# Triggering cryptic natural product biosynthesis in microorganisms

**Kirstin Scherlach and Christian Hertweck***

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Natural products from microorganisms are a crucial source for novel therapeutics. Even so, it seems that many valuable compounds are overlooked when culturing microbes under standardized laboratory conditions. Many biosynthesis genes remain silent and such "cryptic" or "orphan" pathways are only activated under specific conditions. This report gives an overview on the strategies to trigger biosynthetic pathways to yield "cryptic natural products" through external cues, co-cultivation and genomic approaches such as genome-mining, epigenetic remodeling, and engineered pathway activation.

## Introduction

Natural products continue to play a pivotal role in modern drug-based therapy of various diseases.[1] Especially for the treatment of cancer, infectious diseases and lipid metabolic disorders, or as immunosuppressants, secondary metabolites have proved to be a persistent source of innovative therapeutic agents and drug leads.[2,3] Moreover, they can be virulence factors mediating pathogenicity and serve as invaluable tools for elucidating biological targets.[4] Evolved by Nature, these products are characterised by their unique structural heterogeneity, accounting for potency and selectivity, as well as by their innate ability to bind to protein folds.

*Leibniz Institute for Natural Product Research and Infection Biology, (HKI), Beutenbergstr. 11a, 07745, Jena, Germany. E-mail: christian.hertweck@hki-jena.de; Fax: +49 (0) 3641 5320804; Tel: +49 (0) 3641 5321100*

Traditional methods to discover microbial natural products usually involve the collection and cultivation of strains, extraction, bioassay-guided isolation and structure elucidation. Unfortunately, this approach is often frustrating because of a high rediscovery rate. The growing understanding of microbial genomics, however, has provided valuable insights into the principles of natural product biosynthesis and thus offers promising alternatives for the discovery and engineering of new chemical entities.[5] The observation that biosynthetic genes that code for a secondary metabolic pathway are clustered in microbial genomes facilitates their study and manipulation. Furthermore, profound knowledge has been gained for pathways involving polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) assembly lines, which are responsible for the biosynthesis of two large classes of natural products.[6,7]

Recent whole-genome sequencing programmes have revealed that the biosynthetic potential of microorganisms has been greatly underexplored, relying as it does on traditional approaches.[5,8–13]

**Kirstin Scherlach**

*Kirstin Scherlach was born in 1977, and studied pharmacy at the University of Jena. After a research internship at the School of Pharmacy in London, UK, she started her PhD studies at the Leibniz Institute for Natural Product Research and Infection Biology with Prof. C. Hertweck in 2002. Since 2007 she has been working as a postdoc in the same group. Her current research interests focus on the chemistry of microbial natural products and genome mining strategies.*

**Christian Hertweck**

*Christian Hertweck (born 1969) studied chemistry at the University of Bonn and performed his Ph.D. work under the supervision of Prof. Boland at the Max Planck Institute for Chemical Ecology. In 1999 he became a Humboldt postdoctoral fellow of Profs. Floss and Moore at the University of Washington, Seattle. He then set up an independent research group at the HKI in Jena. Since 2006 he has held a chair of natural product chemistry at the Friedrich Schiller University Jena, and is head of the Department of Biomolecular Chemistry at the Leibniz Institute for Natural Product Research and Infection Biology. His current research involves natural products from bacteria and fungi with emphasis on the investigation and manipulation of biosynthetic pathways.*

In fact, the number of genes encoding biosynthetic enzymes in various bacteria and fungi clearly outnumbers the known secondary metabolites of these organisms. One reason for this observation might be that only a subset of biosynthetic pathway genes is expressed under standard laboratory culture conditions and therefore only a minority of potential chemical structures is produced. Such silent genetic loci are referred to as "cryptic" or "orphan" pathways. To gain access to this untapped reservoir of potentially bioactive structures, the biosynthesis of these putative metabolites needs to be induced. However, not all "cryptic" pathways are necessarily silent. It might well be that the corresponding metabolites may be overlooked due to low production rates, a large metabolic background or unpropitious culture conditions. A systematic investigation of the microbial secondary metabolome might therefore represent a first step towards exploitation of the natural biogenetic capability of microorganisms.

