Discovery of aspoquinolones A–D, prenylated quinoline-2-one alkaloids from *Aspergillus nidulans*, motivated by genome mining†

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Received 17th May 2006, Accepted 17th July 2006
First published as an Advance Article on the web 10th August 2006
DOI: 10.1039/b607011f

Motivated by the observation that the *Aspergillus nidulans* genome bears multiple anthranilic acid synthase gene copies, the fungal metabolome was reinvestigated under various fermentation conditions, resulting in the discovery of novel prenylated quinolin-2-one alkaloids, two of which bear unprecedented terpenoid side chains.

Introduction

Filamentous fungi are known to be producers of a large variety of natural products, many of which have found application as important therapeutic agents. Recently, several genomes of fungi have been sequenced, unveiling the molecular basis for their diverse metabolic pathways, *e.g.* in *Aspergillus.*[1] With this valuable information at hand, it is remarkable that the wealth of potential biosynthesis genes that can be found in fungal genomes does not necessarily reflect the complexity of the observed metabolic profile under standard fermentation conditions. The occurrence of 'silent' or cryptic biosynthesis genes might suggest that a multitude of potentially useful metabolites still awaits discovery.[2] This assumption is in full agreement with the one-strain-many-compounds (OSMAC) hypothesis of Zeeck and coworkers, according to which varying growth conditions can dramatically influence the metabolite profile of bacteria and fungi.[3] Thus, mixing both genomic data and analytical techniques can be a powerful approach to the discovery of novel and potentially bioactive natural products, as has been impressively demonstrated for actinomycetes[4–6] and myxobacteria.[7,8] Yet there has been only a single communication on the discovery of novel fungal metabolites by a combined genomic and analytical approach.[9]

Results and discussion

Scanning the publicly available *Aspergillus nidulans* genome sequence[10] (GenBank) for putative biosynthesis genes revealed the presence of at least three copies of genes that probably code for proteins with high similarity to anthranylate synthases (AS). It is known that these enzymes catalyze the transformation of chorismate to anthranilic acid, a key building block in the biosynthesis of tryptophane. However, the presence of multiple copies of putative AS genes prompted us to assume that some of their gene products might be involved in secondary metabolic pathways. In fact, anthranilic acid is also known as a precursor of alkaloids, in particular in the biosynthesis of quinazoline, quinoline and acridine alkaloids. Since no such compounds have been reported for *A. nidulans*, the metabolic function of the putative AS remains to be established. The flanking gene regions did not give a hint to an encoded alkaloid pathway. For this reason, we have reinvestigated the metabolome of *Aspergillus nidulans* HKI 0410. A panel of extracts (40) prepared under 40 different culture conditions (six different media, stationary and submerged cultures and changing cultivation periods) was screened by HPLC-DAD and HPLC-UV-MS, with particular attention being given to nitrogen-containing metabolites. The extracts of the solid state fermentation on rice medium afforded a great variety of known metabolites (*e.g.* shamixanthone, variecoxanthone, sterigmatocystin) and previously unknown compounds with m/z $[M + H]^+ = 466$ and m/z $[M + Na]^+ = 506$, which appeared to be aromatic alkaloids (UV). A larger fermentation resulted in the successful isolation of aspoquinolone A (**1**) and B (**2**) (31 mg and 8.5 mg). A third fraction yielded a mixture of two closely related diastereomers, which could not be separated by any means (aspoquinolone C–D, **3**–**4**, 2 mg). The structures of the compounds were elucidated by extensive 1 and 2D NMR experiments (see ESI†).

