Biosynthesis

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Formation of 1,3-Cyclohexanediones and Resorcinols Catalyzed by a Widely Occuring Ketosynthase**

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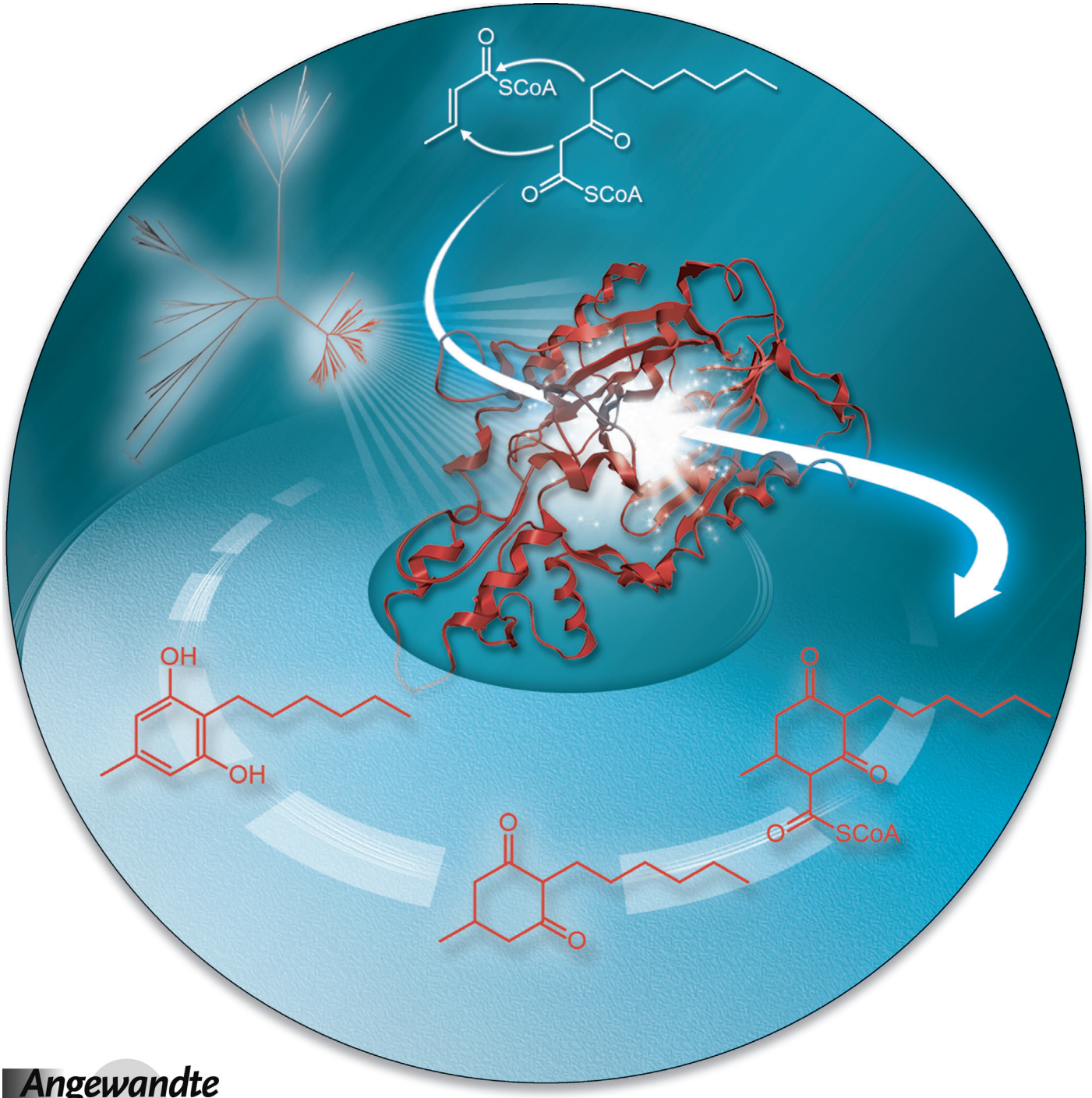


