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The 3,4-dioxygenated 5-hydroxy-4-aryl-quinolin-2(1*H*)-one alkaloids. Results of 20 years of research, uncovering a new family of natural products†

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Aspergillus and *Penicillium* are fungal species known to produce a high diversity of secondary metabolites, many of them endowed with interesting bioactivity. The small but steadily growing family of the naturally occurring 5-hydroxy-4-aryl-quinolin-2(1*H*)-one alkaloids and closely related compounds, which represent the results of various research projects that spanned over 20 years and involved scientists from different continents, are covered here. Emphasis is placed on the isolation and chemical structures of the different compounds, together with their source microorganisms, environmental conditions, country or region of origin, and relevant biological activities. In addition, stereochemical aspects, as well as the proposed biosynthetic pathways for the different members, and the incipient synthetic efforts towards some of the compounds or their key intermediates, are discussed in detail.

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1 Introduction

Historically, the interest in natural products stemmed mainly from their potential as leads for new active pharmaceutical agents[1] or novel valuable materials.[2] More recently, however, they also became a focus to inspire the development of new chemical reagents[3] and synthetic strategies.[4]

Fungi live in complex ecosystems where they have to compete, collaborate and communicate with other organisms without being able to flee from conditions threatening their own existence. Their survival after millions of years of evolution is strongly related to the chemicals they produce;[5] the latter can act as chemical signposts to defend the habitat,[6] serve as signals for communication,[7] or can be supposedly used as weapons to inhibit the growth of competitors.[8]

Filamentous fungi like *Penicillium* and *Aspergillus* are known to be the source of an ample diversity of secondary metabolites.[9] Although a multitude of natural products have been identified during the last decades it is obvious that a plethora of compounds produced by these species of fungus is still awaiting discovery.

The family of 5-hydroxy-4-aryl-quinolin-2(1*H*)-one alkaloids (produced by species of *Penicillium* and *Aspergillus*) and closely related compounds, is reviewed here, with emphasis on the

isolation and structural elucidation of the natural products including stereochemical aspects. Their source microorganisms and relevant biological activities, as well as the proposed biosynthetic pathways, and the synthetic efforts towards some of the compounds or their key intermediates are also discussed.

2 Isolation of the natural products

Quinoline alkaloids are particularly abundant in Nature and have been isolated from plant, microbial and animal sources.[10] Notably, however, the number of examples known to originate from filamentous fungi is small, especially when compared to the large range of quinolines that have been reported from plant origin.

The 3,4-dioxygenated 5-hydroxy-4-aryl-quinolin-2(1*H*)-one alkaloids constitute a relatively new and steadily growing family of natural products, isolated from both terrestrial and marine fungi, of the genera *Aspergillus* and *Penicillium*, that has been uncovered piecewise during the last 20 years of research.

Although naturally-occurring quinolin-2(1*H*)-one derivatives have been known long before, including those from fungal origin and precursors of the 3,4-dioxygenated 5-hydroxy-4-aryl-quinolin-2(1*H*)-one alkaloids, the history of the latter began to take form by the middle of the 1990s, when Nakaya examined the toxicity of substances produced by *Penicillium* sp. NTC-47 toward brine shrimp (*Artemia salina*), with the aim of finding new insecticides.

In 1995, upon cultivating the fungus on okara, the insoluble residue of whole soybean, this scientist disclosed the isolation of two unique heterocycles (Fig. 1), which were designated as NTC-47A (**1**) and NTC-47B (**2**).[11]

This report on the first two members of the then unknown family of 5-hydroxy-4-aryl-quinolin-2(1*H*)-one alkaloids, did not provide spectroscopic information on the isolated compounds nor information related to the stereochemistry of their 3,4-monoprotected glycol feature; oddly, NTC-47A was proposed to contain an unprecedented ten-membered unsaturated cyclic phenyl ether. This author also prepared a dihydro-derivative of NTC-47A by catalytic hydrogenation of the styrenic double bond of NTC-47A, and a dehydro-NTC-47A by dehydration of the natural product. Structures of these two derivatives were not given.

By the same time, Kimura and co-workers were studying fungal metabolites as growth inhibitors of the tea pollen grains[12] of *Camellia sinensis* O. Kuntze.[13]

The aim of their research was to provide useful hints toward the development of new herbicides, and also contribute suitable tools for the analysis of the reproductive functions in higher plants.[14]

As a result, in 1996 this group disclosed the isolation of the penigequinolones A (**3**) and B (**4**) from the mycelial mats of a strain of *Penicillium* sp. no. 410 of undisclosed origin. This 2 : 1 mixture of diastereoisomeric compounds ($[\alpha]_D = +60°$, $c = 1.0$,

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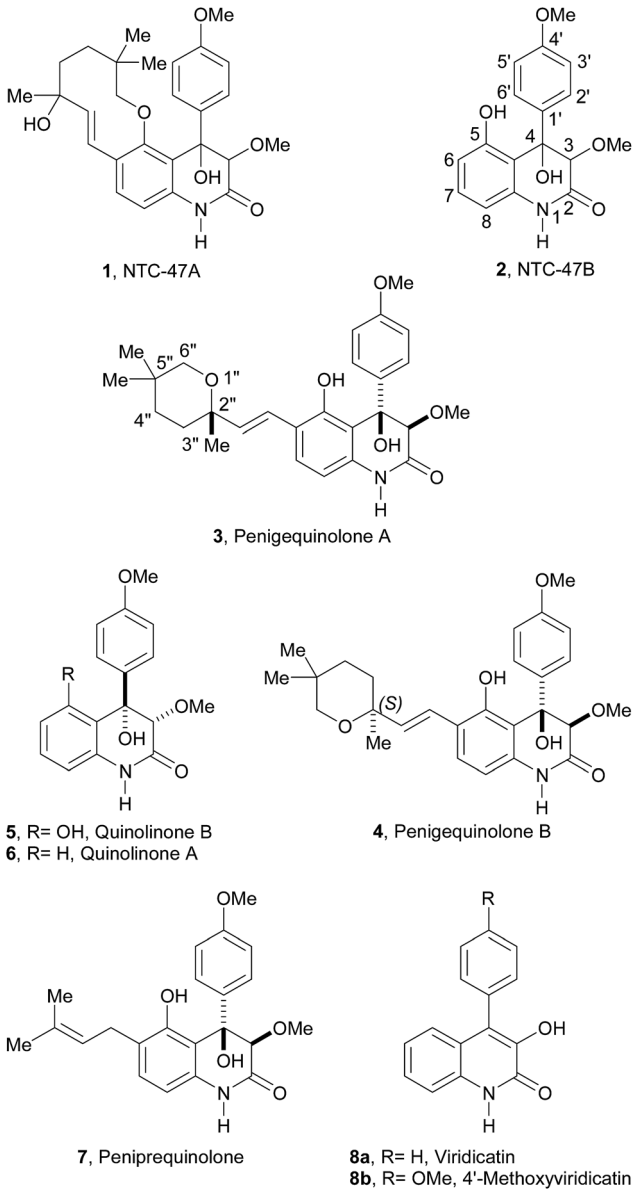


Fig. 1 Chemical structures of compounds **1**–**8b**.

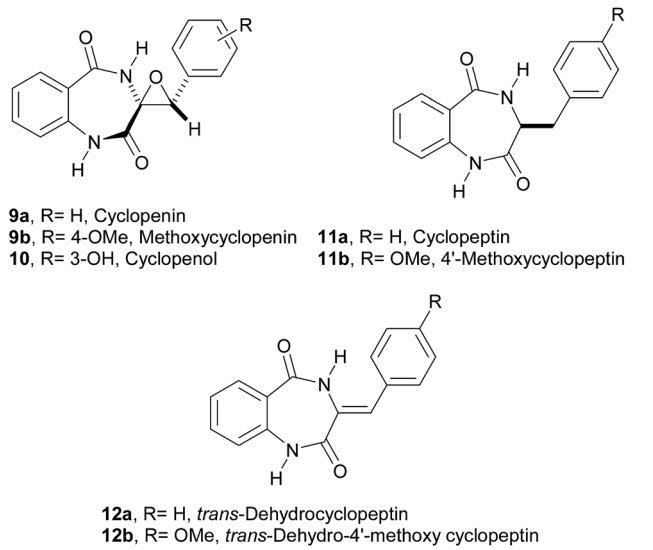


Fig. 2 Chemical structures of cyclopenin (**9a**), methoxy cyclopenin (**9b**), cyclopenol (**10**), and the cyclopeptin derivatives (**11a,b** and **12a,b**).

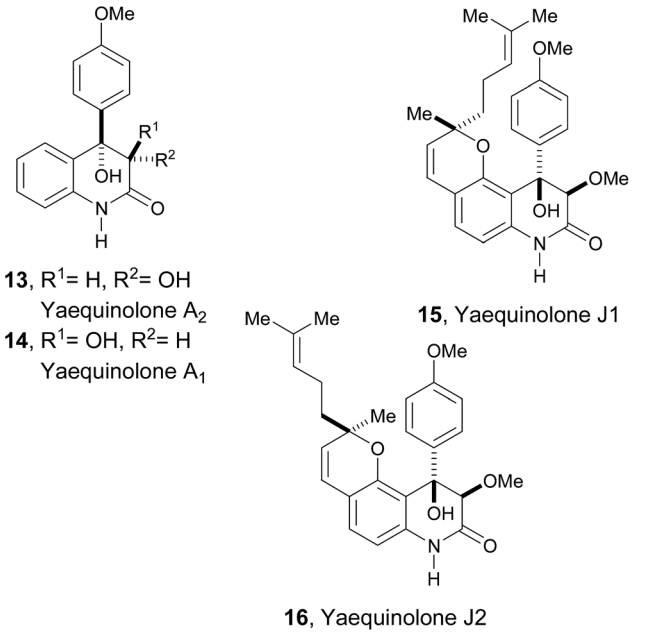


Fig. 3 Chemical structures of the yaequinolones A2 (**13**), A1 (**14**), J1 (**15**) and J2 (**16**).

MeOH) bears a ten carbon isoprenoid side chain featuring a unique 1,4-tetrasubstituted tetrahydropyran ring, which does not seem to have been found previously among natural products.

Interestingly, one of these diastereomeric penigequinolones was detected by LC-MS analysis during a screen of common Danish building materials, for their resistance to colonization by microfungi at various temperatures and humidity levels, with no identifiable apparent producer.[15]

Almost concomitantly, and unaware of the above investigations, studies of a strain of *Penicillium* sp. NTC-47 isolated form a soil sample and toxic to brine shrimp, were carried out with the aim of obtaining insecticidal compounds. Fermentation with okara resulted in 1997 in the isolation of two new levorotatory dihydroquinolinones, to which structures **6** $\{[\alpha]_{20}^{D} = -62$ ($c = 0.3$, MeOH)$\}$ and **5** $\{[\alpha]_{20}^{D} = -55$ ($c = 0.02$, MeOH)$\}$, were assigned based on their spectral data;[16] these compounds were later known as quinolinone A and B, respectively.[17] Taking into account that NTC-47B (**2**) was previously reported as being produced by *Penicillium* sp. NTC-47 fermented with okara, it is not unreasonable to suppose that **5** should be identical with NTC-47B.

P. scabrosum was described as a new species by the group of Frisvad.[18] Morphologically and physiologically, it resembles *P. atrovenetum*; however, both species can be distinguished chemotaxonomically because they do not share secondary metabolites. However, this fungus had been reported to produce viridicatin (**8**), the related compounds cyclopenin (**9**) and cyclopenol (**10**), among other unknown metabolites. Shortly

after, in 1999 it was shown to consistently produce penigequinolones A and B (**3** and **4**).[19]

During a search for new nematicidal agents, Kusano and coworkers studied the culture metabolites of *Penicillium cf. simplicissimum* (Oudemans) Thom.[20] In 2000, they succeeded in isolating peniprequinolone (**7**), along with the known penigequinolones A and B (**3** and **4**),[16] as well as quinolinone A (**6**) {$[\alpha]_{20}^{D} = -54.7$ ($c = 0.5$, $CHCl_3$)} and a heterocycle to which the structure of quinolinone B (**5**) was assigned. However, the relatively lower $[\alpha]_D$ value observed {$[\alpha]_{20}^{D} = +17.1$ ($c = 0.2$, EtOH)} casts some doubts about the purity or relative stereochemistry of the C-3 and C-4 centers of the latter.

In addition, the benzodiazepine derivatives **9**,[21] cyclopeptin (**11a**) and the 4′-methoxy analogue **11b**, with the dehydrogenated compounds **12a, b** were concomitantly isolated (Fig. 2).

Several new members of this family of heterocycles were unearthed in 2005 from *Penicillium janczewskii* Zalessky H-TW5/869, isolated from surface water collected from the German Bight (North Sea), northeast of the island of Heligoland. A German team directed by Sattler reported the isolation of a known compound (Fig. 3), quinolinone A (**6**), along with two new diastereomeric levorotatory quinolinone diols (**13** and **14**),[22] later designated by the group of Tomoda as yaequinolones A2 and A1, respectively.[23]

These C-6 unsubstituted heterocycles were isolated along with the known peniprequinolone (**7**). This constituted the first isolation of **5** and **7** from a marine-derived fungus, and although the enantiomer of **7** was depicted in the publication, no optical rotation data of the latter pair of compounds were provided.

