.0947
Pyranonigrin B (pyranopyrrol B) [57]	*Nigri*	$C_{11}H_{11}NO_6$		253.0586
Pyranonigrin C (pyranopyrrol C)[57]	*Nigri*	$C_{11}H_{11}NO_6$		253.0586
Ochratoxin α	*A. carbonarius, A. niger, A sclerotioniger*	C11H9ClO5		256.0133
Tensyuic acid B [102]	*A. niger*	$C_{12}H_{18}O_6$	204 (8,900), 207 (7,700)	258.1103
Nigerazine A [71]	*A. niger*?	$C_{16}H_{22}N_2O$		258.1727
Nigerazine B [71]	*A. niger*?	$C_{16}H_{22}N_2O$	280 (22,400)	258.1732
Nigerloxin [79]	*A. niger*?	$C_{13}H_{15}NO_5$		265.0945
TAFU-567 [103]	*A. acidus*?	$C_{13}H_{14}O_6$		266.0785
Antafumicin A [104]	*A. acidus*	$C_{13}H_{14}O_6$	217(21,000), 279(15,500), 317(8,720)	266.0786

Table 2 (continued)

Component	Species	Elementary composition	UV/VIS	Monoisotopic mass (Da)
Antafumicin B [104]	*A. acidus*	$C_{13}H_{14}O_6$	217(21,000), 279(15,500), 317(8,720)	266.0786
TMC-256A1 [105]	*A. niger*	$C_{15}H_{12}O_5$	236 (100%), 276 (68%), 368 (12%), 482 (1%)	272.0679
TMC-256C1 [105]	*A. niger*	$C_{15}H_{12}O_5$		272.0679
Rubrofusarin(heminigerone) [44, 106]	*Nigri*	$C_{15}H_{12}O_5$	(225, 28,200) (253sh, 19,600) (278, 49,700) (328, 3,400) (415, 5,200)	272.0679
Tensyuic acid C [102]	*A. niger*	$C_{13}H_{20}O_6$	201 (7,700), 206 (4,500)	272.1260
Tensyuic acid D [102]	*A. niger*	$C_{13}H_{20}O_6$	202 (17,000), 205 (7,700)	272.1260
Asperrubrol [64, 107]	*A. niger, A. tubingensis*	$C_{20}H_{22}O$	242(9,800), 293(11,200), 400(63,200)	278.1672
Asperic acid [65]	*A. niger*	$C_{16}H_{28}O_4$	225, 280	284.1988
Flavasperone [108, 109]	*Nigri*	$C_{16}H_{14}O_5$	225(28,184), 254(47,863), 406(5,495)	286.0841
Tensyuic acid E [102]	*A. niger*	$C_{14}H_{22}O_6$	198 (13,000)	286.1416
Pyrophen [65]	*A. tubingensis, A. niger*	$C_{16}H_{17}NO_4$		287.1158
Funalenone [55]	*A. niger, A. tubingensis, A brasiliensis*	$C_{15}H_{12}O_6$		288.0634
Fonsecin (TMC-256B1) [110]	*Nigri*	$C_{15}H_{14}O_6$		290.0790
Nigerasperone A [111]	*A. niger?*	$C_{16}H_{14}O_6$	210sh (55%), 226 (74%), 255sh (56%), 276 (100%), 325 (14%), 400 (22%)	302.0785
Xanthoherquein [55]	*A. sclerotioniger?*	$C_{15}H_{12}O_7$		304.0578
Fonsecin B (TMC-256B2) [105, 110, 112]	*Nigri*	$C_{16}H_{16}O_6$	231 (80%), 275 (100%), 328 (26%), 406 (21%)	304.0941
Fonsecin monomethyl ether [44, 110, 112]	*Nigri*	$C_{16}H_{16}O_6$	232(28,500), 277(40,500), 317(9,100), 330(10,000), 395(8,400)	304.0947
Asnipyrone B [113]	*A. niger?*	$C_{20}H_{20}O_3$	235(16,600), 265(15,490), 376(33,885)	308.1413
Carbonarin E [114]	*A carbonarius*	$C_{18}H_{17}NO_4$		311.1158
Asperenone [107]	*A. niger, A. tubingensis*	$C_{22}H_{24}O_3$	242(13,800), 300(9,200), 414–416(94,200)	312.1726
4,9-Dihydroxyperylene-3,10-quinone [88]	*Nigri?*	$C_{20}H_{10}O_4$		314.0573
Asnipyrone A [113]	*A. niger*	$C_{21}H_{22}O_3$	240(18,600), 290(19,055), 378(31,625)	322.1570
Atromentin [75]	*A. niger, A. tubingensis*	$C_{18}H_{12}O_6$		324.0628
Cycloleucomelon [57]	*A. niger, A. tubingensis*	$C_{18}H_{10}O_7$		338.0421
Tensidol B [100]	*A. niger, A. tubingensis*	$C_{18}H_{17}NO_6$	206 (100%),	343.1050
Nafuredin [115]	*A. niger?*	$C_{22}H_{32}O_4$		360.2295
22-Deacetylyanuthone A [77]	*A. niger, A. tubingensis*	$C_{22}H_{32}O_4$	238(16,220), 302(480)	360.2302
Aspergillin [54]	*A. Niger*	$C_{19}H_{12}O_8$		368.0527
2,3,4,8,9,10-Hexahydroxy-5*H*,6*H*,7*H*-benzopyrene-1,11-dione [88]	*Nigri*	$C_{19}H_{12}O_8$		368.0527
1-Hydroxyyanuthone A [77]	*A. niger, A. tubingensis*	$C_{24}H_{32}O_5$	234(8,510), 290(220)	400.2251
1-Hydroxyyanuthone C [77]	*A. niger, A. tubingensis*	$C_{24}H_{32}O_5$	232(4,680), 282(468)	400.2251
Yanuthone B [77]	*A. niger, A. tubingensis*	$C_{24}H_{32}O_5$	244(4,470), 290(1,950), 338(830)	400.2251
Tubingensin A [116]	*A. tubingensis* ATCC 76608[b]	$C_{28}H_{35}NO$		401.2713
Tubingensin B [117]	*A. tubingensis* ATCC 76608	$C_{28}H_{35}NO$	218(17,200), 237(25,500), 260(10,100), 299(10,100), 325(2,200), 338(6,700)	401.2720
Yanuthone A [77]	*A. niger, A. tubingensis*	$C_{24}H_{34}O_5$	232(7,950), 310(500)	402.2407
Yanuthone C [77]	*A. niger, A. tubingensis*	$C_{24}H_{34}O_5$	232(13,180), 280(2,040), 322(1,070)	402.2407
Ochratoxin A [118]	*A. niger, A. carbonarius, A. sclerotioniger*	$C_{20}H_{18}ClNO_6$	205(94%), 215(98%), 218(21%), 283(2%), 332(17%)	403.0823
Dihydrotubingensin B [119]	*A. tubingensis* ATCC 76608	$C_{28}H_{37}NO$		403.2870
Dihydrotubingensin A [119]	*A. tubingensis* ATCC 76608	$C_{28}H_{37}NO$		403.2870
10,23-Dihydro-24,25-dehydroaflavinine [120]	*A. tubingensis* ATCC 76608	$C_{28}H_{39}NO$	226(31,600), 284(4,400), 291(4,100)	405.3032
Aflavinine [120]	*A. tubingensis* ATCC 76608	$C_{28}H_{39}NO$	225(18,700), 283(3,010), 291(2,710)	405.3032
Bicoumanigrin (JH 0508 O'; bicumarine) [57]	*A. niger*	$C_{22}H_{18}O_8$		410.0996

Table 2 (continued)