The molecular formula of compound **1** was established to be $C_{27}H_{31}NO_6$ by HRESI-MS, which was supported by 27 signals in the ^{13}C NMR spectrum (attributable to three methyl, two methoxy, a carbonyl, a methylene, nine quaternary, two olefinic, three aliphatic and six aromatic carbons). The IR spectrum indicated the presence of hydroxyl and phenyl groups and an amido and a methylene group respectively ($\nu = 3400$, 1600, 1685 and 2970 cm^{-1}). ^1H and ^{13}C NMR data showed similarity to data obtained for penigequinolones A and B (**5**) suggesting that **1** has a 3-methoxy-4,6-dihydroxy-4-(4′-methoxyphenyl)quinolinone unit, which is substituted at position 7.[11] HMBC long range correlation between H-17 and C-6 and C-8 as well as between H-18 and C-7 confirmed the connectivity. A coupling constant of 16.6 Hz indicated an (*E*)-double bond between C-17 and C-18. The carbon at 5.9 ppm was shown to be a methylene carbon by DEPT experiments suggesting the presence of a cyclopropane ring. The H–H COSY coupling of the methylene protons H-23 with H-20 and H-21 and the HMBC long range correlation between H-23 and C-19 and C-22 established an unusual 2,2,4-trimethyl-3-oxa-bicyclo[3.1.0]hexane ring system. This finding was supported by HMBC cross peaks between H-25 and C-21 and H-24 and C-20. The spectroscopic data of compound **2** closely resembled those of **1**, suggesting that both compounds are isomers. In both

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† Electronic supplementary information (ESI) available: ^1H-NMR and HMBC spectra for **2**, HMBC data for **1**–**4**. See DOI: 10.1039/b607011f

compounds NOE correlations were observed between the protons of a methoxy group (3-OCH_3) and the proton of a hydroxyl group (4-OH), H-3 and the protons at C-12/16 and between H-3 and 4-OH. However, **1** showed a clear correlation of the methylene protons (H-23) and the methyl protons (H-24 and H-26), whereas the diastereomeric configuration of **2** was deduced from NOE interactions between H-24 and H-26, H-24 and H-20, and H-25 and H-23 (Fig. 1). Doubled signals in the ^1H and ^{13}C NMR spectra of the third fraction suggested the presence of a mixture of diastereomers (**3** and **4**, 2 : 1 ratio). From HRESI-MS and ^{13}C NMR data a molecular formula of $C_{27}H_{33}NO_7$ was deduced. Similar UV spectra as well as similar MS^n fragmentation patterns hinted that **3** and **4** possess a similar carbon skeleton to **1** and **2**, which was strongly supported by proton and carbon NMR data. The signals for the highly substituted quinolinone unit appeared at the same chemical shifts. However, compounds **3** and **4** did not show the characteristic methylene signal of the cyclopropyl moiety, but two other methylene signals (δ_C 26.4, 38.0 and 38.5) and a quaternary carbon adjacent to an oxygen atom (δ_C 71.1–71.2) instead. HMBC correlation between the methyl protons H-25 and H-26 and the CH at 85.5 ppm was observed indicating that the carbon next to C-23 must be further substituted. Since all other signals could be assigned and MS^n measurements revealed the facile loss of water, a substitution with a hydroxyl group was concluded. The H–H COSY correlation between H-20 and H-21 and HMBC long range correlations between H-21 and C-19 and between H-20 and C-22 as well as between H-18 and C-20 confirmed the connectivity of the tetrahydropyranyl ring. NOE interactions between H-3 and 3-OCH_3, H-3 and 4-OH and between H-3 and H-12 revealed the same relative stereochemistry of the 3-methoxy-4,6-dihydroxy-4-(4′-methoxyphenyl)quinolinone unit as seen for compounds **1** and **2**. The occurrence of the minor compounds **3** and **4** could give a hint on the biosynthesis of the unusual cyclopropyl units of **1** and **2**. It is very likely that the biosynthetic pathways of the known **5** and the new compounds **1–4** involve the formation of a putative bis-epoxide intermediate, which then would undergo a series of rearrangement reactions (Scheme 1). On the basis of this model, it seems more likely that the configuration of C-19 is the same in **1** and **2**, while the latter differ in the C-20/C-21 stereochemistry.