In addition, the group of Schmeda-Hirschmann isolated in 2005 *Penicillium janczewskii* K. M. Zalessky from the phloem of the Chilean gymnosperm *Prumnopitys andina* (Poepp. ex Endl.) de Laub (Lleuque), Podocarpacea.[24] When cultured in potato-dextrose medium, among the metabolites isolated from this endophytic fungus, in 2005 these authors identified peniprequinolone (**7**), the specific rotation and NMR spectral data of which fully agreed with those previously reported by Kusano.[20]

Further, the group of Tomoda announced in 2006 the discovery of the epimeric yaequinolones J1 (**15**) and J2 (**16**), two novel *p*-methoxyphenyl quinolinone alkaloids fused with an isoprenyl pyran ring. The source of these angular tricycles was the strain *Penicillium* sp. FKI-2140, isolated from a soil sample collected at Ishigakijima Island, Okinawa Prefecture, Japan.[23] These and their congeners were termed antibiotics FKI-2140 A–G.

The unearthing of novel metabolites by a combined genomic/analytical approach is a widely known strategy in actinomycetes[25] and myxobacterial[26] research. Today, it is also one of the most recent approaches[27] toward the discovery of novel bioactive fungal compounds; however, ten years ago its use oriented to this purpose was rather uncommon.

Scherlach and Hertweck scanned the publicly available (GenBank)[28] genome sequence of *Aspergillus nidulans* for putative biosynthesis genes.[29] This study revealed the presence of at least three copies of genes that, as it was conjectured, probably code for proteins which are highly similar to anthranilate synthases (AS).

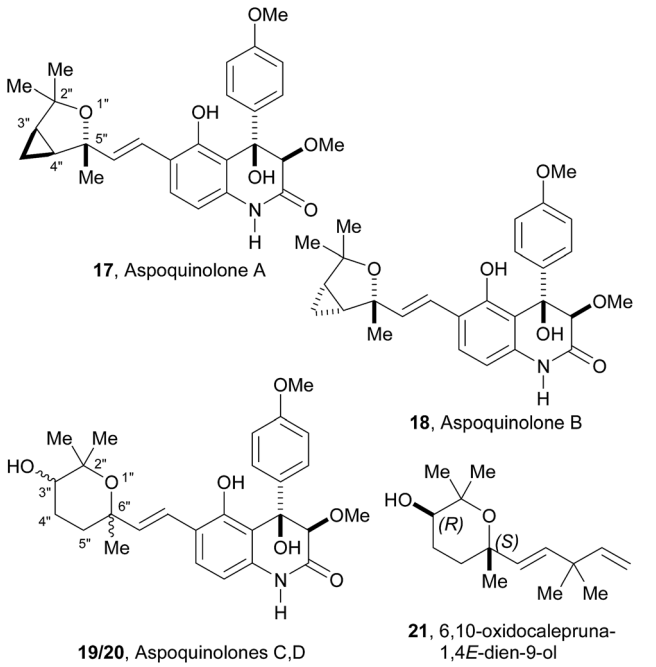


Fig. 4 Chemical structures of the aspoquinolones A–D (**17–20**) and compound **21**.

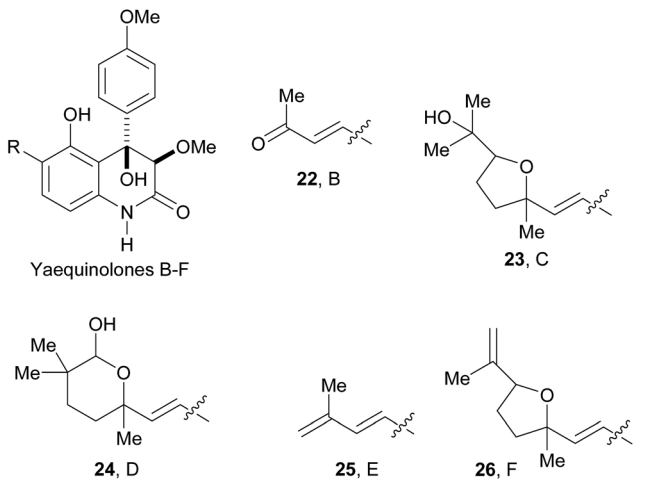


Fig. 5 Chemical structures of the yaequinolones B–F (**22–26**).

The enzyme product (anthranilic acid) is a known precursor in the biosynthesis of alkaloids, including quinolones.

For this reason, they reinvestigated the metabolome of *Aspergillus nidulans* HKI 0410, submitted to solid state fermentation on rice medium, in search of new secondary metabolic pathways and their products. Thus, Scherlach and Hertweck prepared a panel of 40 extracts from 40 different culture conditions (comprising six different media, stationary and submerged cultures and changing cultivation periods) and screened, employing HPLC-DAD and HPLC-MS techniques, for nitrogen-containing metabolites of the fungus.

Their motivation to search for anthranilate-derived metabolites was crowned with the isolation of several new compounds

(Fig. 4), namely the aspoquinolones A (**17**) and B (**18**), along with a mixture of two closely related but inseparable diastereomers, the aspoquinolones C–D (**19**/**20**).

Interestingly, compounds **17–20** (Fig. 4) were the first prenylated quinolones isolated from *Aspergillus* species. A series of aspoquinolones were also isolated from *A. nidulans* DSM 17772 and described in a German patent.[30] Furthermore, a terpenoid pyran residue analogous to the side chain of the aspoquinolones C and D has been reported for the natural product 6,10-oxidocalepruna-1,4*E*-dien-9-ol (**21**).[31]

The group of Tomoda employed the brine shrimp test to screen for insecticidal agents. Studying *Penicillium* sp. FKI-2140, from a soil isolate of subtropical Okinawa, Japan, in 2006 they reported the isolation of several new compounds (Fig. 5), namely the yaequinolones B–F (**22–26**).[17,32] These heterocycles share the fundamental *p*-methoxyphenyl quinolin-2-one skeleton and C-6 side chain containing an isoprenyl derived (C_5 or C_{10}) unit.

In addition, they re-discovered the yaequinolones J1 and J2 (**15** and **16**), as well as yaequinolones A1 (**14**) and A2 (**13**), and other related and known compounds were identified, including the quinolinones A (**6**) and B (**5**), peniprequinolone (**7**), and the mixture of penigequinolones A and B (**3** and **4**).

Intermediates such as 4′-methoxycyclopeptin (**11a**/**b**)[20] and *trans*-dehydro-4′-methoxycyclopeptin (**12a**)[32b] were also reported. The production of a total of 13 compounds including the yaequinolones and other related heterocycles, produced by fermentation of *Penicillium scabrosum* CBS-305.97 was also presented in the patent literature.[33]

The group of Gloer obtained a culture of *Aspergillus* sp. (section Flavipedes; MYC-2048 = NRRL 58570) from the basidioma of the saprophytic plant pathogen *Rigidoporus microspores*, found on a dead hardwood branch in a Hawaiian alien wet forest. After the fungus was cultured by solid-substrate fermentation on rice, and the fermentation mixture was extracted with EtOAc, the extract exhibited antifungal activity against *Aspergillus flavus* (NRRL 6541) and *Fusarium verticillioides* (NRRL 25457), also reducing the growth rate of *Spodoptera frugiperda* (fall armyworm).[34]

Fractionation of the extract resulted in the isolation of several known compounds and two new dihydroquinolin-2(1*H*)-ones (Fig. 6), designated as aflaquinolones A (**27**) and B (**28**). Interestingly, five additional heterocycles (aflaquinolones C–G, **29–33**, respectively) were reported in the same paper, as obtained from an isolate of the marine *Aspergillus* sp. SF-5044 collected in Korea.[34]

The aflaquinolones A–D (**27–30**) lack the 4′-methoxy moiety and their C-6 side chain features different 2,4-dimethyl cyclohexanone/cyclohexanol moieties, with an ethylene motif linking both cycles, resulting from cyclization of a rearranged C_{10} isoprenoid motif. Aflaquinolone B (**28**), which displays the cyclohexanol ring, represents a reduced product of aflaquinolone A (**27**) at its ketonic carbonyl.

On the other side, aflaquinolone E (**31**) lacks the C-6 side chain whereas the remaining congeners seem to be precursor molecules, also missing the C-5 hydroxyl group. Interestingly, aflaquinolone G (**33**) exhibited a *trans* 3,4-diol pattern.

Further members of this family, lacking the 4′-methoxy group, were reported in 2013.[35] They were isolated from *Aspergillus nidulans* MA-143, an endophytic fungal strain obtained from the fresh leaves of the mangrove plant *Rhizophora stylosa* (stilted mangrove), the ubiquitous mangrove of Australia and also very common in the Indonesian Archipelago. The extract of

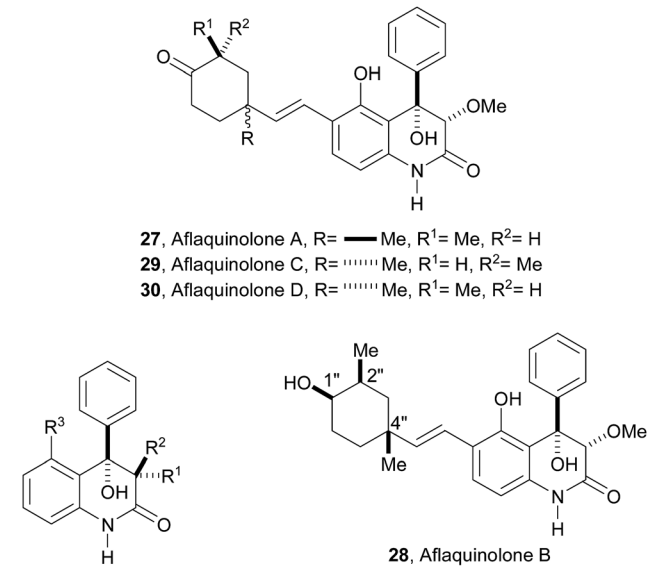


Fig. 6 Chemical structures of the aflaquinolones A–G (**27–33**).

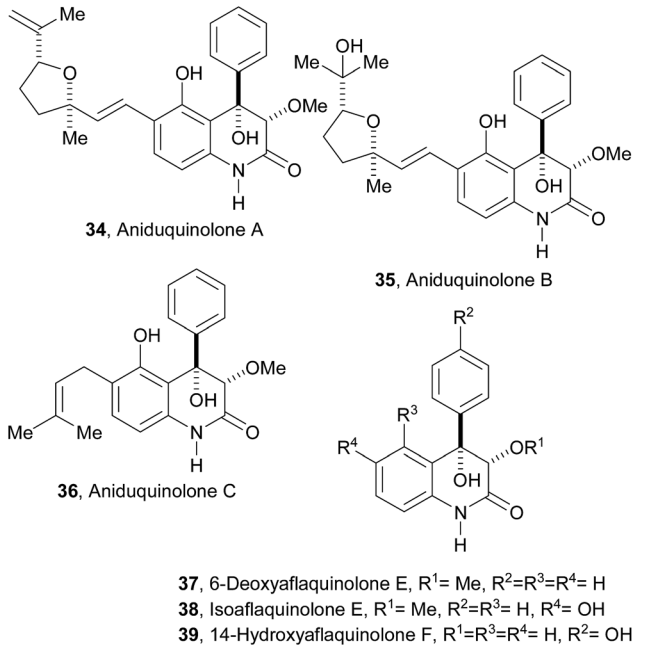
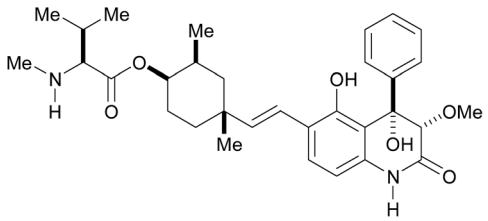
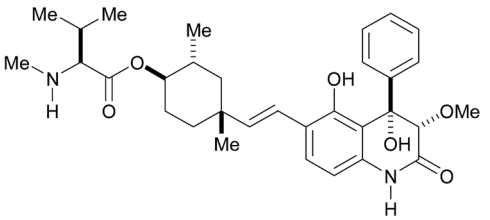


Fig. 7 Chemical structures of the aniduquinolones A–C (**34–36**), 6-deoxyaflaquinolone E (**37**), isoaflaquinolone E (**38**), and 14-hydroxyaflaquinolone F (**39**).



40, 22-*O*-(*N*-Me-L-valyl)aflaquinolone B



41, 22-*O*-(*N*-Me-L-valyl)-*epi*-aflaquinolone B

Fig. 8 Chemical structures of 22-*O*-(*N*-Me-L-valinyl)aflaquinolone B (**40**) and 22-*O*-(*N*-Me-L-valinyl)-21-*epi*-aflaquinolone B (**41**).

this fungal strain was lethal to brine shrimp and also exhibited antibacterial activity.

Extract fractionation afforded the previously known aflaquinolone A (**27**) and six new 4-phenyl-3,4-dihydroquinolone derivatives (Fig. 7), designated as aniduquinolones A–C (**34–36**), 6-deoxyaflaquinolone E (**37**), isoaflaquinolone E (**38**), and 14-hydroxyaflaquinolone F (**39**).

Aniduquinolone C can be regarded as a 4′-demethoxy analogue of peniprequinolone (**7**), whereas the aniduquinolones A (**34**) and B (**35**) carry the same type of C-6 side chain as that found in the yaequinolones F (**26**) and C (**23**), respectively. On the other hand, isoaflaquinolone E (**38**) exhibits a 6-OH group instead of the characteristic hydroxyl moiety on C-5, deoxyaflaquinolone E misses the 5-OH and 14-hydroxyaflaquinolone F displays a 4′-OH motif, which is rare among these compounds.