## Challenging microorganisms through culture conditions, external cues, and stress

Since the early days of fermentation science it has been known that the choice of the cultivation parameters is critical to the number of secondary metabolites produced by microorganisms. Even small changes in the culture medium may not only impact the quantity of a certain compound but also the general metabolic profile of an organism (Fig. 1). In particular, in the fields of antibiotics much effort was directed toward optimizing production rates and directing the product spectrum.[14–20] Manipulating nutritional or environmental factors can promote secondary metabolite biosynthesis and thus facilitate the discovery of new natural products. Zeeck and co-workers, who deliberately elaborated fermentation parameters to increase the number of microbial metabolites, coined the term "OSMAC" (One Strain MAny Compounds) for this approach. By altering easily accessible cultivation parameters, such as media composition, aeration, temperature or shape of culturing flask, they were able to isolate hitherto unknown natural products from various fungi and actinomycetes.[21–23] Applying several culture conditions to an *Aspergillus ochraceus* strain known to biosynthesise only aspinonene resulted in the production and identification of 15 additional compounds of diverse biogenetic origin.[22]

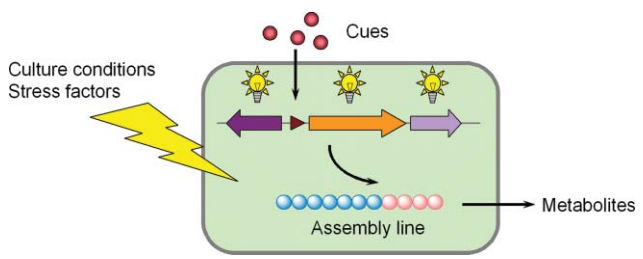
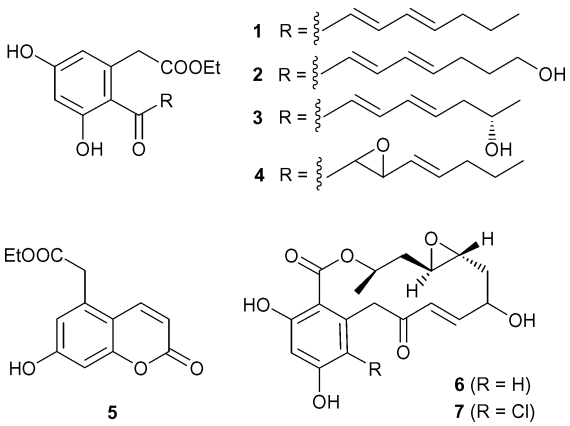
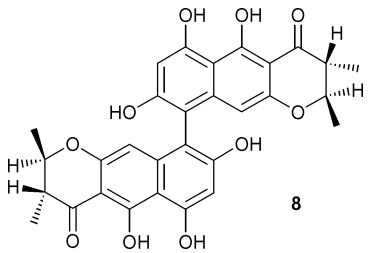



**Fig. 1** Influence of culture conditions, external (chemical) cues, and stress on natural product biosynthesis.

In a similar approach, Paranagama *et al.* investigated the secondary metabolome of two plant-associated fungi. Simply by changing the water used to prepare the medium from tap water to distilled water the production of six new metabolites by *Paraphaeosphaeria quadriseptata* was induced (cytosporones F–I **1–4**, quadriseptin A **5** and 5′-hydroxymonocillin III **6**).[24] In *Chaetomium chiversii* a shift in the production of radicicol **7** to chaetochromin A **8** was shown by changing from a solid to a liquid medium.[24]

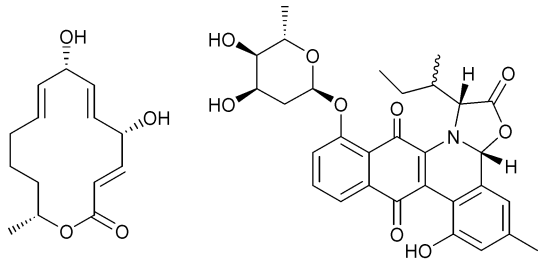



In addition to variation of culture conditions, the influence of various stress conditions on secondary metabolite biosynthesis has been evaluated. Chemical screening of the culture broth of the fungus F-24′201y, obtained by UV mutagenesis of a spirobisnaphthalene-producing *Sphaeropsidales* sp., resulted in the discovery of the 14-membered macrolide mutolide **9**. Interestingly, the production of this compound was also elicited when the fungal cultures were supplemented with tricyclazole, an inhibitor of DHN biosynthesis, which suppresses spirobisnaphthalene formation. These results demonstrate that enzyme inhibitors can have an impact on the activation of biosynthetic pathways.[23]