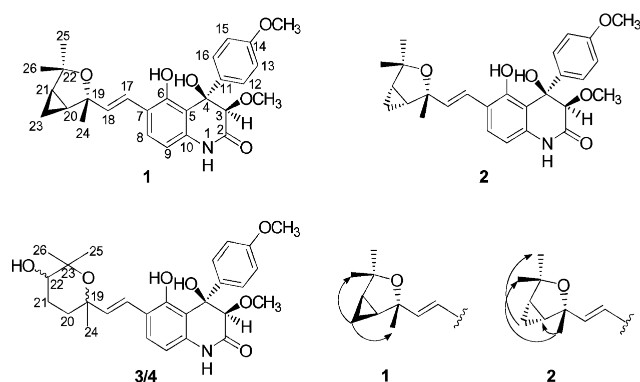
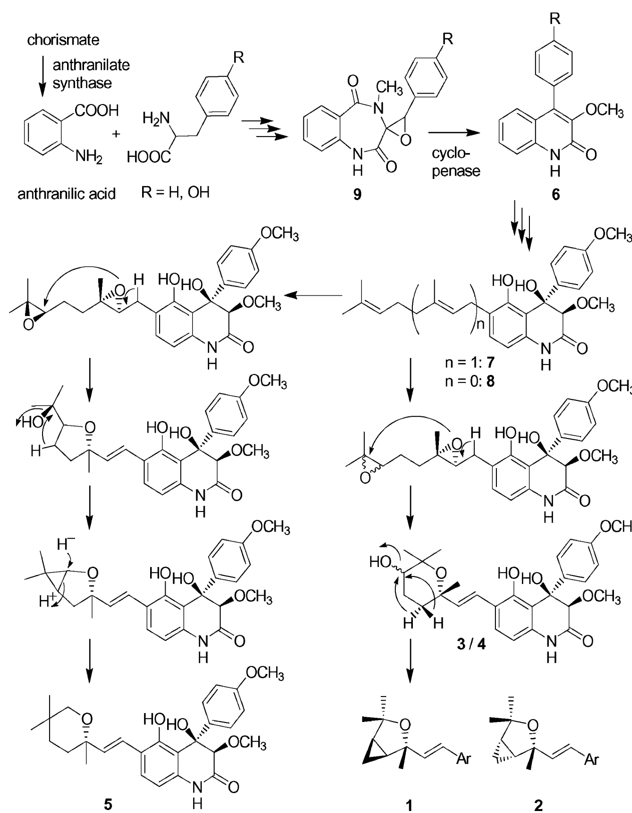


Fig. 1 Structures of aspoquinolines A–D (**1–4**) and key NOE correlations of **1** and **2**.

Quinoline alkaloids are particularly abundant in nature and have been isolated from plant, microbial and animal sources.[12]



Scheme 1 Biosynthetic scheme of fungal quinoline-2-one alkaloids involving an anthranilate synthase.

However, compared to the large range of quinolines that have been reported from plant sources, only very few examples of such compounds are known from filamentous fungi. The only representatives of fungal prenylated quinoline-2-ones are derived from 3-*O*-methylviridicatin (**6**), *i.e.* the nematicidal alkaloid penigequinolone (**5**) and the peniprequinolones (**7**, **8**) from *Penicillium* species.[13,14] Interestingly, in *Penicillium* sp., quinoline-2-one alkaloids are biosynthetically derived from benzodiazepine precursors. The formation of viridicatin by a rearrangement of the benzodiazepine cyclopenin (**9**) catalyzed by the enzyme "cyclopenase" has been demonstrated in *P. viridicatum* and *P. cyclopium* (Scheme 1).[15] It should be noted that although production of benzodiazepines has been observed in a related species,[16] to date no occurrence of prenylated quinoline-2-one derivatives has been reported for *Aspergillus.* However, as biosynthetic studies revealed, in fungi the diazepine heterocycle of **9** is assembled from phenylalanine (or tyrosine) and anthranilic acid,[17] which is synthesized by an anthranilate synthase. Thus the discovery of the novel alkaloids **1–4** clearly justifies our motivation to search for anthranilate-derived metabolites. Furthermore, we found that aspoquinolones A and B exhibit high cytotoxicity against L-929 mouse fibroblast cell lines (GI_{50} 10.6/11.4 μg ml^{-1}) and good antiproliferative effects on human leukemia cell line K-562 (GI_{50} 17.8/21.2 μg ml^{-1}).