In addition, during 2013 the group of Staerk employed the high-resolution radical scavenging profile of an extract of the endophytic fungus *Penicillium namyslowskii* for the identification of anti-oxidative secondary metabolites by high-performance liquid chromatography-high-resolution mass spectrometry-solid-phase extraction-nuclear magnetic resonance spectroscopy (HPLC-HRMS-SPE-NMR).[36] The strain of *P. namyslowskii* was isolated from the flowering plant *Rhododendron tomentosum* Harmaja (Marsh Labrador tea). The plant is used as an ingredient in Labrador tea and its extracts have been shown to repel mosquitos.[37]

Unexpectedly, one of the chromatographic peaks with the highest relative response in the radical scavenging profile belonged to peniprequinolone (**7**), and no other analogues or biosynthetic precursors/successors of this compound were found in the extract.[36]

In 2014 the group of Wang reported the isolation of two 22-*O*-(*N*-Me-L-valyl)aflaquinolone B (**40**) and 22-*O*-(*N*-Me-L-valyl)-21-*epi*-aflaquinolone B (**41**) as new prenylated dihydroquinolone derivatives (Fig. 8), from the mycelia of *Aspergillus* sp. XS-20090B15 derived from the gorgonian *Muricella abnormaliz* collected from the South China Sea, cultured on a rice medium. These were obtained along with aflaquinolones A (**27**) and D (**30**, or a diastereomer of it).[38]

Attached to the C-6 terpenoid side chain, these new compounds contain an *N*-methyl-L-valinyl residue, connected through an ester bond. They represent the first examples of prenylated dihydroquinolone derivatives containing an amino acid residue in their C-6 side chain.

Finally, in 2016 Barkal and co-workers introduced a class of microscale culture platforms to analyze chemical diversity of fungal and bacterial secondary metabolomes. This approach facilitated the exploration of culture microenvironments (including rare media), the use of unusual organic solvents for metabolite isolation and microbial mutants.

Employing *Aspergillus nidulans* and *A. fumigatus* as models, the effects of culture geometry and growth matrix on secondary metabolism were characterized, highlighting the potential of the system for natural products discovery. In this way, heterocycles presumed to be the aspoquinolones A and B were detected in glucose-minimum medium cultures of *A. nidulans*.[39]

3 Stereochemical elucidation and assignment

Kimura and co-workers[13] were the first to propose the relative stereochemistry of a member of this family, on a diastereomeric mixture of penigequinolones (**3** and **4**). In selective PFG-1D-ROESY experiments, ROEs were observed between the protons of the 3-OMe group and 4-OH, as well as between H-3 and 4-OH, and the *ortho* aromatic protons of the *p*-methoxyphenyl moiety (H-2′ and H-5′). These results, together with the fact that no ROE was observed between the protons of the 3-OMe group and H-2′/H-5′, suggested a stereochemistry where both oxygenated functions were oriented in a *syn*-periplanar way (3*R**,4*R**).

These experiments also enabled to propose that in both diastereomers, the conformation of the tetrahydropyranyl moiety was a chair carrying the chain with the double bond in an axial orientation. Although these and other[20] authors isolated a 2 : 1 mixture of diastereomers (by ^{1}H NMR), others have isolated an approximately equimolar mixture (by ^{13}C NMR).[19]

After X-ray studies, Hayashi and co-workers[16] assigned the 3*R**,4*R** configuration to quinolinone A (**6**). Accordingly, the relative configuration of the related quinolinone B (**5**) was also assumed to be 3*R**,4*R**.

NMR experiments (NOE) on peniprequinolone also suggested the 3*R**,4*R** stereochemistry. The same relative configuration (3*R**,4*R**) was proposed for the penigequinolones (**3** and **4**) and quinolinone A (**5**) after observing that the three exhibited the same Cotton effect in their CD spectra.[20] The diastereomeric penigequinolones were separated by HPLC with a chiral column; however, no special efforts were placed in order to study their configurational stability nor the configuration of the C-3″ center.[17]

The relative stereochemistries of the related levorotatory 3-OH diastereomers **13** and **14**, known as yaequinolones A2 and A1, respectively, were determined as 3*R**,4*R** and 3*S**,4*R** respectively, through conformational analysis (AM2, MMDO) and quantitative NOE data obtained from NOESY spectra. The former clearly indicated an almost perpendicular orientation of both aromatic rings and a significant rotational barrier around the C-4–C-1′ bond.[22] The concomitantly found quinolinone B (**5**) and peniprequinolone (**7**) were also drawn in this publication with the 3*R**,4*R** configuration.

The 3*R**,4*R** relative configuration was assigned to both diastereomeric yaequinolones J1 (**15**) and J2 (**16**), on the basis of their corresponding NOE signal enhancements between H-3 and H-2′. This information was employed to establish the relative configuration of the additional stereocenter of these heterocycles. Observation of NOE between H-5′ and H-4″ enabled to assign the relative *S** configuration to C-3″ in yaequinolone J1 (**15**). Analogously, the NOE enhancements observed between H-5′ and the methyl proton H-10″ suggested that the diastereomeric yaequinolone J2 (**16**) should bear the opposite, 3″*R* configuration.[23]

Regardless of the complexity of the substituent and the presence of stereogenic centers, the 6-substituted yaequinolones B–F (**22–26**) were dextrorotatory. Based on NOE experiments, the configuration of the heterocyclic ring was assumed as 3*R**,4*R**.[17] No efforts were made to elucidate the configuration of the additional chiral centers of yaequinolones D–F (**24–26**). Furthermore, yaequinolone D (**24**) exhibits an hemiacetal motif; however, contrary to the penigequinolones (**3** and **4**), only one of the possible diastereomers was isolated.[32a]

The use of a battery of analytical methods on the aflaquinolones A and B (**27** and **28**) made it possible to assign their configurations. The relative configurations of the stereocenters of the heterocycle and cyclohexanone rings were deduced from analysis of ^{1}H NMR coupling constants (*J*). However, the pairs of data could not be correlated to each other.[34]

Electronic circular dichroism (ECD) data were collected for aflaquinolone A (**27**) and matched closely with those of peniprequinolone (**7**) and quinolinone B (**5**), which lacks the C-6 side chain.[38] This similarity suggested that the shape of the ECD curve is dictated largely by the configuration of the heterocyclic moiety. Therefore, it was concluded that these three compounds should have the same absolute configuration in that part of their structure.

Both, the experimental and calculated spectra of aflaquinolone A (**27**) exhibited a positive Cotton effect (CE) below 220 nm, a negative CE near 250–260 nm and a pair of positive CE above 275 nm. This suggested a 3*S*,4*S*-absolute configuration for the heterocyclic moiety, regardless of the configuration of the stereocenters of the cyclohexanone motif. However, this approach was unable to decipher the absolute configuration of the stereocenter of the cyclohexanone portion.

Aflaquinolone B (**28**) displayed a similar ECD spectrum. However, application of Mosher's method to its secondary alcohol and careful spectral analysis of coupling constants enabled assignment of the absolute configuration of C-2″ as *S*. This observation, coupled to the fact that the shapes of the ECD curves were almost identical for the aflaquinolones A–D (**27–30**), were the key to fully elucidate their absolute stereochemistry with the help of additional ^{1}H NMR analysis.

Analogous reasoning helped to establish the absolute configuration of aflaquinolones E (**31**) and F (**32**), whereas in case of aflaquinolone G (**33**), the best match of the ECD spectrum was for a 3*R*,4*S* configuration. In these cases, a small shift of their Cotton effect frequencies was observed, mainly due to the lack of C-5 and C-6 substituents.[34]

Soon after, a quite similar approach was used by the team of Wang to settle the absolute configuration of the dextrorotatory aniduquinolones A–C (**34–36**).[35] In this case, in addition to ^{1}H NMR and NOESY studies, XRD was employed together with ECD to establish the configuration of all of the stereogenic centers of (+)-aniduquinolone A (**34**).

Analogously, according to their ECD spectra, which were almost identical to that of aniduquinolone A (**34**), the absolute configurations of the dihydroquinolone portion in the aniduquinolones B (**35**) and C (**36**) were assigned as 3*S*,4*S*. However, the absolute configuration C-6 side chain of compound **35** remains unassigned.[35]

On the other hand, NOE experiments and analysis of the CD traces, which were identical in shape to those of the aniduquinolones, except that they were blue-shifted due to the absence of olefins attached to C-6, suggested that the configurations of 6-deoxyaflaquinolone E (**37**), isoaflaquinolone E (**38**) and 14-hydroxyaflaquinolone F (**39**) were 3*S*,4*S* in the three cases.[35]

The important contribution of ECD in support of the 3*S*,4*S* configuration of the aflaquinolones and aniduquinolones implies that the congener heterocycles to which originally 3*R**,4*R** relative configurations were assigned, should be actually drawn as the corresponding enantiomeric 3*S**,4*S** derivatives, which probably better represents their absolute configuration.

Two compounds related to aflaquinolone B and bearing the same amino acid residue, were also isolated. The absolute configuration of the dextrorotatory compound **40** was determined by a combination of ECD data analysis, Marfey's method, and chemical conversion. Hydrolysis of the natural product afforded an *N*-methyl amino acid derivative and aflaquinolone B (**28**). The data of the latter were compared with the literature, whilst the absolute configuration of the so-obtained amino acid was assessed as *S* by the use of Marfey's method.

Analogously, after ECD spectral comparison, analysis of the *J* values in the ^{1}H NMR and taking into account NOESY cross-peaks, it was found that a similar compound was its epimer at C-2″.

On the other hand, comparison of spectral data suggested that two additional compounds concomitantly isolated were the aflaquinolones C (**29**) and D (**30**), because their NMR and mass spectral data matched well with the published information. However, aflaquinolone D was tentatively assigned as the 2″*S*,4″*S* isomer, but the 2″*R*,4″*R* diastereomer could not be ruled out.

Interestingly, since aflaquinolone C (**29**) has a 4″*R* configuration established by analysis of the coupling constant, NOESY data and Mosher's method, it does not seem unlikely that,

based on biogenetic considerations, aflaquinolone D (**30**) would have a 4″*R* configuration.

4 Biological activity

Most of the compounds of this family were found as a result of screens oriented toward specific bioactivities, or through bioactivity-guided fractionation of selected extracts.

Compounds NTC-47A (**1**) and NTC-47B (**2**) showed toxicity against brine shrimp (*Artemia salina*).[11] On the other hand, the mixture of diastereometic penigequinolones (**3** and **4**) inhibited the pollen-growth of *Camellia sinensis* O. Kuntze by 40% at 10 μg mL^{-1} and achieved complete inhibition at 100 μg mL^{-1}.[13] Once separated by HPLC, it was observed that both compounds exhibited toxicity in the *Artemia salina* test, with MIC values of 0.19 μg mL^{-1} for each diastereomer.[32a]

The availability of the closely related monoprotected glycols **5** and **6** (ref. 16) allowed the elaboration of some structure–activity conclusions on their toxicity toward brine shrimp. Compound **6** exhibited much lower activity (LC_{50} = 20 μg mL^{-1}) than rotenone (LC_{50} = 0.14 μg mL^{-1}) and okaramine B (LC_{50} = 0.74 μg mL^{-1}). On the other hand, **5** was inactive at a dose of 100 μg mL^{-1}, strongly suggesting that the phenolic hydroxyl is essential for the toxicity of these compounds.

The inseparable mixture of penigequinolones (**3** and **4**, 69.2% at 1000 μg mL^{-1}) and peniprequinolone (**7**, 82.4% at 1000 μg mL^{-1}) were found to be nematicidal[20] with selectivity against the root-lesion nematode *Pratylenchus penetrans*. This species parasitizes several plants, including carrot (*Daucus carota* L.), burdock (*Arctium lappa* L.) and radish (*Raphanus sativus* L. var. *acanthiformis* Makino), causing disease and crop loss. They had hardly any effect at 1000 μg mL^{-1} against the free-living nematode *Caenorahabditis elegans*. On the other hand, compound **5** exhibited lower potency (57.7% at 1000 μg mL^{-1}), suggesting the potential key role of the C-6 side chain in modulation of the bioactivity.

Both, the penigequinolones (**3** and **4**) and peniprequinolone (**7**) accelerated the root growth of rice seedlings in proportion to their concentrations, in the range 100–300 μg mL^{-1}. Compound **7** demonstrated toxicity in the *Artemia salina* test (MIC = 0.78 μg mL^{-1}).[32a] It was also found to act as a strong but non-selective cytotoxic agent,[22] exhibiting cytotoxicity against AGS (human gastric adenocarcinoma cells) cells (IC_{50} = 89 μM) and fibroblasts (IC_{50} = 116 μM).[24]

Compound **7** was also identified as an antioxidant and radical scavenger in the microplate-based high-resolution $ABTS^{\bullet+}$ [diammonium salt of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] test. Moreover, the trolox equivalent antioxidant capacity (TEAC) of peniprequinolone was 0.62 mM trolox equivalents per millimole of metabolite.[36]

The diastereomeric compounds **13** and **14** showed low to moderate general toxicity against a panel of cell lines including MDA-MB 231 (human breast adenocarcinoma), DU-145 (human prostate carcinoma), HT-29 (human colon carcinoma), A549 (human non-small cell lung carcinoma), CAKI-1 (human kidney carcinoma), SK-MEL 2 (human melanoma), and K562 (human myeloid leukemia) cells, with **13** being slightly more potent. In addition, a significantly stronger cytotoxicity against SKOV-3 cells (human ovary adenocarcinoma) was found for **13**.[22] Since structurally, **13** and **14** differ only in their relative stereochemistry, these differences in efficacy may be highlighting a structure–activity relationship.