Several years before, Ayer *et al.* reported the isolation of jadomycin B **10**, a glycosylated benzoxazolophenanthridine antibiotic, from a *Streptomyces venezuelae* ISP5230 culture that had undergone heat shock.[25] The aglycon jadomycin had been found to be produced in a galactose–isoleucine-containing medium at 37 °C.[26] Raising the temperature to 42 °C shifted the production to jadomycin B. Successive experiments revealed further stimulating parameters. Short-time heat shock resulted in even higher jadomycin B formation, as did ethanol treatment and phage infection.[27,28] Only recently, jadomycin B has been shown to be a potent inhibitor of Aurora-B kinase, thus making it a promising anti-cancer drug candidate.[29,30]

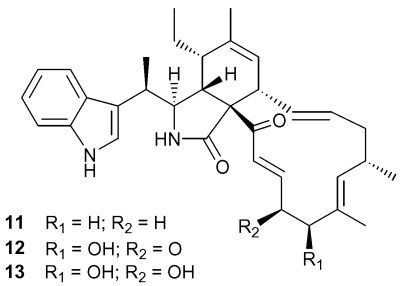
 



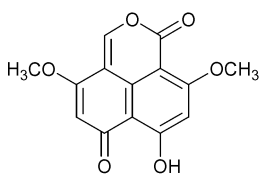
**9** **10**

Crews and co-workers isolated three new chaetoglobosin analogues (chaetoglobosin-510, -540 and -542 **11–13**) from *Phomopsis asparagi* after supplementing the cultures with the potent F-actin inhibitor jasplakinolide.[31]

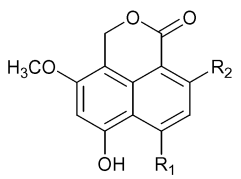
It is conceivable that microbial cultures lack the production of metabolites under non-natural laboratory conditions, and one may consider taking specific environmental requirements of the organisms into account. In an attempt to mimic the fungus' natural habitat, Overy *et al.* cultured several necrotrophic *Penicillium* strains on macerated host tissue media. Thus the production of corymbiferone **14** and the corymbiferan lactones **15–18** was stimulated, which might suggest a functional role of these metabolites in the necrotrophic plant/pathogen environment.[32,33]



**11** $R_1 = H; R_2 = H$
**12** $R_1 = OH; R_2 = O$
**13** $R_1 = OH; R_2 = OH$



**14**



**15** $R_1 = CH_2OH; R_2 = OH$
**16** $R_1 = CH_2OOCH; R_2 = OH$
**17** $R_1 = CH_2OH; R_2 = OCH_3$
**18** $R_1 = CH_3; R_2 = OCH_3$

To facilitate the screening for optimal growth conditions for filamentous fungi, Bills *et al.* developed nutritional arrays. With miniaturized parallel fermentations in microplates, they demonstrated that culture conditions can be varied for large screening populations of fungi. However, the application of this strategy is restricted to preliminary assays for biological activities – scale-up fermentations are required for compound isolation.[34]

## Interspecies crosstalk: Novel metabolites produced in co-cultures

There have been many discussions on the functional role of natural products in microbes.[35] Particularly intriguing is the study of secondary metabolite function in complex situations such as microbial communities or inter-species associations. One could imagine that every natural product might result from the interaction of an organism with its environment. In particular, the interplay between organisms of the same or different species seems to have resulted in huge natural product diversity (*e.g.* pheromones, predator–prey molecules, metabolites of symbiotic associations) (Fig. 2). The idea of an inter-species crosstalk leading to chemical diversity has also been applied to the laboratory. Fenical, Clardy and colleagues investigated whether mixed fermentations can stimulate the production of secondary metabolites. When they cultured a marine *Pestalotia* species together with an unidentified, antibiotic-resistant marine bacterium, the biosynthesis of pestalone **19**, a new benzophenone, was elicited. This compound, which displayed potent antibacterial activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*, could not be detected when either strain was cultured individually.[36]

Fenical *et al.* showed the induced production of two new cyclic depsipeptides from a marine-derived *Emericella* species in competing co-culture. Challenging the fungus with the actinomycete *Salinispora arenicola* enhanced the native production of emericellamide A **20** and B **21** by 100-fold and thus enabled structural elucidation of these compounds.[37] Recently, the emericellamide biosynthetic genes were discovered *via* a gene deletion approach in the model organism *Aspergillus nidulans*, offering the possibility to engineer novel analogues.[38]