Component	Species	Elementary composition	UV/VIS	Monoisotopic mass (Da)
Orlandin [56]	*A. niger*	$C_{22}H_{18}O_8$	311(29,500), 321(25,120)	410.1002
BMS-192548 [52, 121]	*A. niger*?	$C_{21}H_{18}O_9$	280(4,440), 320(35,548), 414(38,822)	414.0950
Nygerone A [76]	*A. niger*	$C_{24}H_{22}N_2O_5$		418.1529
10,23-Dihydro-24,25-dehydro-21-oxo-aflavinine [120]	*A. tubingensis* ATCC 76608	$C_{28}H_{37}NO_2$	224(18,200), 267(4,200), 273(1,400), 283(2,100), 291(1,750)	419.2824
14-epi-14-hydroxy-10,23-dihydro-24,25-dehydroaflavinine [122]	*A. tubingensis* ATCC 76608	$C_{28}H_{39}NO_2$		421.2975
14-epi-14-hydroxy-10,23-dihydro-24,25-dehydro-aflavinine [120]	*A. tubingensis* ATCC 76608	$C_{28}H_{39}NO_2$	226(16,400), 284(2,290), 292(2,100)	421.2980
Demethylkotanin [75]	*A. niger*	$C_{23}H_{20}O_8$		424.1153
Aspernigerin [73]	*A. niger*	$C_{26}H_{32}N_4O_2$		432.2525
Kotanin [56]	*A. niger*	$C_{24}H_{22}O_8$	203(92%), 208(100), 237(35), 259(15%), 296(36%), 306(41%), 317(35%)	438.1309
Aspernigrin B [57, 96]	*A. niger*	$C_{27}H_{24}N_2O_5$		456.1679
Yanuthone D [77]	*A. niger, A. tubingensis*	$C_{28}H_{38}O_8$	226(7,940), 254(3,715), 298(1,150)	502.2567
Malformin A_2 [63, 67]	*A. niger, A. brasiliensis, A. tubingensis*	$C_{22}H_{37}O_5N_5S_2$	End abs.	515.2239
Malformin B_5 [63, 67]	*A. niger, A. brasiliensis, A. tubingensis*	$C_{22}H_{37}O_5N_5S_2$	End abs.	515.2239
Malformin B_2 [63, 67]	*A. niger, A brasiliensis, A. tubingensis*	$C_{22}H_{37}O_5N_5S_2$	End abs.	515.2239
Yanuthone E [77]	*A niger, A. tubingensis*	$C_{29}H_{42}O_8$	234(9,120), 280(1,000), 340(450)	518.2881
Malformin A_1 [63]	*A. niger, A brasiliensis, A. tubingensis*	$C_{23}H_{39}O_5N_5S_2$	End abs.	529.2396
Malformin B_{1a} [63, 67]	*A. niger, A brasiliensis, A. tubingensis*	$C_{23}H_{39}O_5N_5S_2$	End abs.	529.2396
Malformin B_{1b} [63, 67]	*A. niger, A brasiliensis, A. tubingensis*	$C_{23}H_{39}O_5N_5S_2$	End abs.	529.2396
Malformin B_5 [63, 67]	*A. niger, A brasiliensis, A. tubingensis*	$C_{23}H_{39}O_5N_5S_2$	End abs.	529.2396
Malformin B_3 [63, 67]	*A. niger, A brasiliensis, A. tubingensis*	$C_{23}H_{39}O_5N_5S_2$	End abs.	529.2396
Malformin C [123]	*A. niger, A brasiliensis, A. tubingensis*	$C_{23}H_{39}O_5N_5S_2$	End abs.	529.2396
8′-*O*-Demethylnigerone [49]	*A. niger*	$C_{31}H_{24}O_{10}$	284 (56,200), 365 (12,020), 400 (9,775)	556.1369
8′-*O*-Demethylisonigerone [49]	*A. niger*	$C_{31}H_{24}O_{10}$	281 (33,885), 355 (26,915), 389 (7,760)	556.1369
Asperpyrone A [124]	*Nigri*	$C_{31}H_{24}O_{10}$,		556.1369
Dianhydroaurasperone C [125]	*Nigri*	$C_{31}H_{24}O_{10}$	225(22,908), 255(26,915), 280(85,113), 325(3,715), 405(5,754)	556.1369
6′-*O*-Demethylnigerone [106]	*Nigri*	$C_{31}H_{24}O_{10}$	226(39,810), 278(63,095), 408(10,000)	556.1369
Aurasperone D [44, 48, 102, 108, 109]	*Nigri*	$C_{31}H_{24}O_{10}$	235–240(50,119), 280(51,286), 320–325(15,136), 380(7,080)	556.1369
Carbonarin C [114]	*A carbonarius*	$C_{33}H_{27}NO_8$		565.1737
Carbonarin D [114]	*A carbonarius*	$C_{33}H_{27}NO_8$		565.1737
Carbonarin H [114]	*A. carbonarius*	$C_{33}H_{26}O_9$		566.1577
Isonigerone [49]	*Nigri*	$C_{32}H_{26}O_{10}$	UV: 226 (65%), 275 (100%), 327 (17%), 407 (19%)	570.1526
Asperpyrone B [124]	*Nigri*	$C_{32}H_{26}O_{10}$		570.1526
Asperpyrone C [124]	*Nigri*	$C_{32}H_{26}O_{10}$		570.1526
Aurasperone A [47, 102, 108, 109]	*Nigri*	$C_{32}H_{26}O_{10}$	225(51,300), 258(53,700), 280(100,000), 325(8,710), 406(12,880)	570.1526
Fonsecinone A [126]	*Nigri*	$C_{32}H_{26}O_{10}$	228(45,700), 256(44,670), 278(67,600), 325(11,480), 398(7,080)	570.1526
Fonsecinone D [126]	*Nigri*	$C_{32}H_{26}O_{10}$	227(26,900), 279(48,975), 316(8,128), 328(6,920), 403(6,310)	570.1526
Isoaurasperone A [44, 112]	*Nigri*	$C_{32}H_{26}O_{10}$	255(33,884), 275(38,905), 385–390(3,467)	570.1626

Table 2 (continued)