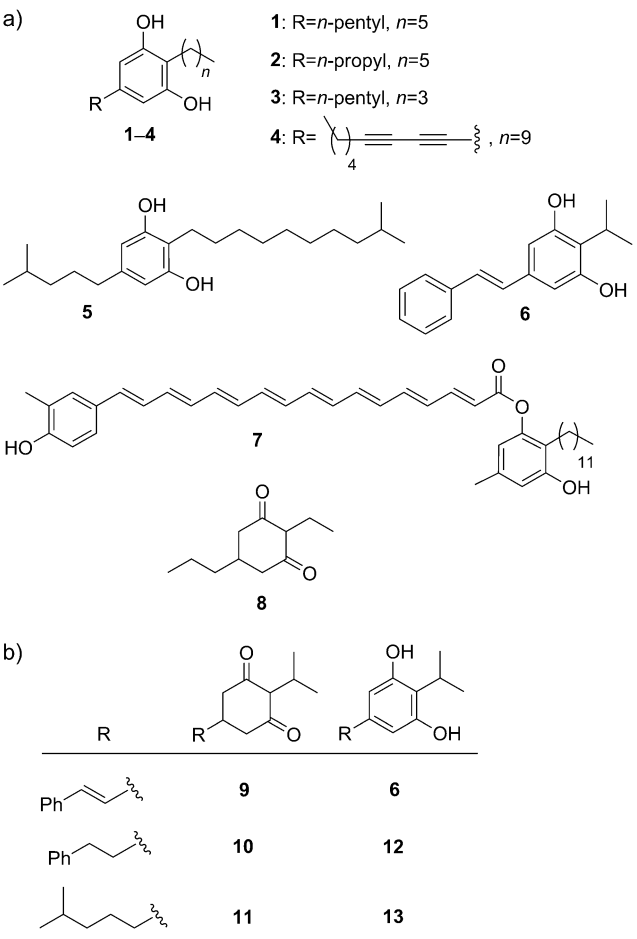
Ketosynthases are essential for fatty acid and polyketide biosynthesis, and several different classes of these enzymes have been described. They can be part of large multifunctional enzymes like the type I polyketide synthases (PKS) and type I fatty acid synthases, or they exist as stand-alone enzymes as in the type II fatty acid synthases, type II PKS, or chalcone synthases (type III PKS).[1] In type II fatty acid and polyketide biosynthesis, functionally different ketosynthase classes are known, which either catalyze the first (KS III or FabH) or the consecutive elongation steps (FabF and FabB).[1,2] Despite their different functions, all ketosynthases are members of the thiolase protein family and catalyze C–C bond formation. However, recently the first member of the KS III class has been described that is a functional malonyltransferase.[3]

Herein we report a novel and widespread class of ketosynthases catalyzing the formation of 2,5-dialkylcyclohexane-1,3-diones (CHDs) from two fatty acid derived precursors. CHDs can be further oxidized to 2,5-dialkylresorcinols (DARs) by an aromatase, the corresponding gene of which is always encoded adjacent to the gene of the ketosynthase. Since different biosynthesis gene clusters in 89 bacteria have been identified as using this novel biosynthesis pathway, CHDs and DARs can be regarded as an unexplored class of natural products. The only known examples for DARs are resorstatin (**1**), DB-2073 (**2**),[4–6] stemphol (**3**),[7] microcarbonin A (**4**),[8] resorcinin (**5**), and isopropylstilbene (IPS) (**6**) and derivatives thereof from entomopathogenic *Photorhabdus* strains (Scheme 1 a).[9,10,11] The flexirubins (**7**), found in several taxa of the gliding bacteria, are DAR derivatives connected to a polyene acyl chain showing antibacterial and anticancer activity.[12,13]

Hitherto, the only example of CHD natural products are the chiloglottones (chiloglottone 1 (**8**)), which were identified in orchids of the genus *Chiloglottis*, where they act as pheromones to fool its pollinator, the wasp *Neozeleboria cryptoides*.[14]