Another interesting example of the synergistic role of microorganisms resulting in metabolite production was reported by Angell *et al.* in 2006.[39] They observed that in a mixed bacterial culture isolated from ocean floor sediments a blue pigment with antibiotic activity was produced. Chemical analyses (X-ray as well as 1D and 2D NMR) revealed the structure as pyocyanin **22**, a phenazine derivative. However, when they analysed single bacterial isolates of the original mixed culture for pyocyanin production the pigment could not be detected. Assuming that microbial interaction was necessary to stimulate pyocyanin formation, they started a split-and-pool approach to identify the species combination responsible. Two bacteria, classified as *Pseudomonas aeruginosa* and *Enterobacter* sp., were found to be the pair involved, with *P. aeruginosa* as the true producer and *Enterobacter* sp. as the "inducer". Pyocyanin biosynthesis was already known to be regulated by quorum sensing in several *P. aeruginosa* strains,[40] suggesting an involvement of small molecules in this example of inter-species crosstalk as well. The assumption was proven by successive co-culturing experiments using a Boyden chamber. Moreover, to establish whether there was a specific interaction between both species or whether any microorganism could induce pyocyanin formation, *P. aeruginosa* was cultured with various other microorganisms, disclosing another *Enterobacter* strain as a possible activator. The results suggest that the isolated *Pseudomonas* strain has lost the ability of self-inducing pyocyanin production and that the "inducing signal" is provided by the *Enterobacter* sp. strain instead.[41] Watanabe and co-workers also gained insight into the primary biological effects of the phenazine metabolite by investigating its role as an inducer of oxidative stress.[39]

The growing understanding of bacterial communication systems may also open new possibilities to trigger natural product biosynthesis. It has been shown that small signalling molecules play important regulatory roles in the onset of antibiotic production in several bacteria.[42,43] For example, the A-factor

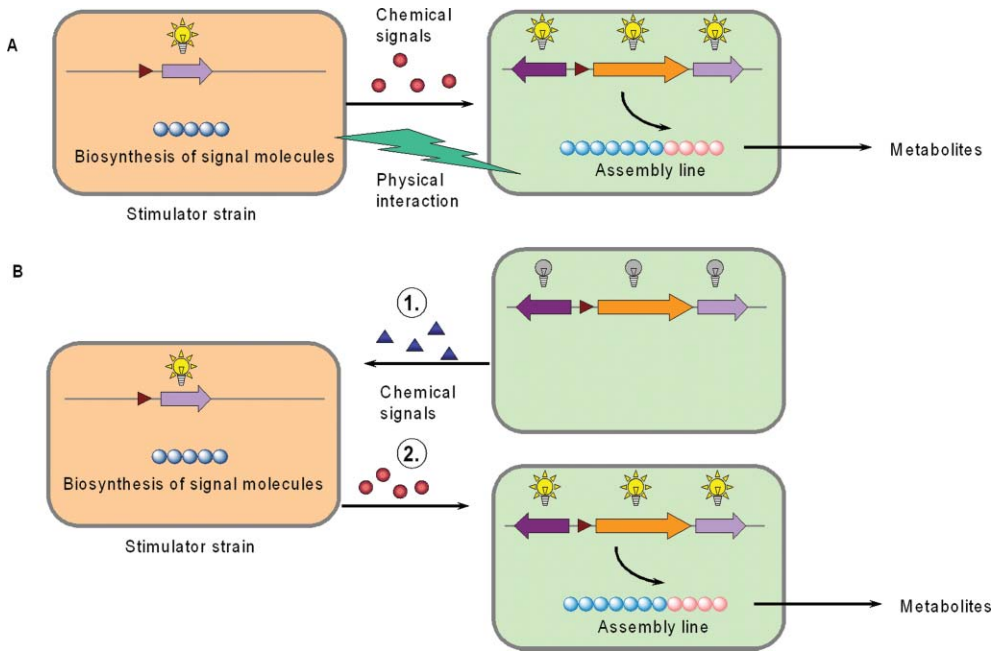
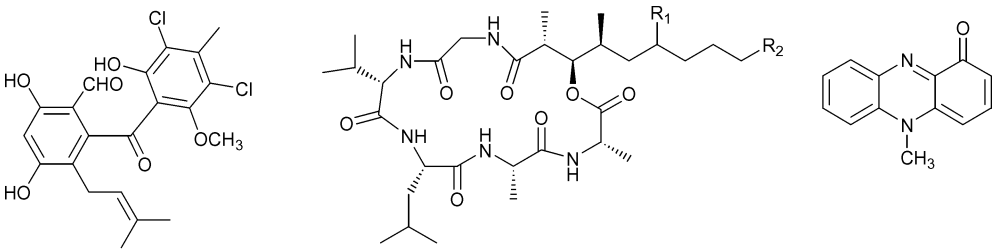



**Fig. 2** Possible routes for secondary metabolite biosynthesis in mixed cultures: (A) One organism stimulates assembly of natural products in the other organism *via* secretion of chemical signals or physical interaction; (B) Exchange of chemical signals: One organism induces biosynthesis of signalling molecules which then stimulate production of cryptic metabolites.