Interestingly, quinolinone B (**5**) exhibited moderate toxicity against *A. salina* (MIC = 25 μg mL^{-1}), whereas its congener quinolinone A (**6**), which lacks OH-5, was essentially inactive (MIC = 100 μg mL^{-1}).[32a] These differences are also indicative of a structure–activity relationship.

The aspoquinolones A (**17**) and B (**18**) exhibited strong 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}).[29] The yaequinolones showed widely variable activity against *Artemia salina* (0.19–100 μg mL^{-1}), with yaequinolone F (**26**) being the most potent member of this class, followed by yaequinolones D (**24**), E (**25**), J1 (**15**) and J2 (**16**), with MIC levels of 6.25 μg mL^{-1}.[23a] The comparatively lower activity of the yaequinolones lacking the C-6 chain suggested that the presence of an isoprenyl-derived side chain in the structures should be responsible for the observed cytotoxic activity.

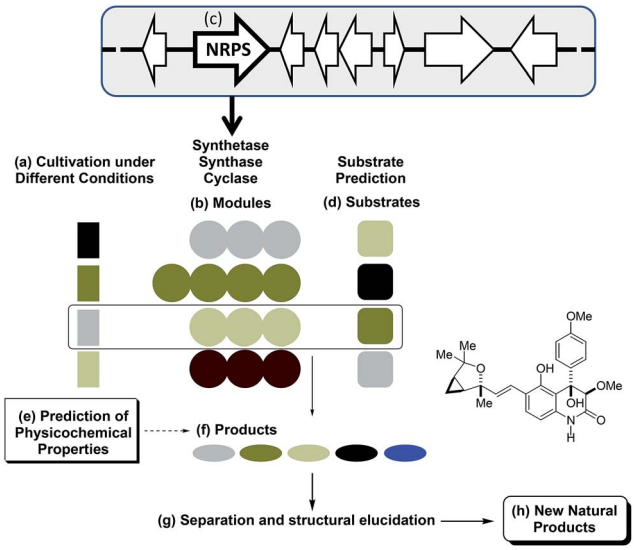
The yaequinolones had no effect on *C. elegans* at 100 μg mL^{-1}, and exhibited no antimicrobial activity in a paper disk assay against a panel of 14 different microorganisms (bacteria, fungi and yeasts), including *Bacteroides fragilis* ATCC23745, *Mycobacterium smegmatis* ATCC607, *Acholeplasma laidlawii* PG8, *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* FDA209P, *Micrococcus luteus* PCI1001, *Escherichia coli* NIHJJ-2 IFO12734, *Pseudomonas aeruginosa* IFO3080, *Xanthomonas campestris* pv. *oryzae*, *Pyricularia oryzae* KF180, *Aspergillus niger* ATCC6275, *Mucor racemosus* IFO4581, *Candida albicans* and *Saccharomyces cerevisiae*.[32a]

The aflaquinolones were evaluated for growth inhibitory activity against a panel of five cell lines, including chronic myelogenous leukemia (K562), human acute promyelocytic leukemia (HL-60), human breast cancer adenocarcinoma (MDA-MB-231), murine melanoma (B16F10), and hepatocellular carcinoma (Hep3B), being essentially inactive (IC_{50} > 80 μM).[34]

On the other side, aflaquinolone A (**27**) was modestly toxic to *Artemia salina* (LD_{50} = 5.5 μM),[35] and aflaquinolone D (**30**) has moderate anti-RSV (Respiratory Syncytial Virus) activity (IC_{50} = 6.6 μM), whereas 22-*O*-(*N*-Me-L-valinyl)-21-*epi*-aflaquinolone B (**41**) exhibited outstanding anti-RSV activity (IC_{50} = 42 nM). Interestingly, the latter is approximately 500-fold stronger than that of ribavirin, the positive control (IC_{50} = 20 μM) and further, compound **41** showed a comparatively higher therapeutic ratio (TC_{50}/IC_{50} = 520).[38]

These results indicate that compound **41** might be a promising drug candidate against RSV, also suggesting the potential of the *N*-Me-L-valinyl residue when designing molecules with promising anti-RSV activity. In addition, the heterocycle **40** and aflaquinolone A (**27**) exhibited no antiviral activity. This may be a result of the configuration of the cyclohexane unit, which may also play a key role in the observed bioactivity.

None of the aniduquinolones showed potent antibacterial or cytotoxic activities; however, aniduquinolones B (**35**) and C (**36**)



Scheme 1 Bioinformatic/experimental approach to the discovery of new natural products, illustrated with the case of the aspoquinolones. Different culture conditions (a) may activate different biosynthetic modules (b) of the NRPS cluster (c), toward the use of given substrates (d), to afford certain metabolite products. Prediction of the physicochemical properties of these products (e) orients the search for their detection (f). Product separation and the corresponding structural elucidation (g) is used to confirm the presence and novelty of the natural products (h).

exhibited modest lethality against brine shrimp ($LD_{50} = 7.1$ and 4.5 μM, respectively).[35]

5 Biosynthetic aspects

The number of sequenced fungal genomes has been growing during the last decade, offering better opportunities to discover new secondary metabolites by genome mining. To date, the sequences of several species of *Aspergillus* (*A. clavatus*, *A. flavus*, *A. fumigatus*, *A. oryzae*, *A. nidulans*, *A. niger*, *A. terreus*, *etc.*) and *Penicillium* (*P. chrysogenum*) have been published, and more genomes are being completed.

This endeavour revealed two important and quite surprising characteristics of the fungal genome. The first one is that the number of genes involved in the biosynthesis of secondary metabolites greatly exceeds that originally expected, most of them being silent under conventional laboratory conditions,[40] whereas the second feature is that most of these genes are located in clusters, so production of certain natural products takes the form of an assembly line.

In fact, two to three dozen non-ribosomal peptide synthetase (NRPS)-associated gene clusters have been consistently found per fungal genome,[41] and only a fraction of them have been linked to known products. As a result, the actual number and diversity of fungal derived natural products and the full potential of a given fungal strain cannot be ascertained, being limited to the current incapacity to grow every fungus in the laboratory, and to the inability to unlock all the silent clusters,[42] due to lack of knowledge of the structure of the corresponding molecular triggers.[43]

Scherlach and Hertweck[29] approached the discovery of new natural products through a combined bioinformatics/experimental strategy (Scheme 1), which involved predicting the physicochemical properties of the targets on the basis of the modular composition of the thiotemplate NRPS, which in turn was used to anticipate their putative substrates.

In this work, genomic analysis of *Aspergillus nidulans* allowed the authors to identify multiple copies of genes highly similar to anthranilate synthases. This prompted the researchers to predict that some of these genes may be involved in the biosynthesis of quinolone alkaloids. After a systematic variation of the cultivation parameters, a screening the extracts for nitrogen-containing metabolites was carried out. Once the presence of new alkaloids was assessed, large scale fermentation experiments were followed in order to obtain enough material for structure elucidation.

Typically, small molecules biosynthesized from modular thiotemplate PKSs and NRPSs are pieced together and processed in an assembly-line fashion based on the number and type of domains within the enzymes.[44] The biosynthesis genes for the 3,4-dioxygenated 5-hydroxy-4-aryl-quinolin-2(1*H*)-one alkaloids are located in clusters that include multidomain and multimodular enzymes like non-ribosomal peptide synthetases (NRPSs). Additional functionalities in NRPS systems include mostly regulatory proteins, oxidases, hydroxylases and transporters which are implicated in the synthesis of the natural products.[45]

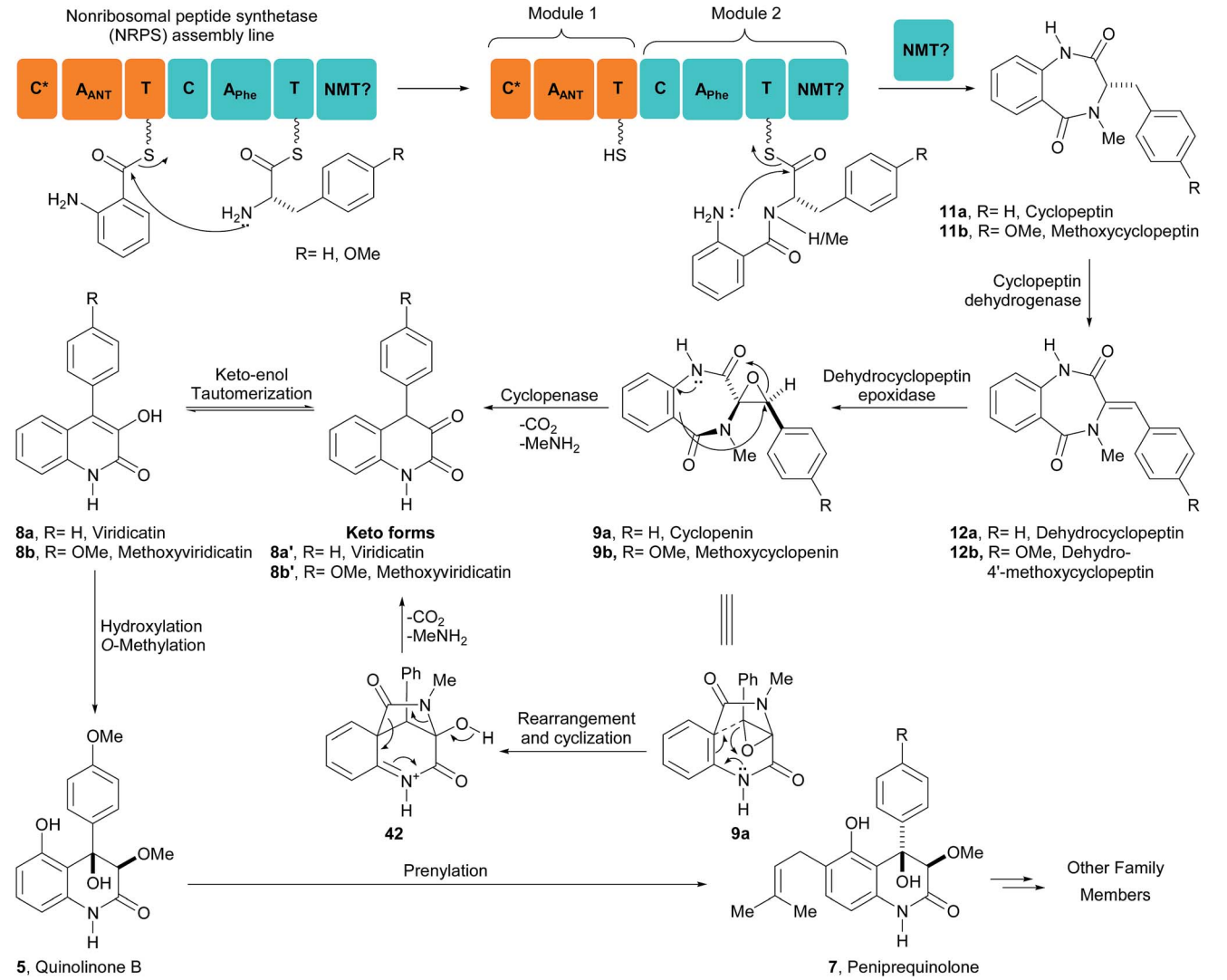
The NRPSs use amino acids as building blocks. Anthranilic acid (*Ant*), a non-canonical amino acid and primary metabolic precursor of L-tryptophan, a proteinogenic amino acid, is the signature building block for this natural product class.

The biosynthesis follows a co-linearity rule, where products are pieced together and processed as in an assembly-line, based on the number and type of domains within the enzymes; different modules take care of the chain-elongation steps and different domains provide control on the choice of the extender unit.

A typical NRPS module consists at least of an adenylation (A) and a thiolation (T) domain (peptidyl carrying protein). The former is responsible for amino acid activation, whereas the latter acts as an anchor for the growing peptide chain. A condensation domain (C) makes the Ant-amino acid peptide bond, creating a dipeptidyl thioester on T_2, the thiolation domain of the second module. Additional domains, like *N*-methyltransferase (NMT) or epimerisation (E) may optionally be present.[46] A small number of tailoring enzymes help to provide efficiently structural diversity by performing three types of maturation chemistry, including oxidation (oxygenations), alkylation (prenyl transfers), and acylation (of amino groups). A highly efficient cyclization chemistry is also part of the biosynthesis.

5.1 Assembly of cyclopeptin and methoxycyclopeptin

The first stages of the biochemical pathway leading to simple quinolin-2(1*H*)-ones in fungi were intensely studied during the decades of 1960 and 1970, being demonstrated in *Penicillium viridicatum* and *P. cyclopium*; however, biosynthetic studies of



Scheme 2 Proposed biosynthetic path toward peniprequinolone (7) and other family members, employing the NRPS assembly lines. A = adenylation, T = thiolation, C = condensation, NMT = *N*-methyltransferase.

the related series of 5-hydroxy and 5-hydroxy-6-prenyl derivatives have been more recent and still rather scarce. The early studies have been summarized elsewhere.[47]

A more modern view on the biosynthesis of the 4-arylquinolin-2(1*H*)-one core and related heterocyclic natural products has been recently presented by Walsh and co-workers.[48] The pathway (Scheme 2) seems to begin in both, *Aspergillus* and *Penicillium*, with the assembly of cyclopeptin (**11**), an *N*-methyl benzodiazepinedione. To that end both, *Ant* and L-phenylalanine (*Phe*) must suffer previous AMP activation.