Conclusions

In conclusion, we successfully identified four novel prenylated quinoline alkaloids from *Aspergillus nidulans* cultures by a genomic and analytical screening approach. In contrast to all known

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fungal quinolin-2-one alkaloids, the cytotoxic aspoquinolones A and B (**1** and **2**) are furnished with an unparalleled terpenoid side chain. Yet the terpenoid pyrane residues of aspoquinolones C and D (**3** and **4**) have been reported for 6,10-oxidocalepruna-1,4E-dien-9-ol.[18] The discovery of the aspoquinolones from *A. nidulans* is an important addition to the large body of metabolomic data that has been available for this well-studied organism. It implies that the fungus features a rare benzodiazepine–quinolinone pathway, which is only set on under defined growth conditions. Further investigation of the aspoquinolone pathway and its encoding biosynthesis genes is in progress in our laboratory.

Experimental

General

NMR spectra were recorded on Bruker Avance DRX 500 and DPX 300 instruments. Spectra were referenced to the residual solvent signals. HPLC-MS measurements were recorded employing a Jasco HPLC with a UV detector (UV 970) and a reversed phase C18 column (Grom Sil 100 ODS 0AB, 3 μm, 250 × 4.6 mm) with gradient elution (MeCN–H_2O 1–99 in 40 min to MeCN–H_2O 100–0, MeCN 100% for 10 min with a flow rate of 0.5 ml min^{-1}) coupled with a Finnigan LCQ benchtop mass spectrometer with an electrospray ion source and ion trap mass analyzer. HRESI-MS were recorded on a Finnigan MAT 95XL sector field mass spectrometer with a compatible ion source. IR and UV spectra were obtained using an FTIR spectrometer Satellite FTIR Mattson (Chicago, USA) and a Specord 200 photometer (Analytik Jena AG, Germany), respectively. Flash chromatography was performed using a CombiFlash® RETRIEVE system by Teledyne Isco, Inc., Lincoln, USA with 120 g Redi*Sep*™ silica columns. Analytical HPLC was performed on a Shimadzu HPLC system consisting of an autosampler, high pressure pumps, column oven and DAD. HPLC conditions: C18 column (Grom Sil 100 ODS 0AB, 3 μm, 250 × 4.6 mm) and gradient elution (MeCN 0.1%–TFA 1–99 in 30 min to MeCN 0.1%–TFA 100–0, MeCN 100% for 10 min), flow rate 1 ml min^{-1}. Preparative HPLC was performed on a Shimadzu HPLC system with a UV detector using a Waters Spherisorb® S5ODS2 250 × 20 column (flow rate 12 ml min^{-1}, detection 232 nm).

Cultivation and extraction

Aspergillus nidulans HKI 0410 was grown on rice medium for seven weeks. Medium: polished rice (250 g) was soaked in distilled water (2 h) and placed in 1000 ml Erlenmeyer flasks. Sufficient water (*ca.* 100 ml) was added to moisten the rice. After sterilization eight flasks were inoculated with the fungus and kept at 25° C for 7 weeks. The mouldy rice was then extracted with chloroform. The combined extracts were concentrated under reduced pressure.