Component	Species	Elementary composition	UV/VIS	Monoisotopic mass (Da)
Isonigerone [49, 106]	*Nigri*	$C_{32}H_{26}O_{10}$	228(50,119), 248(56,234), 279(66,069), 390(9,550)	570.1626
Nigerone [106]	*Nigri*	$C_{32}H_{26}O_{10}$	226(51,286), 278(83,186), 407(14,125)	570.1626
Nigerasperone C [111]	*Nigri*	$C_{31}H_{26}O_{11}$	232 (50%), 282 (100%), 329 (15%), 403 (15%)	574.1470
Aurasperone F [48]	*Nigri*	$C_{31}H_{26}O_{11}$	213, 281, 320, 334	574.1475
Carbonarin A [114]	*A. carbonarius*	$C_{33}H_{26}O_{10}$		582.1526
Carbonarin B [114]	*A. carbonarius*	$C_{33}H_{26}O_{10}$		582.1526
Diketopiperazine dimer [68]	*A. tubingensis*	$C_{33}H_{38}N_6O_4$	240, 300	582.2949
Aurasperone G [48]	*Nigri*	$C_{31}H_{24}O_{12}$	UV: 213, 281, 315, 332, 403	588.1268
Aurasperone E [44]	*Nigri*	$C_{32}H_{28}O_{11}$	230(72,444), 282(147,910), 322(22,387), 330(20,418), 400(19,500)	588.1631
Fonsecinone B [126]	*Nigri*	$C_{32}H_{28}O_{11}$	229(38,900), 255(41,700), 280(70,800), 320(14,125), 328(14,125), 403(9,120)	588.1631
Fonsecinone C [126]	*Nigri*	$C_{32}H_{28}O_{11}$	234(51,285), 254(38,000), 279(60,255), 315(19,500), 327(14,800), 398(7,080)	588.1631
Aurasperone C [44, 47, 102, 108, 109]	*Nigri*	$C_{31}H_{28}O_{12}$	236, 283.5, 323, 336, 412	592.1581
Carbonarin G [114]	*A. carbonarius*	$C_{34}H_{28}O_{10}$		596.1682
10,10′-Bifonsecin B [49]	*Nigri*	$C_{32}H_{30}O_{12}$	231 (84%), 276 (100%), 330 (26%), 411 (24%)	606.1732
Nigerasperone B [111]	*Nigri*	$C_{32}H_{30}O_{12}$	236 (98%), 281 (100%), 316sh (32%), 380 (12%)	606.1732
Aurasperone B [44, 47, 48, 108, 109]	*Nigri*	$C_{32}H_{30}O_{12}$	235(51,000), 282(87,700), 321(20,100), 334(20,000), 410(13,600)	606.1732
Carbonarin F [114]	*A. carbonarius*	$C_{34}H_{28}O_{11}$		612.1632
Asperazine [64]	*A. tubingensis*	$C_{40}H_{36}N_6O_4$	225, 275, 300	664.2798
Fumonisin B_4 [11]	*A. niger*	$C_{34}H_{59}NO_{13}$	Not UV active	689.3986
Fumonisin B_2 [10]	*A. niger*	$C_{34}H_{59}NO_{14}$	Not UV active	705.3930
Biotransformation products (not true metabolites)				
Differenol A (genistein, prunetol, sophoricol) [127]		$C_{15}H_{10}O_5$		270.0528
3′,4′,5,7-Tetrahydroxy-8-methoxy isoflavone [128]		$C_{16}H_{12}O_7$		316.0578
Pisolithin B [88]		$C_8H_8O_4$		168.0417
8-Hydroxygenistein [128]		$C_{15}H_{10}O_6$		286.0472
Flaviolin [129]		$C_{10}H_6O_5$		206.0215
Orobole [127]		$C_{15}H_{10}O_6$		286.0472
Iso-T-2 toxin (Antibase2008)		$C_{24}H_{34}O_9$		466.2197

Extinctions coefficient

The identities of species marked with a *question mark* have not been confirmed.

[a] Series *Nigri*: *A. niger, A. tubingensis, A. acidus, A. brasiliensis, A. carbonarius, A. sclerotiivarbonarius, A. sclerotioniger, A. ibericus, A. vadensis, A. costaricaensis, A. piperis*

[b] This isolate produces an abundance of sclerotia, but has not been unequivocally placed in *A. tubingensis* yet.

End abs. End absorption (UV max. <200nm)

higher mass resolution and 10–100 times better sensitivity than the LC-TOFMS instrument used here.

ESI of OTA mainly yields the protonated molecular ion $[M+H]^+$ in positive mode and $[M-H]^-$ in negative mode, with approximately 10 times higher signal in positive mode. With high in-source fragmentation settings, OTA yields several diagnostic ions for further confirmation. An unidentified fragment ion at *m/z* 358 can be observed along with the sodium adduct $[M+Na]^+$ and the ^{37}Cl $[M+H]^+$ at *m/z* 406 [19]. Care should be taken when using nominal-mass LC-MS, since a very common contaminant from plasticware (presumable a phthalate) with a molecular mass of 386 Da is eluted very close to OTA and makes at predominant $[M+NH_4]^+$ ion at *m/z* 404. OTA can be differentiated from the contaminant on the basis of the chlorine isotope pattern.

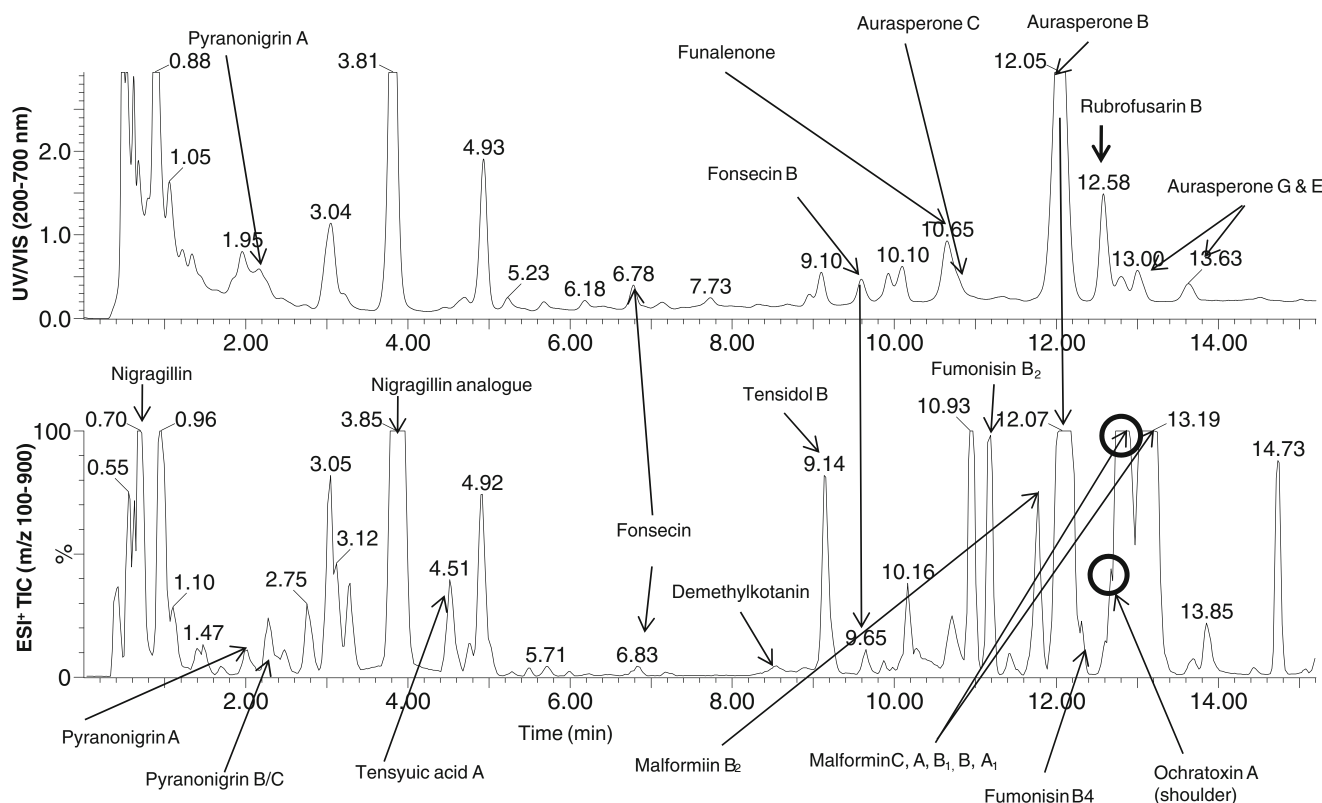

Fig. 2 *Aspergillus niger* extract from 8-day culture on yeast extract sucrose agar analyzed by liquid chroamtography–diode-array detection–time-of flight mass spectrometry. The *upper trace* is the UV/vis (200–700 nm) chromatogram and the lower trace is the total ion chromatogram (*TIC*) [positive electrospray ionization (*ESI*$^+$), *m/z* 100–900]. Separation was done using a Luna C_{18} II column with 15% CH_3CN to 100% CH_3CN in 20 min

Fumonisins

The fumonisins are a group of polyketide-derived mycotoxins, first discovered in 1988 from *F. verticillioides* [36]. This group of compounds is of great importance as they are suspected to be carcinogenic to humans, and are as a consequence regulated mainly in maize-based products. The fumonisins can be divided into four series A, B, C, and P [37], with the B series, mainly FB_1, FB_2, and FB_3 (Fig. 1), as the most abundant naturally occurring fumonisins [37, 38]. The surprising discovery of putative homologues to the *F. verticillioides* fumonisin gene cluster in two different *A. niger* genomes [13, 26] led to the subsequent documentation of actual FB_2 production in *A. niger* [10, 11]. Besides FB_2, FB_4 is also produced in lower amounts, about 10–25% of the amount of FB_2. From a current screening project on grapes and raisins it appears that fumonisins are produced by approximately 75% of all *A. niger* strains, whereas for coffee the figure was 76%, and they are thus much more common within *A. niger* than OTA production (6–10% [7, 8]).