Scherlach and Hertweck have postulated that L-tyrosine (*Tyr*) may also act as a precursor,[29] whereas Tang has proposed the intervention of *O*-methyltyrosine,[49] and *para*-methoxysubstituted analogues of dehydrocyclopeptin (**12a**) and cyclopeptin (**11a**) have been found in the culture broth of *Penicillium* sp. FKI-2140.[32a]

Regardless of the identity of the α-amino acid involved, it is proposed that both building blocks end up forming thioesters with a bimodular NRPS. Then, attack of the more nucleophilic amino group of the α-amino acid to the carbonyl of the tethered *Ant* results in formation of the first amido linkage, which suffers *N*-methylation in the presence of *S*-adenosylmethionine, under *N*-methyl transferase catalysis,[50] before undergoing cyclization by intramolecular attack of the aromatic amino moiety onto the thioester carbonyl.

This path has been demonstrated long ago in *Penicillium cyclopium*.[21c,50,51] However, the group of Watanabe[52] has recently challenged this view, arguing that while NRPS was proposed to form indolebenzodiazepinediones from Ant and L-tryptophan,[53] there are no reports on an analogous NRPS able to condense *Ant* with *Phe*, *Tyr* or derivatives thereof.

They also pointed out to the existence of obscure points in the biosynthesis of intermediates toward viridicatin (**8a**) and the rearrangement of the 6,7-benzodiazepinediones (**9a**/**9b**) into the corresponding 4-arylquinolones (**8a**/**8b**) through the intermediacy of **42**, like the origin of the *S*-adenosyl methionine-derived *N*-methyl group in the skeleton of cyclopeptin (**11a**).

In order to shed light on these matters, a gene cluster of *Aspergillus nidulans* A1149 containing the NRPS gene asqK and

the prenyltransferase gene asqH was examined, along with other genes encoding redox enzymes and terpene cyclases, as a potential gene cluster for the biosynthesis of 4′methoxyviridicatin (**8b**) and related heterocycles.

The transcriptional analysis of each gene in the cluster revealed that, under conventional culturing conditions, only genes asqE, asqF, and asqK were expressed, while the other genes remained silent. Therefore, asqK was overexpressed by placing an extra copy of asqK on a plasmid into *Aspergillus nidulans* A1149.

This resulted in increased production of cyclopeptin (**11a**) and 4′-methoxycyclopeptin (**11b**), upon feeding *Phe* and MeO-*Tyr*, respectively. Furthermore, the latter was approximately tenfold more efficiently incorporated into the benzodiazepinedione framework (**11b**) than *Phe*, suggesting that MeO-*Tyr* is the physiological substrate for AsqK. This also implied the asq gene cluster in the biosynthesis of 4′methoxyviridicatin (**8b**) and hinted that the *N*-methyl group is put in place by the embedded methyltransferase domain of AsqK.

5.2 Rearrangement of the benzodiazepinedione skeleton

Tailoring enzymes prepare this first intermediate for its subsequent rearrangement. Thus, it is proposed that a cyclopeptin dehydrogenase desaturates the side chain to yield (4′-methoxy) dehydrocyclopeptin (**12a**/**12b**), which is then epoxidized to cyclopenin (**9a**) or 4′-methoxycyclopenin (**9b**) by a FAD-dependent dehydrocyclopeptin epoxidase.[51c,54]

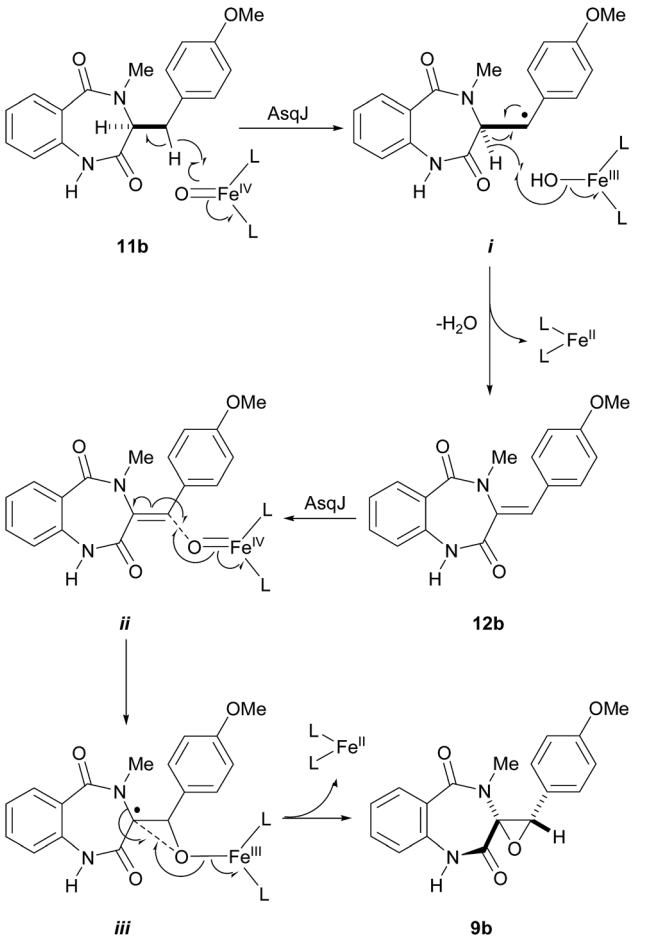
Cyclopenin (**9a**) and 3,4-dioxygenated 5-hydroxy-4-aryl-quinolin-2(1*H*)-one alkaloids were first found together in the culture broth of *Penicillium cf. simplicissimum* (Oudemans) Thom.[20] Shortly after, precursors of the benzodiazepinone were isolated along with 4-aryl-quinolin-2(1*H*)-one alkaloids in the extract of *Penicillium* sp. FKI-2140.[32a]

Next, the enzyme cyclopenase[32b,55] catalyzes the amidic nitrogen-assisted ring-opening of the epoxide of cyclopenin (**9a**) to the quinolin-2(1*H*)-one derivative viridicatin (**8a**), with concomitant loss of CO_2 and $MeNH_2$ and through the intermediacy of its keto form (**8a′**), which readily undergoes keto–enol tautomerism. This proposal suggests that the source of the 3-OH of viridicatin is the oxygen of the epoxide ring of compound **9a**. This rearrangement has been carried out under non-enzymatic conditions, releasing MeN=C=O.[56]

The group of Watanabe[52] examined the role of the redox enzymes associated to AsqE, AsqF, AsqG, AsqI, AsqJ, and AsqL in the quinolone formation steps. Sensing the bioconversion of 4′-methoxycyclopeptin (**11b**), the formation of 4′-methoxy dehydrocyclopeptin (**12b**) was observed in the presence of AsqJ. The process demanded the cofactors required by an α-ketoglutarate-dependent dioxygenase.[57]

Long incubation times resulted in formation of the epoxidized 4′-methoxycyclopenin (**9b**), along with 4′-methoxyviridicatin (**8b**), revealing that AsqJ successively catalyzes the radical-mediated dehydrogenation and epoxidation reactions.

The last stage of this sequence, which includes the rearrangement to 4′-methoxy viridicatin (**8b**), proceeds spontaneously at 30 °C in the absence of enzymes. Analogous



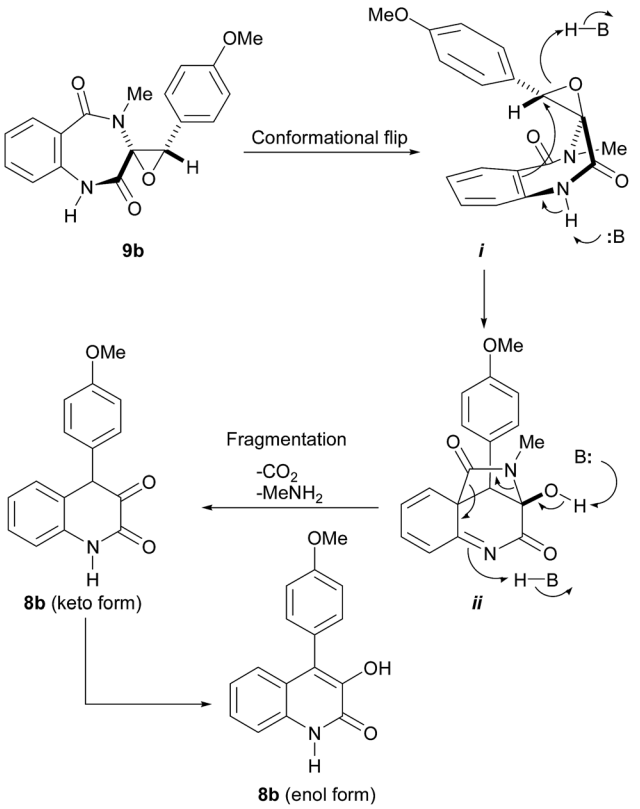
Scheme 3 Proposed mechanism for the AsqJ-catalyzed transformation of 4′-methoxycyclopeptin (**11b**), into dehydrocyclopeptin (**12b**) and epoxy 4′-methoxycyclopenine (**9b**).

experiments demonstrated that AsqJ is capable of sequentially converting cyclopeptin (**11a**) into cyclopenin (**9a**), being unable to further catalyze the transformation of the latter into viridicatin (**8a**).

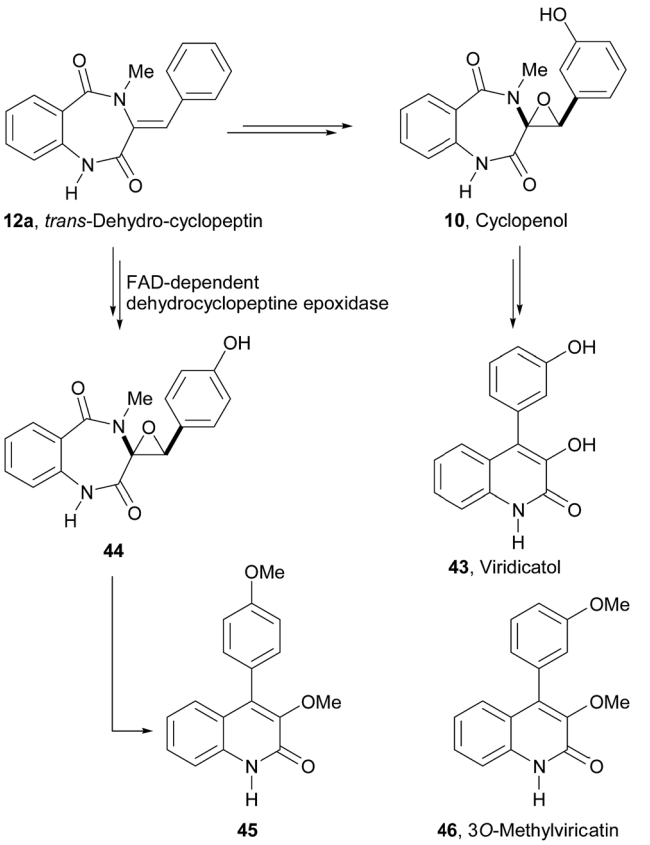
The proposed mechanism for the sequential desaturation–epoxidation (Scheme 3) suggested for the Fe^{II}/αKG-dependent dioxygenases,[57,58] in which a highly reactive Fe^{IV}-oxo species is formed by coordination of the Fe^{II} form at the active center with α-ketoglutarate and oxygen.

This species may abstract a hydrogen atom from the benzylic methylene, generating a radical intermediate (**i**), which in turn can abstract the tertiary α-carbonyl hydrogen atom thus stereoselectively giving access to a *Z*-alkene intermediate (**12b**). The order of the hydrogen abstractions may be reversed.

In the epoxidation sequence, the reactive Fe^{IV}-oxo species transfers an oxygen atom to the substrate through intermediates **ii** and **iii**, converting the dehydro intermediate into the corresponding epoxide. Both reactions cause the release of CO_2 and succinate, and the Fe^{II} form active center of the enzyme is generated at the end of each stage. The reactions are not coupled, and the enzyme requires additional molecules of α-ketoglutarate and oxygen to proceed towards the next reaction cycle.



Scheme 4 Proposed mechanism for the rearrangement of the epoxide of 4′-methoxycyclopenin (**9b**) into 4′-methoxyviridicatin (**8b**).



Scheme 6 Biosynthetic oxidation of **12a**. Pathways to **43** and **45**.

It has been recently proposed[59] (Scheme 4) that the mechanism of the rearrangement of 4′-methoxycyclopenin (**9b**) into 4′-methoxy viridicatin (**8b**) involves ring opening of the spiro-epoxide of **9b** favoured by the stabilization at the benzylic position provided by the 4′-methyl ether functionality.

In this case, the molecule suffers a conformational flip to yield intermediate **i**, which moves closer both aromatic moieties and enables the nucleophilic attack of the oxirane ring at the benzylic carbon atom to afford intermediate **ii**. In turn, the latter undergoes fragmentation, with loss of $MeNH_2$ and CO_2, to afford the keto form of **8b**, which interconverts to the more stable enol form.