Isolation and characterization of aspoquinolones

The crude extract was subsequently separated by flash chromatography on silica gel with chloroform–methanol mixtures of increasing polarity as eluents giving 17 fractions. Fraction six was further purified by SEC on Sephadex® LH-20 (chloroform–MeOH 90–10) and preparative HPLC (gradient mode MeCN–H_2O 1–99 in 30 min to MeCN–H_2 O 83–17, MeCN 83% for 10 min). Thus compounds **1** and **2** were obtained as a mixture which was finally separated by a second step of preparative RP-HPLC (isocratic mode MeCN–H_2O 50–50). A third fraction was isolated as a white amorphous powder which showed a single peak on the HPLC (RP18 column, MeCN–0.1% TFA), however, ^{1}H NMR and ^{13}C NMR data indicated the presence of two closely related diastereomers (compounds **3** and **4**).

Aspoquinolone A (1). White amorphous solid: UV λ_{max} (MeOH)/nm (ε): 201 (34200), 217 (27900), 279 (15100), 323 (14000). IR (ATR, solid) ν_{max}/cm^{-1} 3400, 2970, 2935, 1685, 1600, 1509. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H [ppm] (*J* [Hz]) 0.46 (2 H, m, 23-H), 1.20 (3 H, s, 26-H), 1.29 (3 H, s, 24-H), 1.30 (3 H, s, 25-H), 1.45 (1 H, m, 21-H), 1.77 (1 H, m, 20-H), 3.58 (3 H, s, 3-OCH_3), 3.67 (1 H, d, *J* 1.5, 3-H), 3.74 (3 H, s, 14-OCH_3), 4.51 (1 H, s, 4-OH), 6.30 (1 H, d, *J* 8.2, 9-H), 6.39 (1 H, d, *J* 16.6, 18-H), 6.80 (2 H, d, *J* 8.9, 13-H, 15-H), 6.80 (1 H, d, *J* 16.6, 17-H), 7.16 (2 H, d, *J* 8.8, H-12, H-16), 7.30 (1 H, br s, NH), 7.34 (1 H, d, *J* 8.3, 8-H), 9.07 (1 H, s, 6-OH). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C [ppm] 5.9 (C-23), 26.0 (C-24), 26.0 (C-26), 26.2 (C-20), 28.3 (C-21), 29.2 (C-25), 55.3 (14-OCH_3), 58.8 (3-OCH_3), 78.8 (C-4), 81.4 (C-22), 82.5 (C-19), 84.3 (C-3), 106.7 (C-9), 110.8 (C-5), 114.3 (C-13/C-15), 121.0 (C-17), 122.3 (C-7), 127.5 (C-8), 127.8 (C-12/C-16), 129.1 (C-11), 134.1 (C-10), 137.7 (C-18), 155.3 (C-6), 160.3 (C-14), 165.2 (C-2). (+)-ESI-MS m/z 466 $[M + H]^+$, m/z 488 $[M + Na]^+$. HRESI-MS: m/z $[M + H]^+$ = 466.2217 (calcd. for $C_{27}H_{32}NO_6$ 466.2204).