Analysis of fumonisins produced by *Fusarium* spp. has previously been extensively reviewed [29, 37, 38]. In this review, FB_2 and FB_4 will therefore only be dealt with briefly in respect to the emerging data from analysis of *A. niger* infected commodities for which new analytical methods needs to be developed. Owing to the lack of suitable chromophores or flourophores, fumonisins cannot be detected directly using UV detection or FLD. This is probably why they were not detected in culture extracts of *A. niger* related species until recently. The fumonisins ionize excellently in positive ESI (ESI$^+$) and also quite well in negative ESI (ESI$^-$), yet about approximately tenfold lower than in ESI$^+$ using our instruments. Bartok et al. [37] have listed comprehensive LC-MS/MS data on the fumonisins.

With the increasing sensitivity of LC-MS/MS, especially with triple quadrupole mass spectrometers, multimethods with no purification are emerging for low parts per billion levels of well-ionizing mycotoxins. An example is the screening method of Sulyok et al. [39, 40] which was used to detect fumonisins down to 3–17 µg/kg and OTA down to approximately 1 µg/kg. The method was used to detect very high levels of FB_2 (33 mg/kg) in dark bread, where the absence of FB_1 as well as a black infecting fungus on the bread together indicate that the contaminant was an *A. niger* rather than a *Fusarium* species. FB_2 and FB_4 probably produced by *A. niger* were also reported in [41] to have been detected by LC-TOFMS.

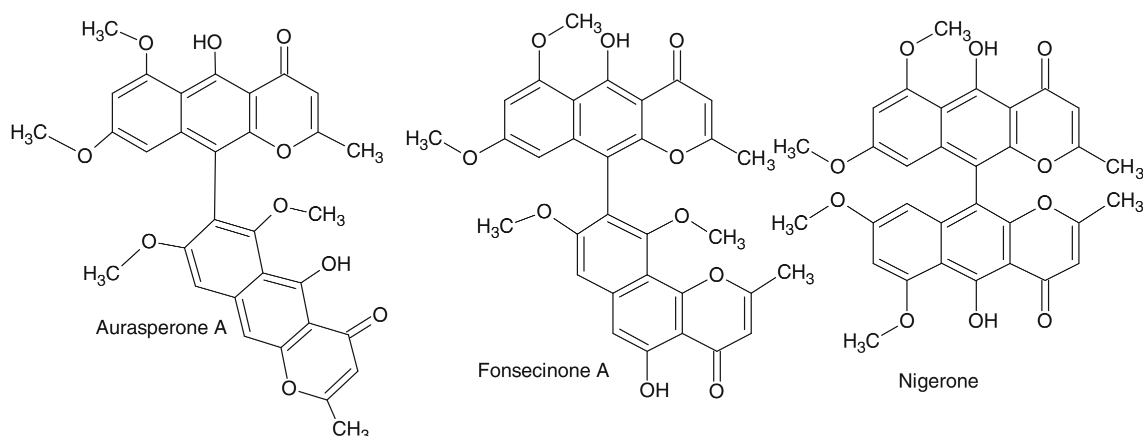

Fig. 3 Structures representing the three major groups of bis(naptho-γ-pyrone)s in the *A. niger* group

From CH_3CN extracts of strawberries, we were able to detect fumonisins with LC-MS/MS down to 10 µg/kg and OTA down to 1 µg/kg, and on berries artificially infected with *A. niger* we found up to 25 mg/kg FB_2 and 2 mg/kg FB_4, as well as up to 1 µg/g OTA, whereas malformins were barely detected (K.F. Nielsen, unpublished results).

For analysis of green coffee, the EN 13585:2001 protocol (70% CH_3OH, strong anion exchange, SAX) for extraction and purification of fumonisins in maize could be directly adapted [11]. Furthermore, OTA which cooccurred in the extract was simultaneously retained and detected by LC-MS/MS [21]. Immunoaffinity purification of fumonisins was not a successful strategy for extracts from green and roasted coffee and resulted in viscous dark samples. A commercial ELISA kit (developed for maize) was also tested, but in green coffee it gave totally random results with many false positives and absolutely no correlation to LC-MS/MS results [11].

Since some *A. niger* strains produce very high amounts of citric acid and other small organic acids (up to 50 g/L) under some conditions, it has not always been possible to use SAX purification. Recoveries from SAX have been seen as low as 0% for OTA and fumonisins owing to competition with small organic acids. In consequence, we are currently testing strong cation exchange. Surprisingly, we have not found publications on cation-exchange purification of fumonisins, which is probably a reflection of the first fumonisin methods, where derivatization with *O*-phthalaldehyde and subsequent LC-FLD was the preferred analytical strategy [29, 37, 38]. And since both *O*-phthalaldehyde and cation exchange target amines, these two methods are not orthogonal and thus not selective as a pair. However, with LC-MS as the detection principle, cation exchange should statistically be preferred since there are far fewer basic than acidic compounds in microorganisms.

Naptho-γ-pyrones

Quantitatively, the NGPs represent the most abundant family of secondary metabolites in the *A. niger* group under all conditions observed (including chemostate cultures).

The biological effects of several NGPs have been investigated in various systems [14], and they have, for instance, been shown to be antibacterial, antifungal [42], antitumoral [43], and cytotoxic [42, 43]. Ghosal et al. [44] reported acute toxicity (interperitoneal) at the 10–50 mg/kg level; however, to the best of our knowledge no data on the bioavailability of these compounds exist. Thus, these compounds cannot currently be considered mycotoxins *sensu stricto*, since this requires toxicity via a natural route of exposure.

The NGP group of compounds comprises a series of aurasperones, fonsecinones, and nigerones, as well as monomers such as flavasperone and rubrofusarin B (Figs. 3 and 4). Reanalysis of in-house LC-DAD and LC-MS data showed

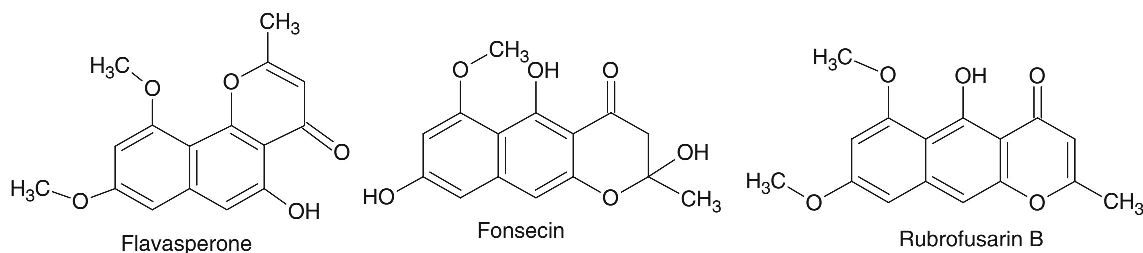

Fig. 4 Structures representing the three major groups of monomeric naptho-γ-pyrones in the *A. niger* group

that all species within the "*A. niger* clade" are able to produce NGPs (*A. acidus, A. brasiliensis, A. carbonarius*, *A. costaricaensis*, *A. ibericus*, *A. niger*, *A. piperis*, *A. sclerotiicarbonarius*, *A. sclerotioniger*, *A. tubingensis,* and *A. vadensis* [5]), whereas species not producing NGPs include the "*A. ellipticus/A. heteromorphus* clade" and the "*A. aculeatus* clade" (comprising *A. homomorphus, A. aculeatinus, A. aculeatus, A. japonicus*, *and A. uvarum*). However, in losing the ability to produce black conidia, *A. lacticoffeatus*, a light-brown-spored naturally occurring mutant of *A. niger*, has also lost the ability to produce NGPs, which is also the case for white-spored industrial strains we have analyzed. So there appears to be a link between black melanin production and NGP production in the *A. niger* group. Moreover, NGPs have been shown to be present in aerosolized spores [45].