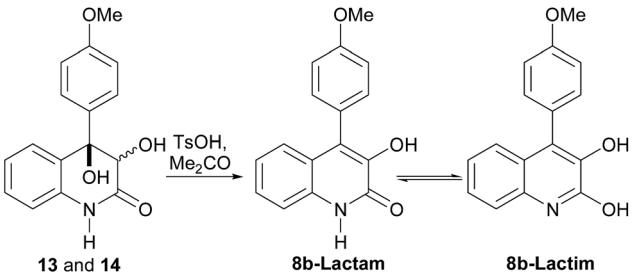
It has also been shown that the overall transformation does not require enzymatic catalysis; however, when the same rearrangement is carried out with cyclopenin (**9a**), which misses the 4′-OMe substituent, and therefore lacks its assistance, it proceeds at a much slower pace.[55,56a,b]

This explains the need of a cyclopenase among fungi producing viridicatin (**8a**) in order to observe more complex products; however, the cyclopenase is absent in the *asq* cluster.[52]

Being the first non-heme fungal α-ketoglutarate and iron-dependent dioxygenase involved in alkaloid biosynthesis, AskJ is an exceptional enzyme. This enzyme is found similar to the dioxygenase that catalyzes the dehydrogenation–epoxidation sequence involved in the biosynthesis of pentalenolactone in *Streptomyces*.[60]

Different crystal structures of the protein, in the absence and in presence of synthesized substrates, surrogates, and intermediates that mimic the various stages of the reaction cycle, have been recently reported.[59] Interestingly, its exquisite dehydrogenation specificity towards *N*-methylated substrates and its epoxidation ability of *Z*-configured olefins were also demonstrated, thus also confirming that the *N*-methyl group is an essential requirement for the enzyme.

In the biosynthesis of the 3,4-dioxygenated 5-hydroxy-4-arylquinolin-2(1*H*)-one alkaloids, the production of 4′-methoxy viridicatin (**8b**) should be followed by 3-*O*-methylation, an hydratase-mediated introduction of the OH-4, an hydrolase-assisted hydroxylation of C-5 and a final C-6 prenylation with a prenyltransferase,[61] to yield the so-called "peniprequinolones" by the groups of Hertweck[29] and Walsh.[48]



Scheme 5 Acid-catalyzed dehydration of **13** and **14**. Lactam–lactim tautomerization of **8b**.

The reverse of the action of the hydratase was reported by the group of He (Scheme 5),[22] finding that the acid-catalyzed dehydration of a 1 : 1 mixture of the diastereomeric diols **13** and **14** with TsOH in acetone affords 4′-methoxyviridicatin (**8b**), as the sole product.[62]

5.3 Installation of 4-OH, 5-OH and other hydroxylations

In some instances, the *meta*-hydroxylation of the mono-substituted phenyl ring takes place at this stage (Scheme 6), to afford cyclopenol (**10**). Rearrangement of the latter affords viridicatol (**43**), the 3′-hydroxy analog of viridicatin. Compound **43** has been isolated from different *Penicillium* and *Aspergillus* species in Russia, China, Japan and Australia,[63] and has been synthesized chemically.[64]

On the other hand, enzymatic studies employing *Penicillium cyclopium* indicated that the FAD-dependent dehydrocyclopeptin epoxidase can effect concomitant hydroxylation of the phenyl ring of cyclopenin (**9a**) to a *para*-substituted phenol (**44**). The transformation occurs in three steps,[21c,51d] generating suitable intermediates toward *O*-methylated products.

Without more details, and opposite to observations by other researchers,[52] the group of Walsh[48] proposed that **44** is processed in *Penicillium* species by tandem *O*-methylation of the 3-OH group and that supported by the 4-phenyl moiety, setting at this stage the formation of the 4′-OMe motif (**45**).

It is currently agreed that the oxygen functionality on C-3 of viridicatin (**8a**) derives from the epoxide of cyclopenin (**9a**). However, the hydration step of viridicatin and its 4′-methoxy analogue (**8b**), which establishes the relative and absolute stereochemistry of the stereocenters of the nitrogenated heterocyclic ring, ranks among the most obscure steps of the biosynthesis of the 5-hydroxy-4-arylquinolin-2(1*H*)-ones and their 6-substituted congeners. The same is true for the hydroxylation of C-5 and the *O*-methylation of 3-OH to afford for instance **46**, which are necessary phases in the biosynthetic path.[48]

Little is known about these stages; however, most probably, introduction of the required pair of additional –OH groups could be sequentially done by hydratase and hydroxylase action.[48] Scherlach and Hertweck performed a biosynthetic proposal for the aspoquinolones; however, they omitted an explanation for these transformations.[29]

On the other hand, Kusano and co-workers proposed that 4′-methoxyviridicatin (**8b**) would experience an "hydroxylation on C-4", followed by a second hydroxylation on the aromatic ring.[20] This model was taken by the group of Uchida for the proposal of a biosynthetic sketch, which unfortunately did not provide further mechanistic precisions.[17]

5.4 The prenylation stage

Prenylation of L-tyrosine, has been occasionally observed;[65] however, the 5-hydroxy-4-arylquinolin-2(1*H*)-ones are also scaffolds which act as substrates of *C*-prenyltransferases. Recently, the group of Tang demonstrated that prenylation of quinolinone B (**5**) takes place stepwise instead of adding a geranyl moiety at once.

In this unprecedented fashion, two dimethylallyl pyrophosphate (DMAPP, **47**) units are sequentially added to **5** to afford an heterocycle carrying an oxidized 10-carbon isoprenoid unit. This intermediate is primed for subsequent rearrangement reactions.[49]

5.5 Further modifications of prenylated quinolones

Scherlach and Hertweck[29] also proposed a biogenetic link between the 3,4-dioxygenated 5-hydroxy-4-aryl-quinolin-2(1*H*)-one alkaloids and 3-*O*-methyl viridicatin (**46**) and its 4′-methoxy analog (Scheme 7) and made a biogenetic proposal toward the penigequinolones (**3** and **4**), yaequinolone C (**23**) and the aspoquinolones A–D (**17–20**) as taking place from a C-10 peniprequinolone, the product of a Friedel–Crafts type geranylation of a 4′-methoxyviridicatin derivative. However, this proved to be wrong.

Recently, the group of Tang, who previously characterized the cyclopenase AsqJ that converts methoxycyclopeptin into 4′-methoxyviridicatin exposed an alternative biosynthetic proposal.

They sequenced the genome of *Penicillium thymicola* IBT5891, which is a producer of penigequinolones,[66] and located the putative *pen* cluster (Fig. 9), which contains PenN, the expected non-ribosomal peptide synthetase (NRPS), PenM, the homologue of AsqJ, and a series of methyltransferase and redox enzymes.

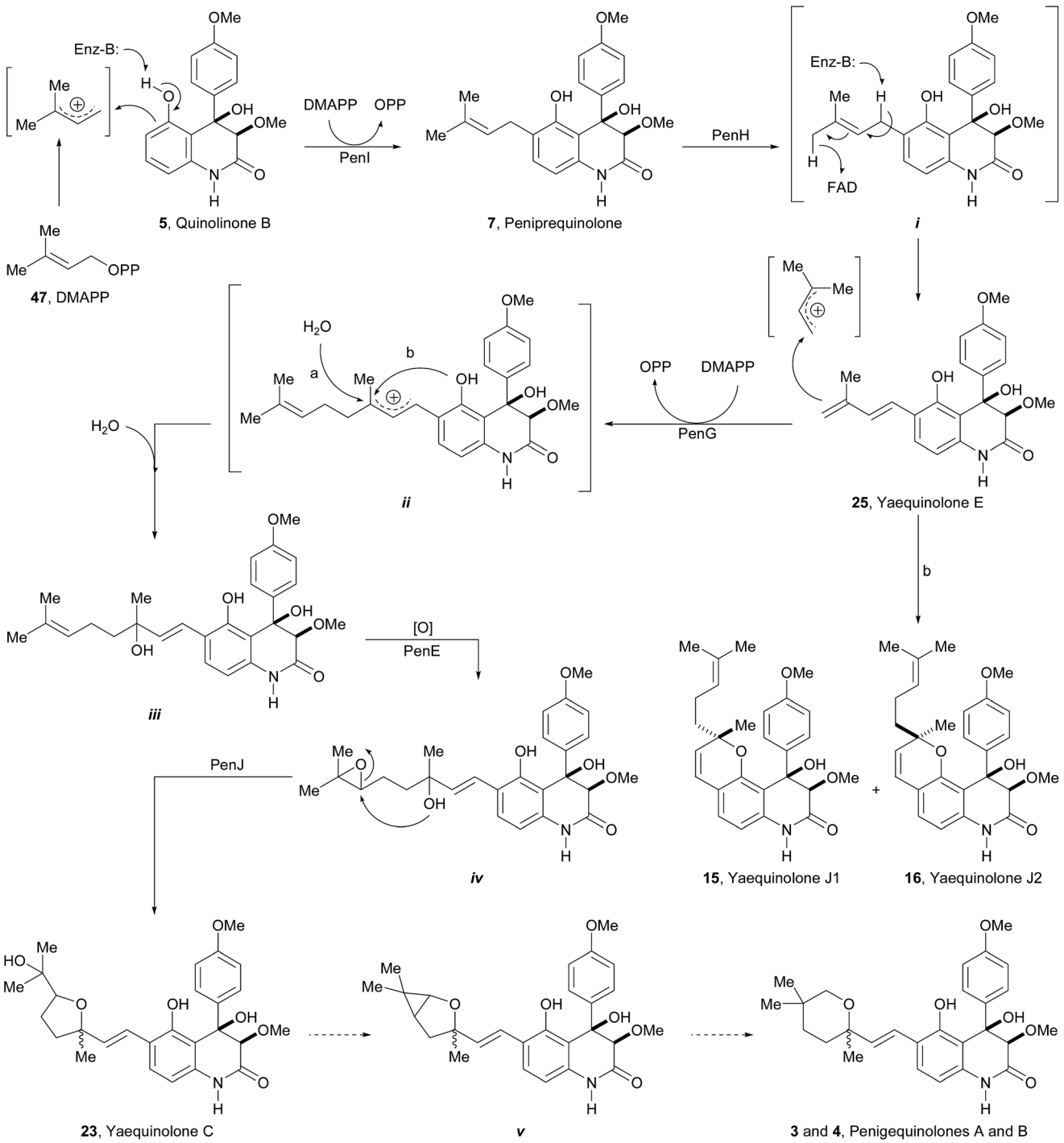
This group observed that the *pen* gene cluster encodes two aromatic prenyltransferases, namely PenG and PenI,[67] instead of a single geranyltransferase, as would be expected to build the geranyl precursors of yaequinolones and penigequinolones (**3** and **4**).

The confirmation of their biosynthetic roles was obtained after deletion of penN, which resulted in biosynthesis of quinolinone B (**5**) and concomitant lack of production of penigequinolones (**3**, **4**).

Employing quinolinone B (**5**) as substrate for biochemical analyses, this group observed that prenylation of the quinolone core takes place with DMAPP (**47**) and not with geranyl pyrophosphate (GPP), under PenI catalysis (Scheme 7).

Further, they demonstrated that peniprequinolone (**7**) is a biosynthetic intermediate and that chain elongation takes place in a stepwise fashion, where **7** is dehydrogenated to yield yaequinolone E (**25**) through the intermediacy of **I** and under PenH catalysis. This enzyme is a membrane-bound flavin monooxygenase, likely to be a FAD-dependent dehydrogenase.[68] A second prenyl unit is then added in a head-to-tail fashion, to afford a quinolone intermediate **ii**, carrying a ten-carbon isoprenoid unit. The latter can undergo cyclization with the phenol moiety, to afford the diastereomeric yaequinolones J1 (**15**) and J2 (**16**), as shunt products.

On the other hand, it was observed that compound **ii** could undergo a PenG-catalyzed hydration, to afford tertiary alcohol **iii**, while the subsequent PenE-mediated oxidation of **iii** results in the epoxy-alcohol **iv**. Pen-E is also a flavin monooxygenase.



Scheme 7 Putative biosynthetic pathway of the penigequinolones (**3** and **4**) from quinolinone B (**5**) and shunt products **15** and **16**.

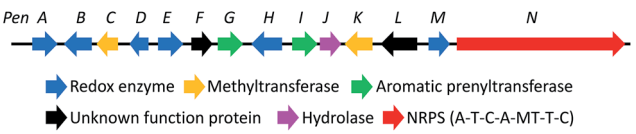
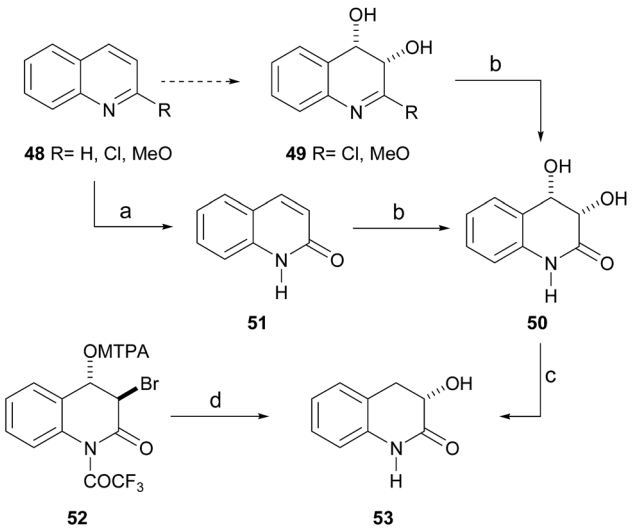


Fig. 9 Organization and proposed function of the Pen gene cluster in *Penicillium thymicola*, as proposed in ref. 66.

The heterocyclic epoxy-alcohol **iv** is a precursor of the tetrahydrofuran derivative yaequinolone C (**23**), which is produced by the intramolecular nucleophilic opening of the oxirane ring, a spontaneous or PenJ-catalyzed 5-*exo-tet* cyclization process.

The same authors also proposed that **23** is the precursor of the yet unobserved cyclopropane derivatives **v**, which in turn should be the precursors of the penigequinolones A and B (**3** and **4**).