**19**

**20** $R_1$ = H; $R_2$ = $CH_3$
**21** $R_1$ = $CH_3$; $R_2$ = $CH_2$-$CH_2$-$CH_3$

**22**

(2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) is required for streptomycin production in *Streptomyces griseus*, while other γ-butyrolactones control formation of virginiamycin, minimycin or actinorhodin.[44,45] Hormaomycin, a peptide lactone produced by *Streptomyces griseoflavus*, affects not only aerial mycelium formation, but also has an impact on the production of secondary metabolites.[46,47] Takano *et al.* have investigated whether such systems can be adapted to the laboratory for inducing secondary metabolite biosynthesis.[48,49]

## Genomics-based approaches: from *in silico* prediction to pathway engineering

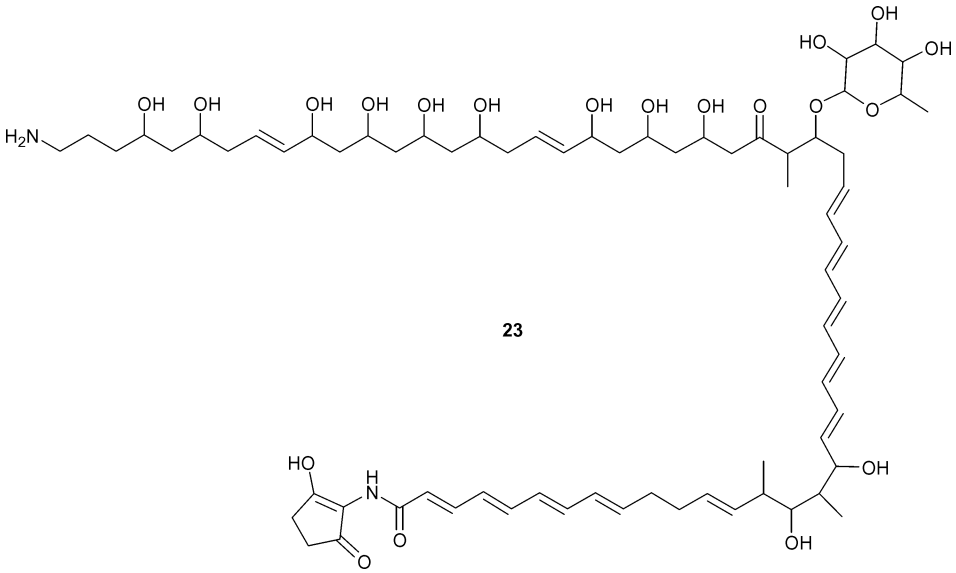
The various approaches summarized above for triggering the biosynthesis of new natural products are heavily dependent on finding the right condition under which the cryptic genes are expressed in laboratory cultures. However, from the discrepancy between detected secondary metabolite gene clusters in fully sequenced genomes and the number of corresponding natural products identified to date, one can assume that a multitude of genes either lack expression, or that the appropriate physical or chemical stimuli to induce the pathway have not yet been discovered. To make this resource accessible several strategies are conceivable.

### Genomics-inspired screening for novel natural products

Combining the chemical screening of extract libraries with a genome-guided approach has emerged as a new powerful tool to discover novel natural products. *In silico* prediction of putative chemical structures encoded by orphan genetic loci enables the directed search for relevant metabolites.[50–54] Zazopoulos *et al.* analysed the genomes of several actinomycetes for genes putatively involved in the biosynthesis of enediyne anti-tumour agents. Optimised growth conditions finally induced the expression of the putative gene clusters, resulting in the production of enediynes.[55]

Scanning the genome of *Streptomyces aizunensis* for type I PKS genes, McAlpine *et al.* predicted the production of a polyketide with a characteristic UV absorbance at 300 nm. Through triggering the biosynthesis by variation of culture conditions, the compound could be isolated. The structure of ECO-02301 **23** was elucidated with the help of bio-informatic analysis of the gene cluster and confirmed by spectroscopic techniques.[56]