Of 140 *A. niger* isolates we have analyzed by LC-DAD and LC-DAD-TOFMS, 134 (96%) were found to produce at least one NGP (usually aurasperone B in the highest amount). Comparable levels were seen for *A. tubigensis* (169 of 177, 95%), *A. ibericus* (three of three, 100%), and *A. acidus* (44 of 47, 94%) [46]. These data are thus conflicting with data reported by Bouras et al. [47], who only found NGPs in a low proportion of the strains investigated. This can be ascribed to differences in strain age, medium, and incubation conditions, since it has been our experience that the NGP levels are highest in fresh isolates and may decrease somewhat over time.

With LC-ESI-TOFMS, simple ionization patterns have been observed. In ESI^+, $[M+H]^+$ was the most abundant ion, and $[M+Na]^+$ and $[M+Na+CH_3CN]^+$ were the main adduct ions. In ESI^-, mainly $[M-H]^-$ and low-abundance $[M-2H+Na]^-$ ions was observed, the latter ion matching an acidic functionality (here a phenol group). Identical patterns were observed by Bouras et al. [47, 48], who also reported numerous specific fragment ions (aurasperones F and G) from MS/MS experiments (ion trap). These can also serve as potential MRM transitions for trace analysis.

The NGPs contain a fully conjugated system, giving rise to very characteristic UV/VIS spectra (Table 2, Fig. 5). However as seen in Table 2, and in the figures in Zhang et al. [49], some spectra are similar, and for the ones with the same elementary composition (e.g., ten different compounds with the formula $C_{32}H_{26}O_{10}$) it may even be difficult to separate them by their MS/MS spectra as they are mostly positional isomers which will probably fragment in a similar way. Thus, the retention time is the only way to differentiate NGPs if no reference standards are available or if no NMR validation can be done. An alternative is to compare the whole profiles with the one in Fig. 2 and the ones in Zhang et al. [49], since the order of elution of the NGPs should be the same if low-pH reversed-phase LC is used.

Since the NGPs constitute a large proportion of the total peak area and they absorb strongly in the 200–450-nm region, they often obscure the detection of compounds such as the malformins and ochratoxins. During preparative isolation of fumonisins, it has been observed that NGPs are not retained on SAX columns, but are retained on mixed-mode reversed-phase SAX columns (e.g., Oasis MAX), as secondary interactions with a polymeric backbone will cause strong retention of these weakly acidic phenols [50, 51]. These columns can then serve as a prefractionation step to selectively remove the NGPs.

Study of the occurrence of NGPs and other related compounds in food and feed samples is limited to the study of Ghosal et al. [44], who detected flavasperone, rubrofusarin, isoaurasperone, and aurasperones E, A, and D in artificially infected mangos at a total level of 60 mg/kg. NGPs were detected using thin-layer chromatography (280-nm absorption) after organic extraction and aurasperones A and D were found to be the predominant ones (approximately 33% each). Aurasperones (erroneously named tetracyclic compounds [52]) and orlandin were also found from *A. niger* infected building materials [53]. Since other metabolites originating

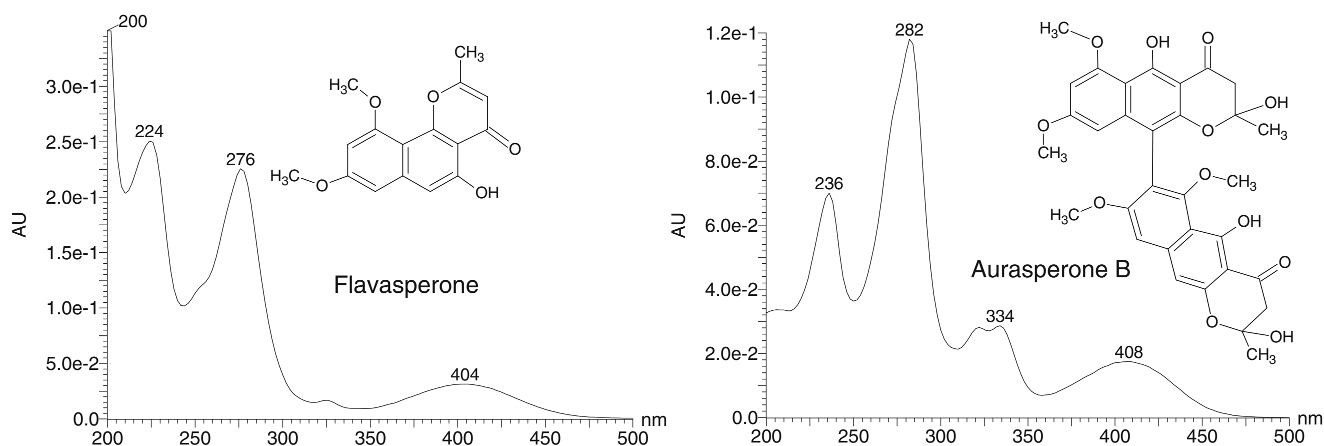

Fig. 5 UV spectra (pH 3.2) of monomeric (flavasperone) and dimeric (aurasperone B) naptho-γ-pyrones

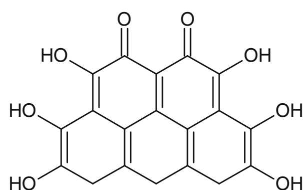

Fig. 6 Structure of the green pigment aspergillin

from the *A. niger* group can be found in food (OTA and FB_2), NGPs are most likely to be naturally occurring in various food matrices, and hereby it would be recommendable to develop analytical protocols for detection of NGPs in such food types as well as investigating their bioavailability and subsequent toxicity.

Occasionally, we have been asked to analyze yellow samples from submerged fermentations with different, mainly industrial *A. niger* strains. With LC-DAD-TOFMS we have each time detected aurasperone B as the only secondary metabolite in the sample. In most cases, ESI^- has been necessary for MS verification since foam suppressors [silicon and/or poly(ethylene glycol) oils] also suppress ESI^+ signals.

In addition, a green pigment has been observed in *A. niger* fermentations. This is aspergillin (Fig. 6), a highly oxygenated polyketide with a hexahydroxyl pentacyclic quinoid structure [54]. Together with another kind of melanin made from monomers, the black pigment is made up from a complicated mixture of aspergillin and proteins [54].

Bicoumarins

The bicoumarins (Fig. 7) represent another prominent family of compounds from *A. niger*, consisting of a group of heterocyclic dimers derived from cinnamic acid lactone, further categorized on the basis of the type of connection between the coumarin moieties. Like the NGPs, the bicoumarins contain a fully conjugated system and thus have very characteristic UV absorption profiles (Fig. 8) that make them easy to tentatively identify in an extract as a group. However, many variations with the same molecular formula make it difficult to make a positive identification on the basis of UV or MS data alone. Bicoumarins seems to be present in most *A. niger* strains, although they sometimes coelute with the aurasperones, which can obscure their detection. Orlandin, kotanin, and desmethyl-orlandin (based on LC-DAD, and LC-DAD-TOFMS) are consistently produced by *A. niger and A. tubingensis.* Another polyketide is funalenone [55], which is consistently (based on LC-DAD-TOFMS) produced by *A. niger* but also by *A. tubingensis* and *A. brasiliensis.*

The bicoumarins have been found to have some inhibitory effects on plant growth [56] and a moderate cytotoxic effect in human cell lines in vitro [57], but at present they are considered nontoxic [56, 59]. Yet, potential bioactivities of compounds of this type have not been investigated much and as a consequence no methods and data exist for their analysis.