Aspoquinolone B (2). White amorphous solid: UV λ_{max} (MeOH)/nm (ε): 201 (33000), 217 (27100), 279 (14400), 323 (13200). IR (ATR, solid) ν_{max}/cm^{-1} 3400, 2970, 2935, 1685, 1600, 1509. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H [ppm] (*J* [Hz]) 0.40 (1 H, m, 23-Hα), 0.47 (1 H, m, 23-Hβ), 1.21 (3 H, s, 25-H), 1.41 (3 H, s, 26-H), 1.49 (3 H, s, 24-H), 1.56 (1 H, m, 21-H), 1.60 (1 H, m, H-20), 3.58 (3 H, s, 3-OCH_3), 3.66 (1 H, d, *J* 1.5, 3-H), 3.74 (3 H, s, 14-OCH_3), 4.49 (1 H, s, 4-OH), 6.19 (1 H, d, *J* 16.2, 18-H), 6.28 (1 H, d, *J* 8.2, 9-H), 6.74 (1 H, d, *J* 16.2, 17-H), 6.80 (2 H, d, *J* 8.9, 13-H, 15-H), 7.15 (2 H, d, *J* 8.9, H-12, H-16), 7.25 (1 H, br s, NH), 7.34 (1 H, d, *J* 8.2, 8-H), 9.06 (1 H, s, 6-OH). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C [ppm] 7.2 (C-23), 26.6 (C-25), 28.1 (C-20), 28.7 (C-24), 29.0 (C-21), 30.2 (C-26), 55.3 (14-OCH_3), 58.8 (3-OCH_3), 78.8 (C-4), 81.2 (C-22), 83.3 (C-19), 84.3 (C-3), 106.6 (C-9), 110.7 (C-5), 114.3 (C-13/C-15), 121.1 (C-17), 122.4 (C-7), 127.9 (C-8), 127.9 (C-12/C-16), 129.1 (C-11), 134.0 (C-10), 134.3 (C-18), 155.4 (C-6), 160.3 (C-14), 165.2 (C-2). (+)-ESI-MS m/z 466 $[M + H]^+$, m/z 488 $[M + Na]^+$. HRESI-MS: m/z $[M + Na]^+$ = 488.2060 (calcd. for $C_{27}H_{31}NO_6Na$ 488.2044).

Aspoquinolone C/D (3/4). White amorphous solid: UV λ_{max} (MeOH)/nm (ε): 201 (30600), 279 (11800), 323 (10600). IR (ATR, solid film) ν_{max}/cm^{-1} 2991, 2950, 1748, 1700, 1509. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H [ppm] (*J* [Hz]) 1.12 (3 H, s, 26-H), 1.22/1.24 (3 H, s, 25-H), 1.38/1.39 (3 H, s, 24-H), 1.72 (1 H, m, 20-Hα), 1.88 (2 H, m, H-21), 1.98 (1 H, m, 20-Hβ), 3.59/3.58 (3 H, s, 3-OCH_3), 3.67 (1 H, d, *J* 1.5, 3-H), 3.74 (3 H, s, 14-OCH_3), 3.83/3.86 (1 H, m, H-22), 4.51/4.50 (1 H, s, 4-OH), 6.25/6.24 (1 H, d, *J* 16.6, 18-H), 6.30 (1 H, d, *J* 8.2, 9-H), 6.74 (1 H, d, *J* 16.6, 17-H), 6.80 (2 H, d, *J* 8.9, 13-H, 15-H), 7.15 (2 H, d, *J* 8.8, H-12, H-16), 7.30 (1 H, br s, NH), 7.34 (1 H, d, *J* 8.3, 8-H), 9.08/9.10 (1 H, s, 6-OH). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C [ppm] 24.2/24.4 (C-26), 26.4 (C-21), 27.3 (C-25), 27.4/27.5 (C-24), 38.0/38.5 (C-20), 55.3 (14-OCH_3), 58.8

(3-OCH_3), 71.1/71.2 (C-23), 78.8 (C-4), 83.2/83.0 (C-19), 84.2 (C-3), 85.5/85.6 (C-22), 106.7 (C-9), 110.8 (C-5), 114.3 (C-13/C-15), 120.8/121.1 (C-17), 121.9 (C-7), 127.9 (C-8), 127.9 (C-12/C-16), 129.0 (C-11), 134.1/134.3 (C-10), 135.3/135.5 (C-18), 155.4 (C-6), 160.3 (C-14), 165.2 (C-2). (+)-ESI-MS m/z 506 $[M + Na]^+$, (−)-ESI-MS m/z 482 $[M - H]^-$, HRESI-MS: m/z $[M + Na]^+$ = 506.2180 (calcd. for $C_{27}H_{33}NO_7Na$ 506.2149).

Acknowledgements

We are grateful to the EC for generous funds in the FP5 EUKETIDES programme. We thank M.-G. Schwinger for strain cultivation, Dr F. A. Gollmick and F. Rhein for NMR measurements, and Dr H.-M. Dahse for cytotoxicity assays.

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