**23**

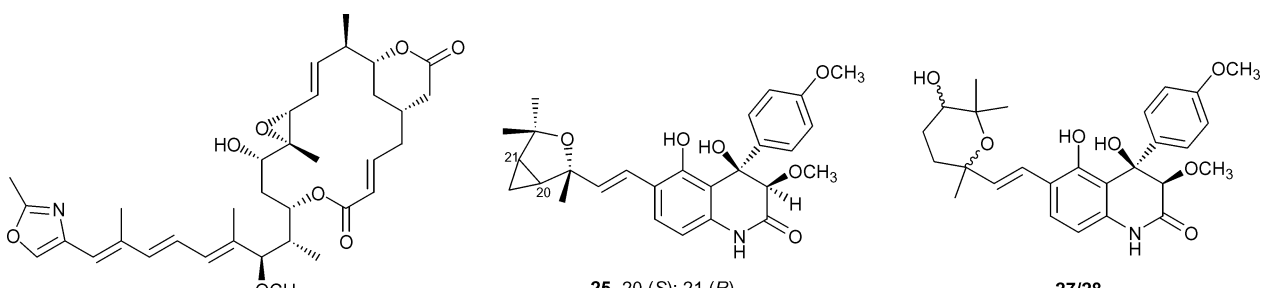
Analysis of the genome of the plant commensal *Pseudomonas fluorescens* Pf-5 revealed a cryptic hybrid PKS-NRPS gene cluster,[57] showing high homology to the recently identified rhizoxin biosynthesis gene cluster.[58] The latter was cloned and sequenced from endofungal bacteria of the genus *Burkholderia*, which turned out to be the true producers of the toxin, and not, as had been believed for decades, the fungal host *Rhizopus microsporus*.[59,60] Because of the similarity of the gene clusters, the biosynthetic abilities of *P. fluorescens* Pf-5 for rhizoxin formation were investigated. Monitoring the metabolic profile under various growth conditions finally confirmed this assumption, and yielded a variety of structurally related antimitotic agents of the rhizoxin complex, *e.g.* **24**.[61]

A combined genomic and analytical approach also led to the discovery of new fungal metabolites. Scanning the *Aspergillus nidulans* genome sequence 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 (ASs). It was known that these enzymes catalyse 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 allowed the assumption that some of their gene products might be involved in secondary metabolic pathways. As anthranilic acid is also known as precursor of alkaloids, in particular in the biosynthesis of quinazoline, quinoline and acridine alkaloids, the secondary metabolome of *A. nidulans* was investigated, paying attention to nitrogen-containing compounds. Out of several tested cultivation conditions a solid-state fermentation on rice finally afforded four novel prenylated quinoline alkaloids (aspoquinolones A–D **25–28**).[62]

## Epigenetic remodeling of the secondary metabolome

Recently, a new strategy to elicit the biosynthesis of fungal natural products has been reported. The discovery of the putative nuclear transcriptional regulator LaeA, which controls secondary metabolite production in *Aspergillus*, suggested the existence of global regulatory mechanisms by which fungi ensure the appropriate production of secondary compounds at certain developmental stages or under specific environmental conditions.[63–66] It has been shown that the transcription of fungal genes is often controlled by epigenetic regulation such as histone deacetylation and DNA methylation. Keller and co-workers demonstrated that deletion of genes (*hdaA*) encoding an *A. nidulans* histone deacetylase (HDAC) caused transcriptional activation of two secondary metabolite gene clusters. Likewise, the treatment of other fungi with HDAC inhibitors resulted in the overproduction of several metabolites.[67]

Cichewicz and colleagues hypothesized that epigenetic modifiers could be rationally employed for modulating secondary metabolite production (Fig. 3). In initial experiments they treated twelve fungi with several DNA methyltransferase and HDAC inhibitors in a dose dilution series. Eleven strains were found to respond, with production of new or enhanced accumulation of constitutively produced natural compounds as demonstrated by comparative



**24**

**25** 20 (*S*); 21 (*R*)
**26** 20 (*R*); 21 (*S*)

**27/28**

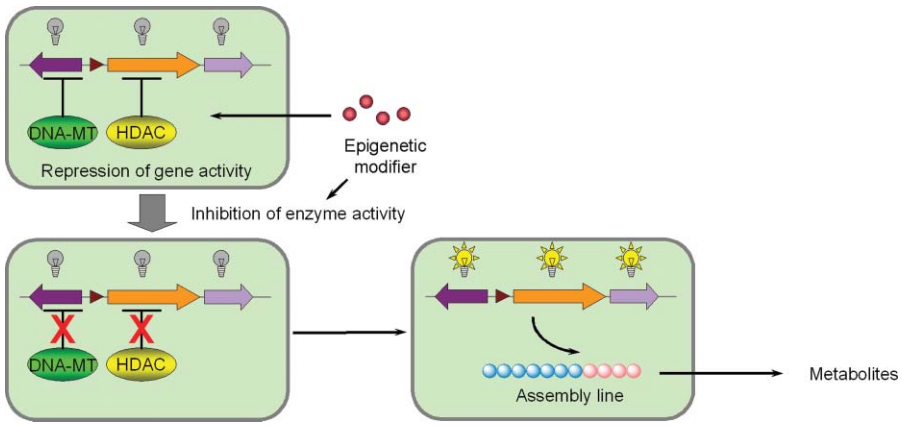



**Fig. 3** Triggering secondary metabolite biosynthesis by epigenetic remodeling; Epigenetic modifiers inhibit DNA-methyltransferase (DNA-MT) and histone deacetylace (HDAC).