Malformins

The malformins (Fig. 9) got their name owing to their ability to cause malformation in plants [60], and have also been reported to be antibacterial [61] and to inhibit interleukin-1β binding to various human cells [62]. The malformins, which are cyclic pentapeptides with a sulfur bridge, have often been called mycotoxins since they have been shown to be toxic after peritoneal injection. However, since they were not toxic by oral administration [63] they cannot currently be considered mycotoxins *sensu stricto.*

In the *A. niger* group, only *A. brasiliensis*, *A. niger*, and *A. tubingensis* produce malformins [5, 64, 65], with *A. brasiliensis* probably being the best producer (confirmed by both LC-DAD-TOFMS and LC-MS/MS). Since they are produced by several abundant food contaminants, a

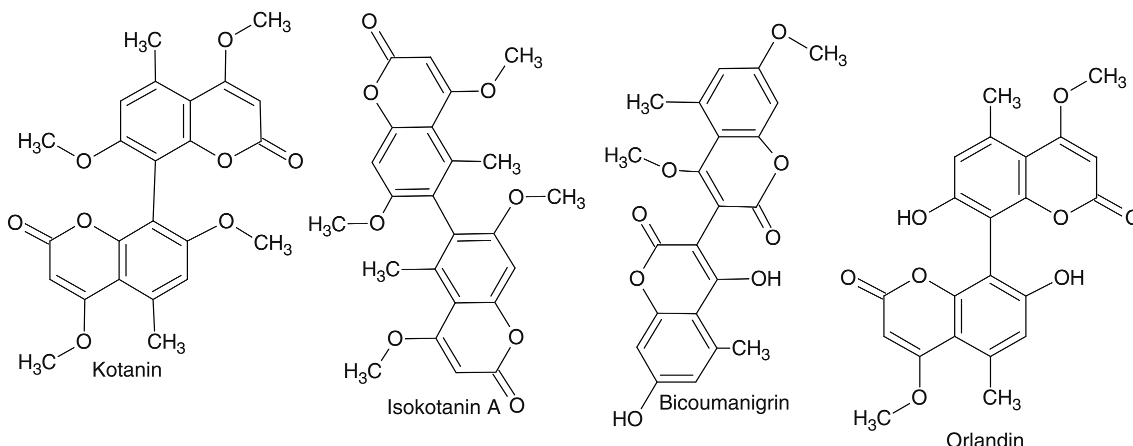

Fig. 7 Structure of selected bicoumarins

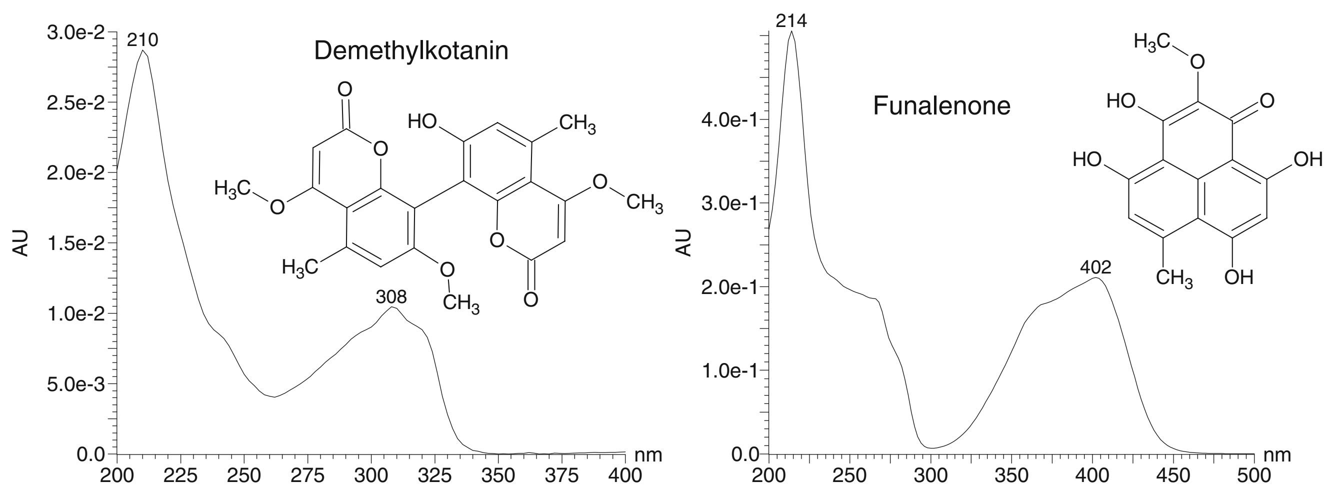

Fig. 8 Structure and UV spectrum of selected bicoumarins

toxicological evaluation, including bioavailability testing, is highly needed.

Even though malformins can be detected using LC-UV detection [19], in our experience they cannot be detected in *Aspergillus* extracts using this method owing to many interfering peaks. That is why LC-MS is the obvious choice for their detection [19, 66]. The sulfur bridge should also enable high-sensitivity electrochemical detection.

Chromatographic conditions used for analysis of the malformins are all based on acidic reversed chromatography [19, 66, 67], as normal phase and paper LC were not efficient [67]. Kim et al. [67] used a purification scheme based on acidic isocratic water–methanol (35:65 v/v) LC on an octadecyl silica column.

In ESI^+, the malformins have a high tendency to form $[M+NH_4]^+$, $[M+Na]^+$, and $[M+K]^+$ adducts [19], often with the abundance of $[M+H]^+$ 20% lower than that of the $[M+NH_4]^+$ ion. If high in-source fragmentation settings are used, sodium and potassium adducts will dominate. In ESI^+, *m/z* 417 is often observed from in-source fragmentation, and it is also a specific fragment for MS/MS analysis (MRM) along with *m/z* 372 and 304. In ESI^-, the malformins do not ionize as well as in ESI^+ (tested on both MS instruments used) and are mainly detected as the $[M+HCOO]^-$ and $[M+Cl]^-$ adducts, with the abundance of $[M-H]^-$ 20% lower than that of the other two adducts.

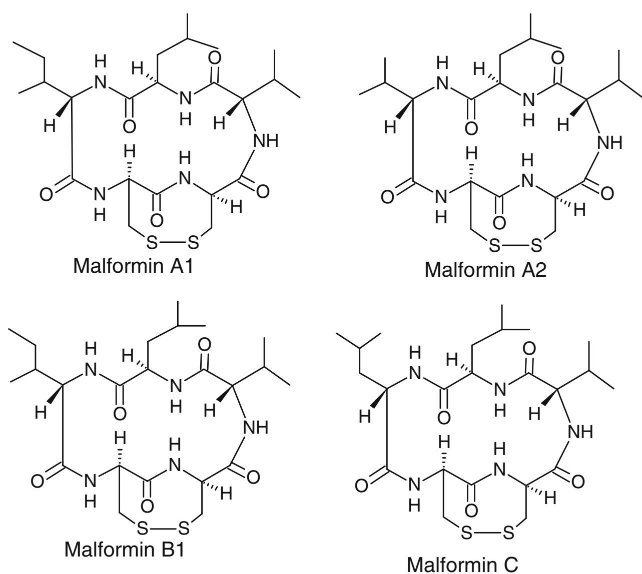

Fig. 9 Structures of selected malformins

Asperazines

Asperazine (Fig. 10) is a complex diketopiperazine dimer, first isolated from a marine-derived *A. tubingensis* (reported as *A. niger)* by Varuglu et al. [64]. An analogue was later described by Ovenden et al. [68], also from an *A. tubingensis* strain. Asperazine was previously found to be cytotoxic in vitro against leukemia [64], but a later investigation reported it to be noncytotoxic [69].