Scheme 8 *Reagents and conditions*: (a) H_2O; (b) *Pseudomonas putida* UV4, O_2 (R = H, 2%; R = OMe, 13%); (c) (1) TBDMSOTf; (2) $LiAlH_4$; (3) Ac_2O; (d) (1) AgOAc; (2) NaOH, MeOH.

6 Synthetic aspects

The synthesis of polysubstituted 3,4-dioxygenated quinolin-2(1*H*)-ones is still a considerable challenge. Although the significance of this skeleton is obvious, available methods and strategies for accessing these targets are relatively scarce and their overall results are not always satisfactory, being considered a synthetically demanding task by itself among these heterocycles.

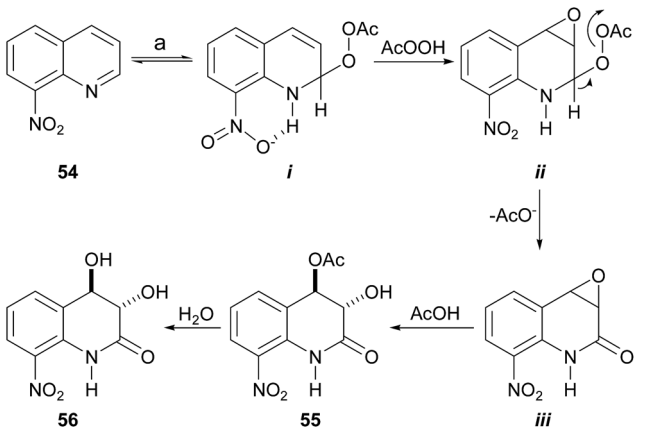
Biotransformations have shown to provide limited access to 3,4-dihydroxyquinolin-2(1*H*)-ones, whereas these compounds have occasionally been obtained with no better luck by chemical means, and not always with the relative stereochemistry found among the natural products.

A photochemical strategy has also been described, but it seems to be restricted to the synthesis of compounds carrying two substituents on C-3. To date, however, the best approach toward the 3,4-dioxygenated motif is the intramolecular aldol-type reaction of 2-methoxyacetyl derivatives of 2′-aminobenzophenones.

6.1 Biotransformations leading to 3,4-dioxygenated quinolin-2(1*H*)-ones

The functionalization of the 3,4-position using enzymatic catalysis, employing the toluene-dioxygenase (TDO) system present in a mutant strain of the bacterium *Pseudomonas putida* UV4 was the first approach of this type of functionalization (Scheme 8).[69]

The stereochemistry of the product **53** resulting from deoxygenation of quinolone (**50**) was established as 3*S*,4*S* by X-ray crystal structure analysis, and confirmed by stereochemical correlation to the Mosher ester [2-methoxy-2-trifluoromethyl-2-phenylacetate (MTPA)] derivative **52** with known absolute configuration. The diol product **50**, which was obtained from **51**, proved to be remarkably stable; however, when heterocycles



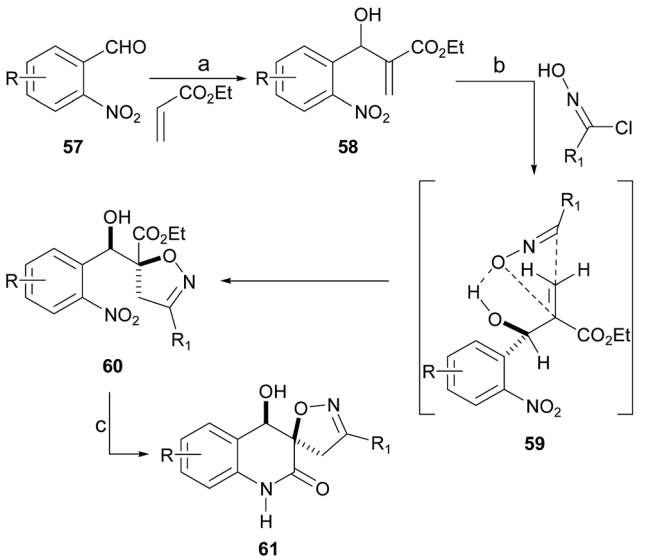
Scheme 9 *Reagents and conditions*: (a) 30% H_2O_2, AcOH, 60 °C, 8 h (**55**, 9.9%; **56**, 5.6%).

48 (R = Cl, OMe) were used as substrates, the intermediates **49** readily hydrolyzed to afford **50.**

Noteworthy, the enzyme-catalysed oxidation of pyridine rings containing alkyl, aryl and thioalkyl substituents has been found to take place at the exocyclic substituents,[70] suggesting that the *cis*-dihydroxylation of the heterocyclic ring is not a preferred metabolic step; hence the low yields of *cis*-diol **50** are not surprising.

6.2 Chemical syntheses of 3,4-dioxygenated quinolin-2(1*H*)-ones

Among the chemical alternatives, the reduction of 3-hydroxyquinolin-2,4-diones looks the most straightforward one. These heterocycles, which are chemically accessible with relative ease,[71] are also metabolites of the *Pseudomonas* species;[72]



Scheme 10 *Reagents and conditions*: (a) DABCO, neat, r.t., 10–24 h; (b) Et_3N, Et_2O, −78 °C → r.t., 3 h (60–91%); (c) In/HCl, 70 °C, THF/H_2O (3 : 2), 5–7 min (55–79%). R = H, 3,4-$(OMe)_2$; R_1 = Ph, 2F–C_6H_4, 4Cl–C_6H_4, 4Me–C_6H_4.

however, they are very reactive compounds, prone to undergo molecular rearrangements that result in the formation of new heterocyclic compounds.

Podesva and co-workers[73] synthesized 6-chloro-3,4-dihydro-3,4-dihydroxy-1-methyl-3-phenylquinolin-2(1*H*)-one by the reduction of the corresponding 3-hydroxyquinoline-2,4-dione with $NaBH_4$, and the group of Kappe prepared 3,4-dihydro-3,4-dihydroxy-3-(pyridin-2-yl)methylquinolin-2(1*H*)-one by hydrogenation of the respective 3-hydroxyquinoline-2,4-dione in the presence of a Pd/C catalyst.[74]

Unfortunately, in both cases the configuration of the product was not described. However, Klásek *et al.* recently demonstrated that the $NaBH_4$ reduction of 3-alkyl/aryl-3-hydroxyquinolin-2,4-diones proceeds with high diastereoselectivity to give *cis*-3-alkyl/aryl-3,4-dihydro-3,4-dihydroxyquinolin-2(1*H*)-ones, through the convincing formation of a cyclic carbonate.[75]

On the other hand, the *trans* diol **56** and the corresponding acetate **55** were isolated in only 5.6% and 9.9% yield respectively upon treatment of 8-nitroquinoline (**54**) with 30% H_2O_2 in warm AcOH (Scheme 9), formed through the epoxy and acetate intermediates **i–iii**.[76]

In addition, treatment of a chiral dihydroquinolin-2(1*H*)-one derivative with $RuCl_3/NaIO_4$ has also been employed to *cis*-dihydroxylate the 3,4-double bond,[77] affording the expected product with excellent diastereo- and enantioselectivity.[78]

Furthermore, as shown in Scheme 10, compound **58** the Baylis–Hillman product of the reaction of 2-nitrobenzaldehyde (**57**) and ethyl acrylate was employed as a substrate for the 1,3-dipolar cycloaddition with different nitrile oxides, which were generated *in situ* from the corresponding hydroximoyl chlorides.[79]

This resulted in substituted 2-isoxazolines **59**, which were obtained with high diastereoselectivity in favor of the *syn* isomers,[80] as a result of hydrogen bond interactions between the nitrile oxide and the hydroxy group of the Baylis–Hillman adducts **58** in the transition state (**60**). The reductive cyclization of the heterocycles with In/HCl afforded *cis*-3,4-dihydroxy-quinolin-2(1*H*)-one derivatives **61**.[81]

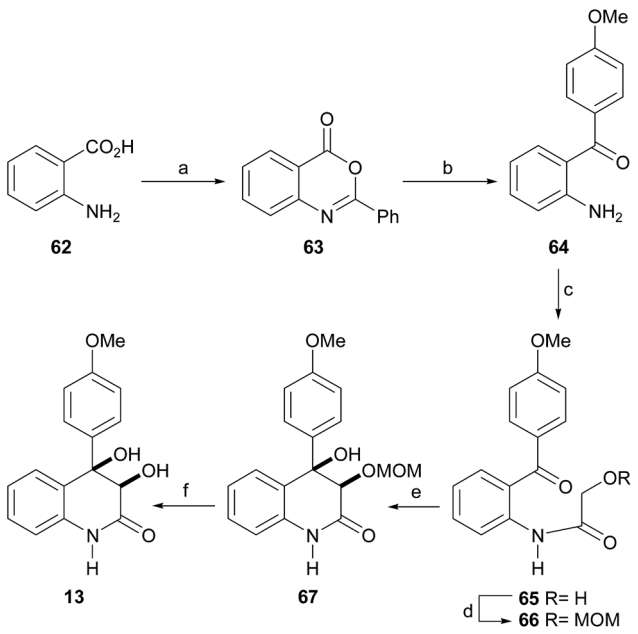
6.3 The total synthesis of (±)-yaequinolone A2 (13)

She and Pan disclosed a short synthesis of (±)-yaequinolone A2 (**13**),[82] the first member of this family being synthesized (Scheme 11), which began with the preparation of the known 2-aminobenzophenone **64**. This task was performed in 84% overall yield by reaction of the 2-phenylbenzoxazinone **63** with *p*-anisylmagnesium bromide.[83] In turn, the oxazinone **63** is an anthranilic acid (**62**) derivative.

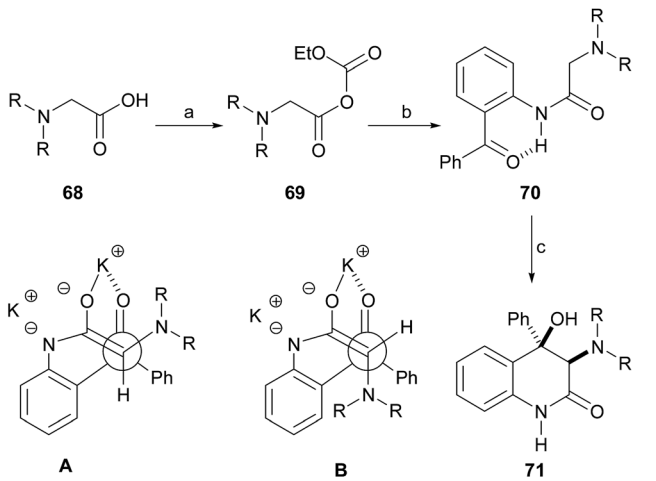
The electron-deficient aniline was activated as a *N*-sulfinylaniline[84] and coupled to glycolic acid, to afford α-hydroxyanilide **65**,[85] the hydroxyl group was protected as the MOM-ether derivative (**66**). The key aldol cyclization toward **67** was carried out under KO^{t}Bu promotion, affording essentially a single diastereomer (>99 : 1).[86]

The observed diastereoselectivity can be a result of formation of the more stable *Z*-enolate. Since deprotection under protic conditions afforded mainly the dehydration product, the use of $AlCl_3$ and NaI offered a mild and fast alternative,[87] which afforded **13** without concomitant dehydration.

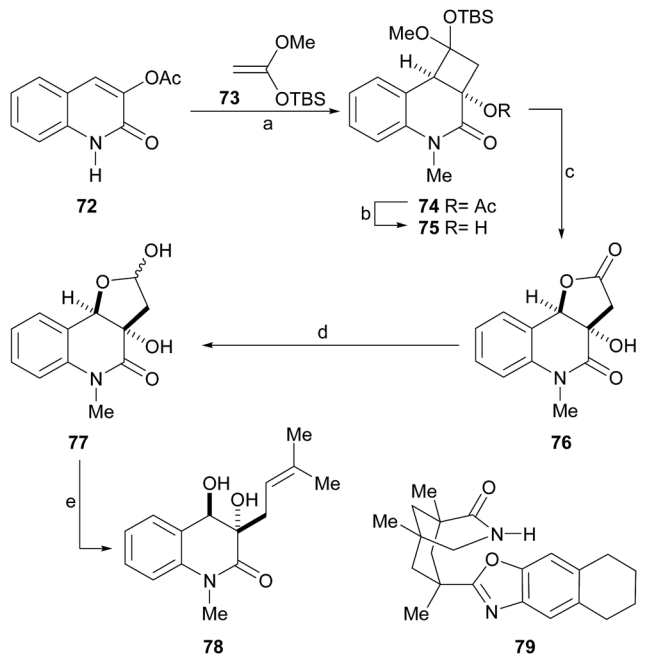
The same synthetic strategy was proposed in the patent literature and clues toward the synthesis of many 5-hydroxyquinolin-2(1*H*)-ones were given; however, besides the synthesis of yaequinolone A2, only some analogues of the natural products were prepared.[33c]



Scheme 11 *Reagents and conditions*: (a) BzCl, Na_2CO_3, THF, 0 °C → r.t., overnight; (b) (1) 4OMe–C_6H_4MgBr, CH_2Cl_2, −70 °C → −40 °C, 2 h; (2) 10M NaOH, MeOH, reflux, 8 h (82% overall); (c) (1) $SOCl_2$, imidazole, −40 °C → 20 °C, 30 min; (2) glycolic acid, Et_3N, CH_2Cl_2, 0 °C, 4 h (73%); (d) MOMCl, $^i Pr_2NEt$, DMAP (cat.), CH_2Cl_2, 0 °C → r.t., 48 h (72%); (e) KO^{t}Bu, THF, r.t., 2 h (92%); (f) $AlCl_3$, NaI, MeCN–CH_2Cl_2 (2 : 1, v/v), 0 °C, 5 min (70%).