metabolic profiling. Two strains were investigated in more detail. Addition of 5-azacytidine to cultures of *Cladosporium cladosporioides* stimulated the production of several oxylipins **29–31**, treatment with suberoylanilide hydroxamic acid afforded two new perylenequinones (cladochromes F **32** and G **33**) along with four known cladochromes **34–37** and calphostin B **38**. Interestingly, two of these compounds (cladochromes A **34** and B **35**) had initially been reported as products of a *Cladosporium* infection of *Cucumis sativus* seedlings and could not be obtained from single-culture fermentations. The second isolate to be investigated was a *Diatrype* species. Administration of 5-azacytidine triggered the formation of two new polyketides, lunalides A **39** and B **40**.[68] Very recently the same group demonstrated the potential of the chemical epigenetics methodology by identifying a cryptic metabolite of *A. niger*. Nygerone A **41** was only produced in fungal cultures supplemented with suberoylanilide hydroxamic acid.[69]

### Activation of pathway-specific regulatory genes

A more targeted approach is the activation of pathway-specific regulatory genes (Fig. 4). Such genes encoding putative activator proteins are present in many secondary metabolite gene clusters. Their overexpression may allow the concerted expression of all pathway genes. The advantage of this technique is that only a small gene needs to be handled and an ectopic integration is sufficient, bypassing all limitations of homologous recombination. The success of the strategy has recently been demonstrated. Mining the genome of *A. nidulans* for cryptic secondary metabolite genes revealed the presence of a putative hybrid PKS-NRPS gene. As no corresponding natural product could be identified, it was assumed that this gene locus is silent under standard fermentation conditions. Within the cluster, the presence of a putative activator gene was noticed, and homologous overexpression of this gene

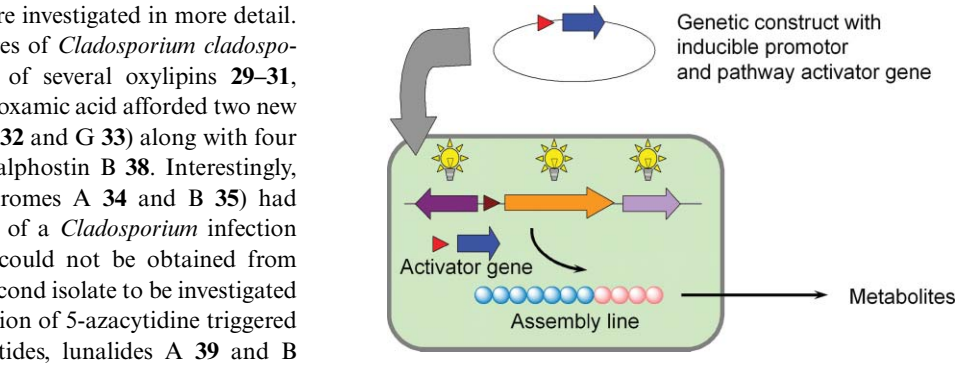
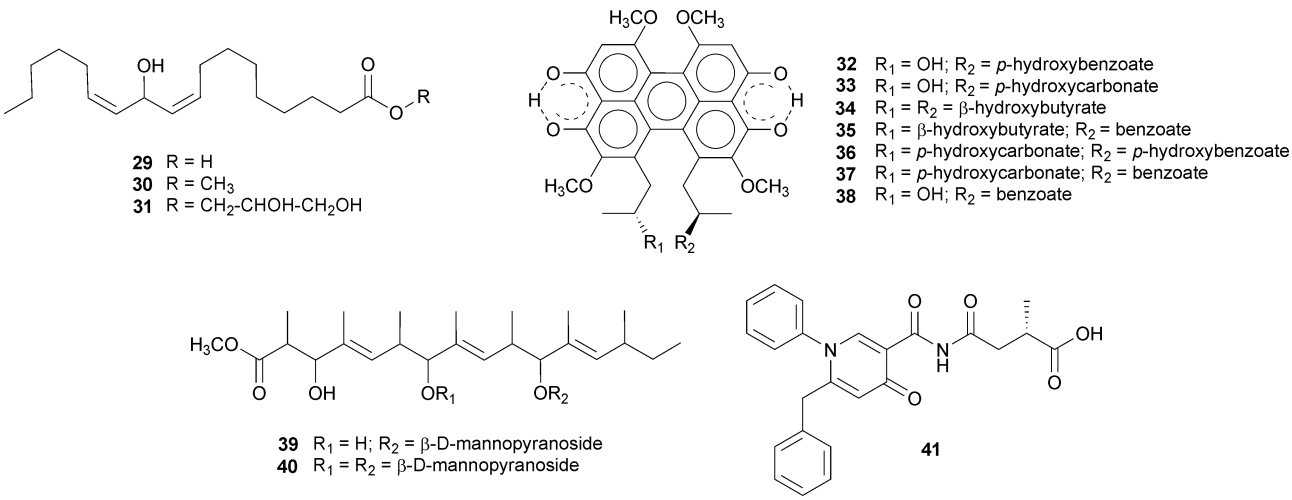
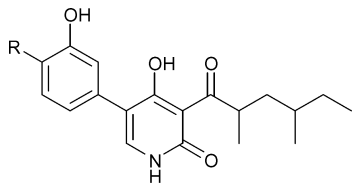