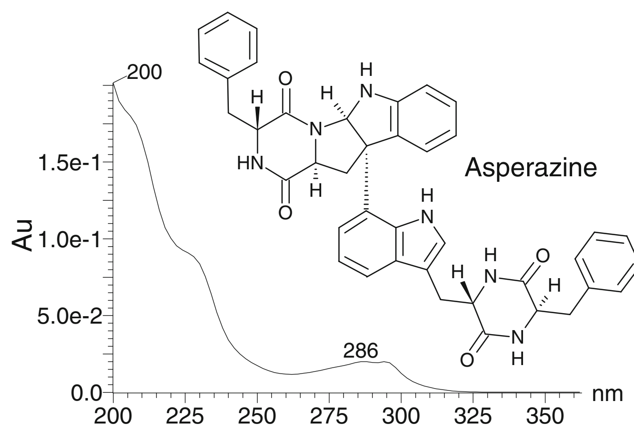

Fig. 10 UV spectrum and structure of asperazine

Reanalysis of extract data (approximately 66% by LC-TOFMS and all by LC-FLD) from 140 *A. niger*, 177 *A. tubingensis*, one *A. vadensis* [5], and 47 *A. acidus* (*A. foetidus*) [46, 70] strains for the production of asperazine showed that none of the *A. niger* strains produced asperazine, whereas a consistent production was observed in *A. acidus* and *A. tubingensis*. Owing to this limited distribution within the group, asperazine seems to be a valuable chemical marker that can be used to distinguish less toxic species such as *A. acidus* and *A. tubingensis* from otherwise similar but more toxic species such as *A. niger*.

As most alkaloids, asperazines ionize very well in ESI$^+$, with $[M+H]^+$ as the only ion detected. Furthermore, asperazines show strong fluorescence at 230–450 nm (acidic conditions), and can usually also be detected from their distinct UV spectra using LC-DAD (UV/vis) (Fig. 10). Only acidic reversed-phase separations have been reported for their separation [5, 64, 70].

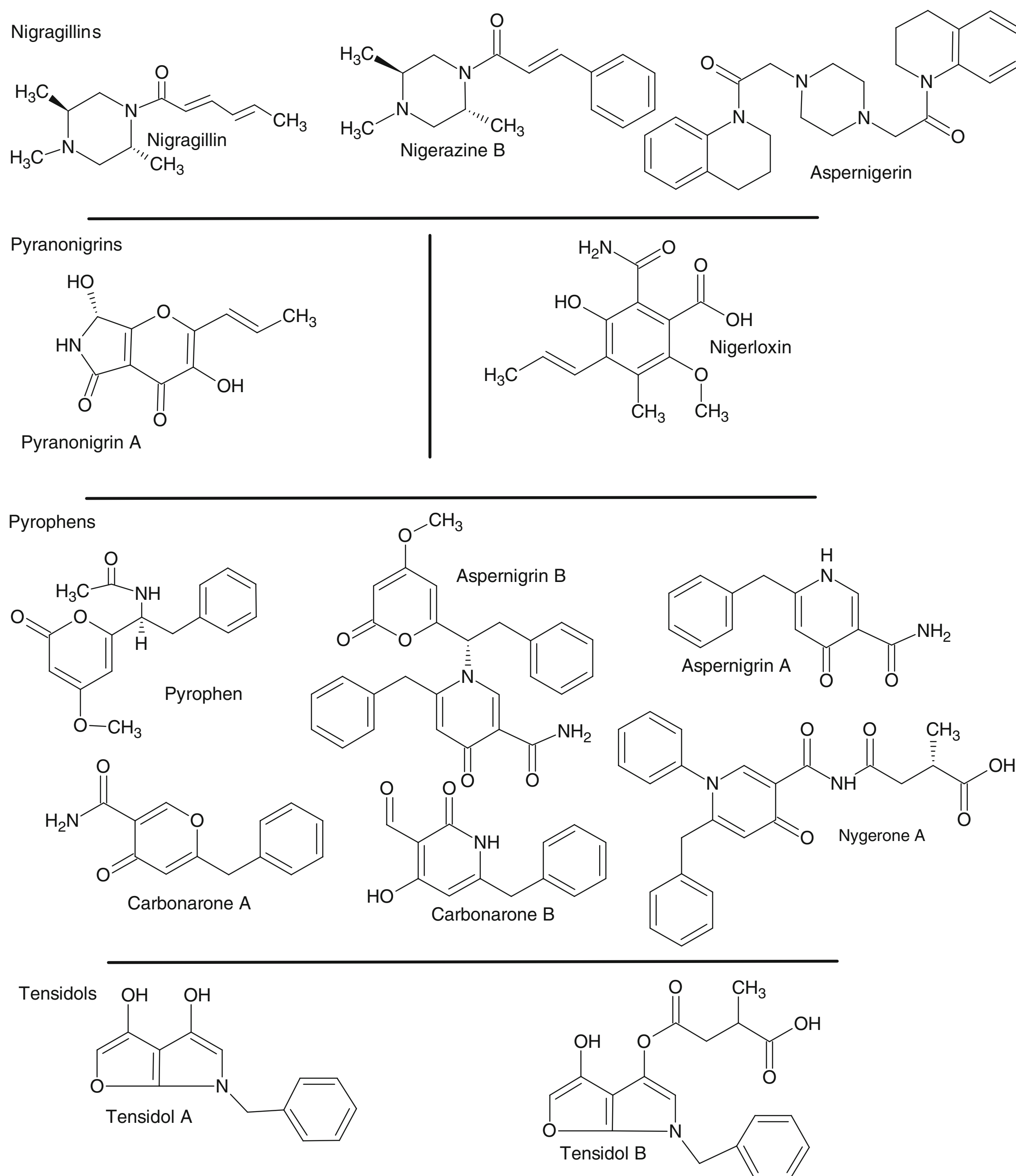

Fig. 11 Structures of the five classes of alkaloids

Other alkaloids

Species in the *A. niger* group produce a number of other nitrogen-containing compounds besides the fumonisins, ochratoxins, malformins, and asperazines. These alkaloids can be grouped into nigragillins, pyranonigrins, nigerloxin, pyrophens, and tensidols (Fig. 11).

Very few data are available for these compounds, with respect to both biological activities and which species they are found in. To our knowledge no studies exist on their presence in food and feeds, which is no surprise since they are, as is also the case for the NGPs, bicoumarins, and malformins, not commercially available.

Owing to their inherent properties as alkaloids, all of the compounds in this class have been found to ionize very well in ESI^+ and form strong $[M+H]^+$ ions, and except for the tensidols, they have very poor or no signal in ESI^-. In addition, many of them have characteristic chromophores suitable for LC-DAD.

The nigragillin-like group of alkaloids in the black aspergilli comprise nigragillin, nigerazines A and B [14, 71], aspernigrin B [57, 72], and aspernigerin [73]. Previous reports on nigragillin production within the *A. niger* group are restricted to *A. niger* (reported as *A. phoenicis*) [74]. Nigragillin was found on *A. niger* infected building materials, where the overall profile appeared similar to that of most rich-solid agar-based substrates [53].