Scheme 12 *Reactions and conditions*: (a) $ClCO_2Et$, Et_3N, CH_2Cl_2, r.t.; (b) 2-PhC(O)–$C_6H_4NH_2$, CH_2Cl_2, 40–50 °C (80%, overall); (c) KO^{t}Bu, 2 h (89–97%); R,R = –$(CH_2)_5$–; Et, Et; Bn, Bn; Bn, H; *o*-$C_6H_4(CH_2)_2$.



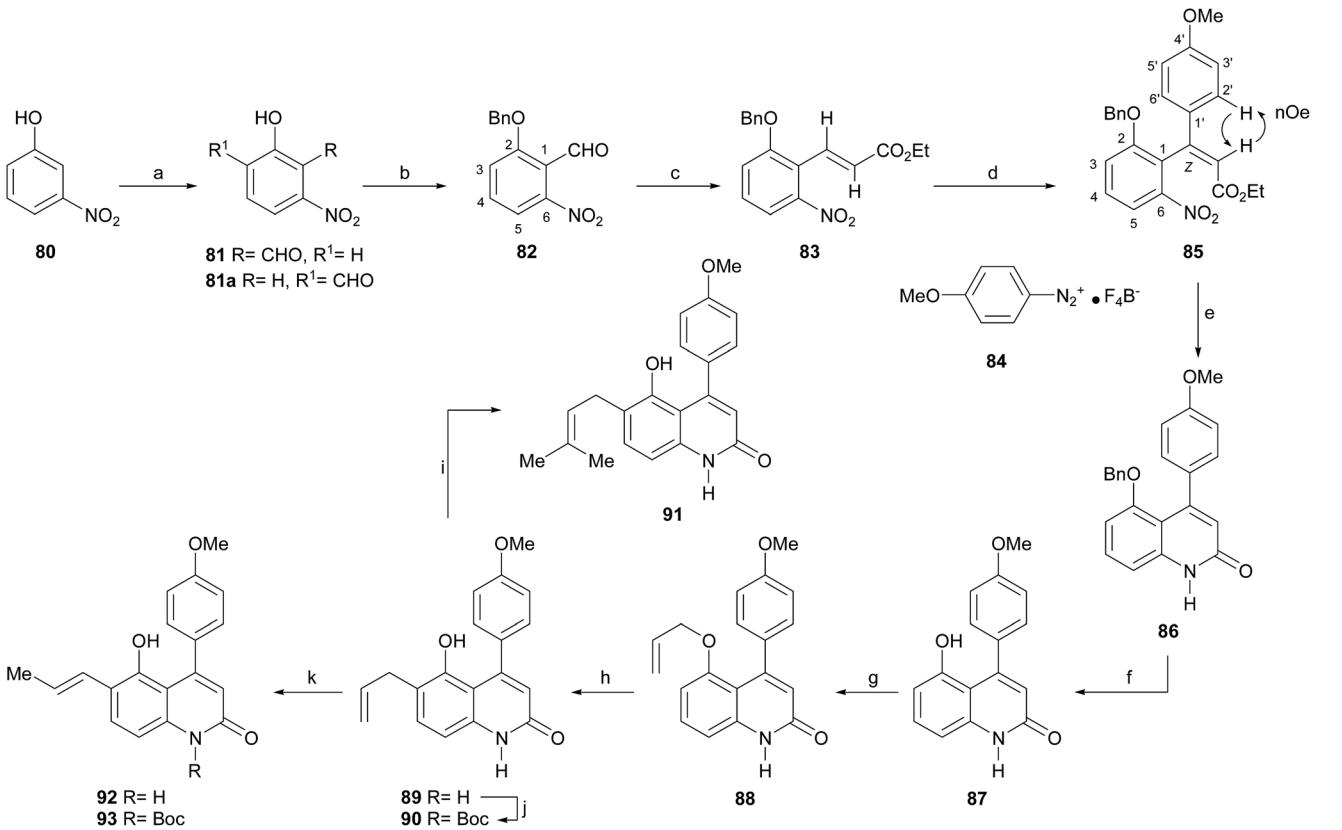
Scheme 13 *Reagents and conditions*: (a) NaH, MeI, **73**, **79**, THF (74%, dr = 74 : 26); (b) KCN, EtOH (97%; *dr* = 70 : 30); (c) *m*-CPBA, $BF_3 \cdot Et_2O$, CH_2Cl_2 (75%); (d) DIBAL-H, CH_2Cl_2 (61%; *dr* = 86 : 14); (e) $^iPrPPh_3^+Br^-$, BuLi, THF (55%).

A quite similar approach was pioneered by the group of Soloshonok for the synthesis of analogous compounds (Scheme 12).[86] Commercially available amino acids **68** were treated with ethyl chloroformate and the intermediate mixed anhydrides **69** were further condensed with *o*-aminobenzophenone to afford the amides **70** in 80% isolated yields.

The final intramolecular aldol condensation reaction to afford **71** was accomplished in high yields in the presence of 7 equiv. of KO^tBu, with almost complete diastereoselectivity (>99 : 1). A similar approach was employed for the preparation of 5-fluoro-4-hydroxy-3-methoxy-3,4-dihydroquinolin-2(1*H*)-one.[88]

Transitions states **A** and **B** were proposed in order to account for the observed diastereoselectivity of the cyclization. In both cases, the enolate and the carbonyl oxygens are located closely to each other, enabling the reaction to take place under minimum charge separation conditions.

However, from the steric point of view, transition state **A** should be more prevalent than transition state **B**, where the substituted amino group may be exposed to unfavourable repulsive interactions with the two phenyl rings. Further stabilizing interactions between the substituted amino group and the oxygen of the enolate should take place in transition state **A**, where they are located in the *cis* position.



Scheme 14 *Reagents and conditions*: (a) HMTA, TFA, 90 °C, 12 h (46%); (b) BnCl, K_2CO_3, EtOH, 70 °C, overnight (99%); (c) $PPh_3{=}CHCO_2Et$, CH_2Cl_2, r.t., 5 h (88%); (d) 4-MeO–C_6H_4–I, $Pd(OAc)_2$, Et_3N, 100 °C (55%), or **84**, $Pd(OAc)_2$, MeOH, 70 °C, 6 h (80%); (e) Fe^0, AcOH, 110 °C, 18 h (74%); (f) H_2, 10% Pd/C, EtOAc–EtOH (1 : 1), 1 atm, r.t., 4 h (89%); (g) $BrCH_2CH{=}CH_2$, K_2CO_3, EtOH, 70 °C, overnight (65%); (h) 1,2-Cl_2–C_6H_4, MW, 190 °C, 2 h (75%); (i) 2-methylbut-2-ene, Grubbs II (5 mol%) CH_2Cl_2, reflux, 2 h (sealed tube, 77%); (j) Boc_2O, Et_3N, DMAP, CH_2Cl_2, r.t., 20 h (63%); (k) $RhCl_3 \cdot 3H_2O$, EtOH, r.t., 31 h (50%).

6.4 Other naturally-occurring 3,4-dihydroxyquinolin-2(1*H*)-ones. Isolation and synthesis of pinolinone (78)

Although the 3,4-dihydroxyquinolin-2(1*H*)-one pattern is infrequent, the 3,4-dihydroxyquinolin-2(1*H*)-ones derived from fungi are not the only examples of natural products carrying this motif. The 3-prenyl derivative (−)-pinolinone (**78**) was isolated from the dried roots of *Boronia pinnata* Sm. (Rutaceae), a shrub found in New South Wales, Australia. Compound **78** is an additional member of this class, seemingly the only one of plant origin.[89]

A six-step synthesis of this natural product in enantiomerically enriched form has been recently reported,[90] employing 3-acetoxyquinolone (**72**) as a competent starting material (Scheme 13).[91] The key step was an enantioselective intermolecular [2 + 2] photocycloaddition under irradiation at 366 nm, controlled by the chiral template **79**,[92] in which the silyl enolether of methyl acetate (**73**) served as the electron-rich reaction partner.[93]

After *N*-methylation to **74**,[94] the acetate group was mildly hydrolyzed with KCN in EtOH (**75**),[95] and then smooth Lewis acid-assisted removal of the mixed ketal combined with a concomitant Baeyer–Villiger oxidation[96] of the resulting cyclobutanone intermediate,[97] afforded the racemic γ-lactones **76**. Chiral HPLC separation of the enantiomeric lactones **76** afforded a product with ee = 99%.

The last stages included partial reduction of the lactone with excess DIBAL-H[98] and Wittig olefination[99] of the resulting mixture of lactols **77** afforded an optically active levorotatory final product (**78**), to which the 3*S*,4*R* configuration was attributed based on literature precedents.[100] The synthesis also served to confirm that the natural product carries the same absolute configuration.

6.5 Synthesis of a key intermediate toward peniprequinolone (7) and congeners

We have recently reported the synthesis of **92**, a potential key intermediate toward the members of this family of natural products carrying a prenylated side chain on C-6, through a strategy which involves a reductive cyclization[101] in order to form the heterocyclic ring (Scheme 14).

To that end, 3-nitrophenol (**80**) was subjected to a Duff formylation with hexamethylenetetraamine (HMTA) in F_3CCO_2H at 110 °C during 12 h, affording the expected aldehyde **81** as an 85 : 15 mixture with its isomer **81a**, in combined 54% yield.[102] In turn, compound **81** was submitted to a Williamson etherification with BnCl in refluxing EtOH, employing K_2CO_3 as base, to render **82** in almost quantitative yield.

A Wittig reaction[103] with ethyl (triphenyl-λ^5-phosphanylidene)-acetate, afforded 88% of cinnamate **83**,[104] serving to introduce the two-carbon moiety required to build the quinolin-2(1*H*)-one ring. After considering unsatisfactory the yields provided by the Heck arylation,[105] a Heck–Matsuda protocol was employed,[106] with the tetrafluoroborate diazonium salt of *p*-anisidine (**84**) as the source of the required 4-methoxyphenyl moiety. This furnished 80% of the (*Z*)-β,β-diarylacrylate **85**.[107]

Next, exposure of **85** to elemental iron powder in glacial AcOH at 110 °C cleanly gave 74% of its reductive lactamization product **86**,[108] whereas selective debenzylation of the latter to **87** and further selective *O*-allylation[109] rendered 65% of **88**.[110] Submission of the ether **88** to a microwave-assisted Claisen rearrangement in 1,2-dichlorobenzene, afforded 75% of the 6-allyl derivative **89**.

Interestingly, the 6-allyl-1*H*-quinolin-2-ones have elicited great synthetic and pharmaceutical interest, having also been used as intermediates toward drugs for treating cardiac diseases, protecting against the UV rays, scavenging active oxygen species, and inhibiting enzymes as well as lipid peroxidation.[111]

Olefin cross metathesis of **89** with 2-methylbut-2-ene, under Grubbs II catalyst promotion in refluxing CH_2Cl_2 furnished 77% of **91**, a peniprequinolone (**7**) analog, lacking its 3,4-glycol monoether feature.[112] However, unexpectedly, the isomerization of the terminal double bond of **89** toward **92**, proved to be challenging,[113] probably due to the formation of unstable quinomethide intermediates.[114]

Therefore, the *N*-Boc derivative **90** was prepared in 63% yield;[115] and when exposed to $RhCl_3 \cdot 3H_2O$ in absolute EtOH, gave 50% of the desired *E*-propenyl derivative **93**.

6.6 Synthesis of analogues and derivatives

Intellectual property protection of a plethora of analogues and derivatives is the subject of a recent patent of Novartis, which unfortunately provides very little chemical information.[51c]

Accordingly, some patent protected compounds which can be accessed by rather simple chemical modifications of a natural product and seemingly through chemical synthesis are reported. The physical characterization employing HPLC retention times under selected chromatographic conditions, as well as low resolution MS data of the compounds are also provided.

7 Conclusions and prospects

This review summarizes 20 years of research, leading to the discovery of the 3,4-dioxygenated 5-hydroxy-4-aryl-quinolin-2(1*H*)-one alkaloids as a new family of natural products, produced by some *Aspergillus* and *Penicillium* species of terrestrial and marine origins.

Although the structures of these fungal metabolites are rather complex, the biosyntheses of some members have been elucidated by different research groups, finding that Nature employs a rather reduced set of enzymes and simple biochemical mechanisms to perform complex tasks, including marvellous rearrangements and ring size modifications.

However, these studies have also revealed part of the sophisticated strategy employed by Nature to generate optically active metabolites derived from L-phenylalanine or L-tyrosine, through the intermediacy of compounds lacking stereogenic centers, like viridicatin and 4′-OMe viridicatin.

Further, they have also unveiled the existence of silent genes and genes with still uncharacterized functions, and that the wide array of compounds isolated in some cases from the same fungal species provides clues to the biochemical pathways involved in their biosynthesis.

The increasing ease with which fungal genomes are being completed and made available, and the informatics tools that enable a facile characterization of the biosynthetic gene clusters promise more discoveries of both, new metabolites and novel enzymatic pathways. Further, the application of novel techniques based on the exposure of fungi to different culture conditions, which allow access to new natural products with remarkable biological activity is also a promising way toward new discoveries.

Finally, although 20 years of discovery have almost placed together a whole family of interesting molecules, endowed with a range of promising biological activities, some of the still-missing key intermediates are pieces of a puzzle that await to be uncovered. On the other hand, the chemical synthesis of most of these compounds is also an objective that remains unconquered.

8 Acknowledgements

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