**Fig. 4** Triggering natural product biosynthesis by activation of pathway-specific regulatory genes.



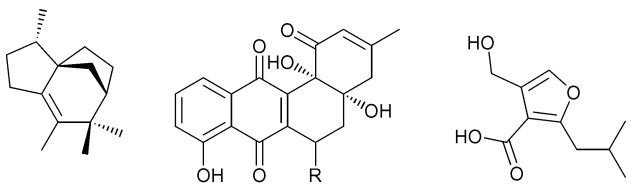
under the control of an inducible promoter led to the activation of the entire biosynthetic pathway. Comparative profiling of the metabolite production of the transformant strain under inducing and non-inducing conditions then revealed the production of new metabolites, and scale-up fermentation enabled the isolation of two novel pyridone alkaloids (aspyridones A **42** and B **43**).[70] This strategy might be applicable to other orphan gene clusters and thus represents a promising new option to promote the discovery of numerous novel metabolites.[13,71]




**42** R = H
**43** R = OH

### Heterologous expression of orphan biosynthesis genes

Transferring orphan genetic loci or parts thereof into heterologous hosts proved to be a successful method to induce expression of silent genes and thus identify new natural products (*e.g.* **44**–**47**, Fig. 5).[72–76] However, the application of this strategy shows some limitations such as difficulties in handling large gene clusters or finding appropriate expression hosts. Especially in fungi, the heterologous production of biosynthetic enzymes can be very cumbersome,[77] emphasizing the need to find alternative or complementary techniques.




**44** **45** R = H **46** R = OH **47**

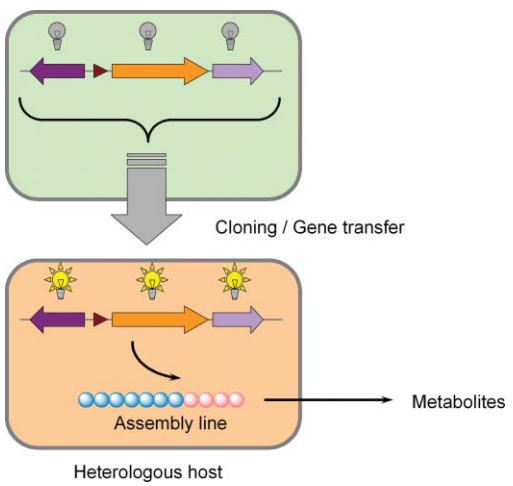



**Fig. 5** Harnessing the biosynthetic potential by heterologous expression.

## Conclusion

There will be a constant need for new chemical structures to meet the growing requirements of modern medicine. Particularly, increasing bacterial resistance to currently used antibiotics will be a major promoter for drug discovery processes. To bypass the limitations of existing methods for natural product discovery, we are compelled to search for alternative strategies. Our growing understanding of the biosynthetic machinery has enabled the advent of new genome-based approaches to make up for the shortcomings of traditional methods. In particular, the study of the role that secondary metabolites play in the natural habitat of an organism seems to be a promising strategy. The deeper insight into natural product function we get, the better chances we have of making use of this resource. Nature has provided a huge pool of yet-to-be discovered drugs; the challenge is to find ways to take advantage of this biosynthetic potential.

## Acknowledgements

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