We have found that nigragillin is easy to detect using LC-DAD-TOFMS, forming $[M+H+CH_3CN]^+$ besides $[M+H]^+$. Notably, to obtain retention in reversed-phase LC, the start gradient should not exceed more than 10% CH_3CN.

Results based on LC-DAD and LC-TOFMS show that all the species (but not all strains) in the "*A. niger* clade" produce pyranonigrins, mainly as pyranonigrin A [5]. The structure of pyranonigrin was first reported by Hiort et al. [57], who later revised it [58] and recently Schlingmann et al. [75] revised it again.

Pyranonigrin ionizes strongly in ESI^+ as $[M+H]^+$, with some $[M+Na]^+$ and $[M+Na+CH_3CN]^+$ adducts also being formed. Owing to the relatively high polarity of these compounds, it is necessary to start a chromatographic gradient in the reverse phase at maximum 10% CH_3CN to obtain proper baseline separation from other early eluted compounds.

According to our data, pyrophens and tensidols are restricted to production in *A. tubingensis* and *A. niger*. On the basis of UV data, both produce several pyrophen analogues, all with the same ion pattern of $[M+H]^+$, some $[M+Na]^+$, and $[M+NH_4]^+$ adducts. The tensidols have consistently been detected in *A. niger* and *A. tubingensis* [15], where tensidol B is produced in much higher quantities than tensidol A. They ionize strongly in ESI^+ as $[M+H]^+$, with some $[M+NH_4]^+$ and $[M+Na+CH_3CN]^+$ ions. Tensidol A loses the 2-methylbutanedioic acid moiety to form fragment m/z 230. In ESI^- $[M-H]^-$ was observed along with some $[M-H+H_2O]^-$ which must be formed via hydrolysis of one of the double bonds.

A novel analogue of pyrophen, nygerone A, was recently described by Henrikson [76] as a cryptic gene product which was induced by addition of histone inhibitors. Potentially, more new metabolites from *A. niger* or other black aspergilli could be unveiled by accessing cryptic gene clusters.

Remaining compounds

Very few terpenes have been isolated from the *A. niger* group, and they all contain a polyunsaturated chain as seen in Fig. 12. No data exist on the natural occurrence of terpenoids and investigation of biological activity has mainly been restricted to antimicrobial activities [77].

Reports of gibberellic acid [78] seem to be unlikely as the identity was only confirmed by a simple unspecific spectrometric assay. Another example is nigerloxin [79], where we have not found the compound in any extracts from the whole group when searching data files of approximately 150 extracts analyzed by LC-TOFMS for the $[M+H]^+$, $[M+Na]^+$, and $[M+H-NH_3]^+$ ions. The latter ion was included since screening of our reference standard

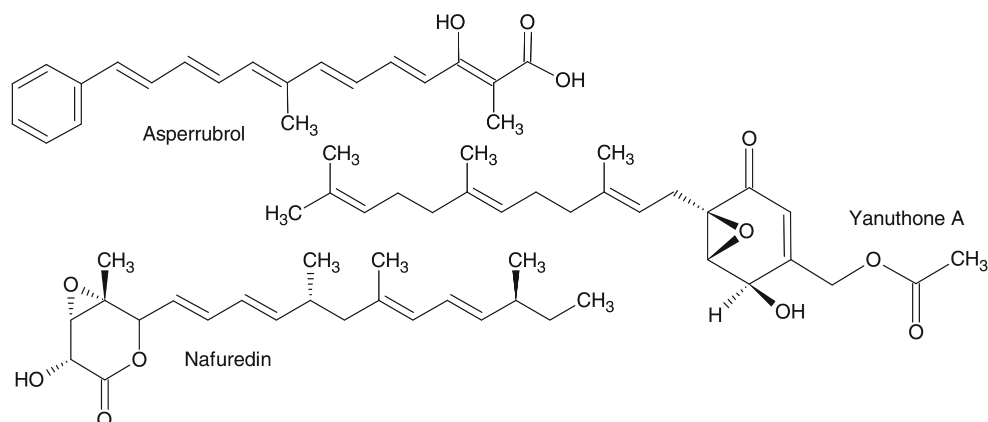

Fig. 12 Structure of selected terpenes

library has shown that NH_3 loss (using in-source fragmentation) is only observed from primary amides.

Biotransformation

This review is not intended to cover this area; however, it needs to be mentioned as some papers do not clearly state that the compounds detected from, for example, *A. niger* are actually biotransformed (small chemical changes to a complex molecule from other species) rather than fully biosynthesized by the fungus. Some of the well-known compounds derived from biotransformation are mentioned at the end of Table 2 and include compounds from animal feed or transformation of the feed material (genisteins, the flavonoids, isoflavone, pisolithin B, orobole), whereas iso-T-2 toxin is a transformation of the well-known *Fusarium* toxin T-2 toxin fed to the culture.

Analytical methods for mycotoxin screening

Summarizing the analytical observations, it appears that validated analytical methods only exist for OTA and the fumonisins. For the remaining compounds produced by the black aspergilli, the existing papers are mainly descriptions of the isolation procedure and structural elucidation, and no reference standards are commercially available. However, it should be possible to include them in an analytical scheme on a nonquantitative scale by using *A. niger* extracts for tuning MRM transitions when using triple quadrupole instruments, as, for example, is done for *F. avenaceum* metabolites in infected apples [80].

Profiles of culture extracts can also be compared with those of crude extracts of infected food and feed samples obtained using newer LC-TOFMS and Orbitrap instruments [41, 81]. These are getting increasingly more and more sensitive, and are a more interesting alternative to MRM analysis as they allow reprocessing of data files for more compounds [82]. With this approach, it is our recommendation to simultaneously monitor compounds within a series, for example, both FB_2 and FB_4, several of the malformins (A–C), or several NGPs, as compounds within a class are usually always coproduced. This will help pinpoint suspicious samples, where, for example, only one malformin is detected.

Given that these compounds will be produced in infected crops, monitoring of NGPs, tensidols, fumonisins, malformins, bicoumarins (kotanins), and asperazines should enable the detection of growth of black aspergilli to species level.

Even though LC combined with DAD and accurate mass determination is a strong tool for identifying mycotoxins and other fungal metabolites, a correct elementary composition, characteristic UV spectra, and the same elution profile [47, 83] are not sufficient for unambiguous identification of positional isomers of, for example, the NGPs or malformins. Detection in the original strain will provide a very strong tentative identification; however, for absolute identification NMR data are required. Improvements in the area of LC-NMR have made it an option even for the analytical chemist, as it is now possible to obtain data in the nanomole range [84]. Combining accurate mass determination with a few NMR recognizable features will make it possible to quickly identify positional isomers of known compounds [84].

In addition, it should be possible to quantify compounds in a fraction by NMR, since signals are proportional the number of moles in the tube. Subsequently one can calibrate against other (standardized) tubes containing accurate amounts of other compounds [85].

Conclusion

In conclusion, species within *Aspergillus* section *Nigri* are excellent producers of a large number of diverse secondary metabolites. Several new metabolites and maybe even new biosynthetic pathways are expected to be discovered in the near future now that the full genome of *A. niger* has been sequenced and soon also the full genomes of other black aspergilli will be sequenced.

Currently LC-DAD with accurate mass determination provides the easiest and most efficient strategy for tentative mapping of secondary metabolites in *A. niger* and its close relatives, especially if compared with already published chromatographic profiles, MS/MS data, and UV whole spectra. If absolute identification and positional isomer identification is needed, LC-NMR will be necessary.

For determination in food and feed, direct analysis of diluted crude extracts using LC-MS/MS analysis or LC-high-resolution mass-spectrometric detection is suggested. If sample pretreatment is needed, care should be taken when using anion exchange since *A. niger* can produce extremely high amounts of organic acids which can outsalt acidic target metabolites.

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