When we analyzed the secondary metabolome of the γ-proteobacteria *Photorhabdus* sp. PB 68.1 and *Photorhabdus temperata* subsp. *thracensis*, we identified three novel CHD derivatives (**9**–**11**, Scheme 1 b). Their structures were elucidated by HR-ESI-MS (Table S4 in the Supporting Information) and detailed 1D and 2D NMR analysis and (Table S5, Figure S1). As NMR assignments were difficult owing to keto–enol tautomerism, compounds **9**–**11** were methylated to allow full NMR assignment (Table S6, Figure S1). Additionally, the DAR compounds **12** and **13** were isolated from *Photorhabdus* sp. PB 68.1, and their structures were confirmed by NMR spectroscopy (Table S5, Figure S1). According to the current hypothesis, DARs are formed by a DarA (also named StlC) catalyzed condensation of two ACP-bound β-keto-ACP-precursors (ACP = acyl carrier protein).[5,10] However, this mechanism would not allow the formation of CHD compounds like **9** and **10** as intermediates. Alternatively, a consecutive Claisen condensation and Michael addition of a β-keto- and an α,β-unsaturated-precursor would allow the formation of a CHD compound as proposed in the biosynthesis of **8**,[14] subsequently leading to the formation of DARs through oxidation.

We decided to analyze the activity of DarAB by using heterologously produced proteins from our model organism and flexirubin producer *Chitinophaga pinensis* DSM 2588. As previously described for *darABC* (*darC* encodes an acyl



Scheme 1. a) Structures of known DARs (**1**–**7**), a DAR derivative (**7**), and the only example of a CHD natural product (**8**). b) CHD derivatives (**9**–**11**) and DAR compounds (**12**, **13**) identified during the analysis described herein.

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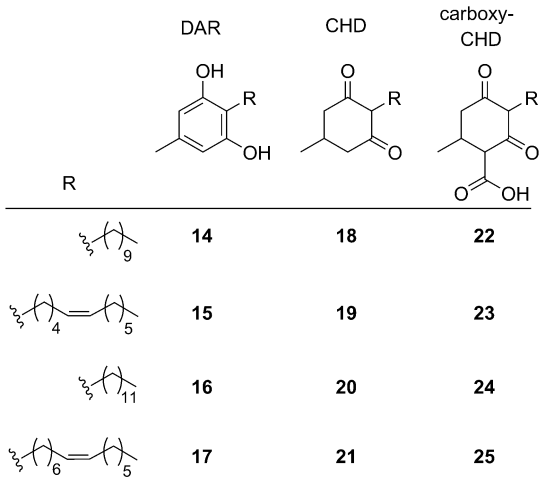
carrier protein) from *Pseudomonas chlororaphis* subsp. *aurantiaca*,[5] the heterologous expression of *darABC* from *C. pinensis* (*cpin_6851*, *cpin_6850*, and *cpin_6845*) in *Escherichia coli* led to the production of the DAR compounds **14**–**17** (Scheme 2, Figure 1 a), the structures of which were determined by HR-MS (Table S4) and NMR spectroscopy (Table S7, Figure S2). On the other hand, the expression of *darBC* did not result in the formation of DAR (Figure 1 b) but led to the identification of the CHD compounds **18**–**21** (Figure 1 d), the structures of which were also determined by HR-MS (Table S4) and NMR analysis (Table S8, Figure S2). Regarding **18**–**21** to be precursors of **14**–**17**, supports the DAR biosynthesis via CHD formation. Thus, the ketosynthase DarB catalyzes the cyclization of a β-keto-acyl precursor with a α,β-unsaturated acyl precursor, thereby resulting in the formation of a 4-carboxy-CHD, which is subsequently decarboxylated and oxidized. As expected, 4-carboxy derivatives of all CHDs were detected after heterologous expression of *darBC* (**22**–**25**; Figure 1 f); their structures were determined from MS data only, as they were produced only in trace amounts. No carboxy-CHDs were detected when *darA* was additionally overexpressed (Figure 1 e), thus indicating that DarA converts carboxy-CHD or the carboxy-CHD-thioester intermediates into the corresponding DARs. The CHDs themselves (Figure 1 d) might result from spontaneous thioester hydrolysis and decarboxylation of carboxy-CHDs.



Scheme 2. Structures found after heterologous expression of *darABC* from *C. pinensis*.

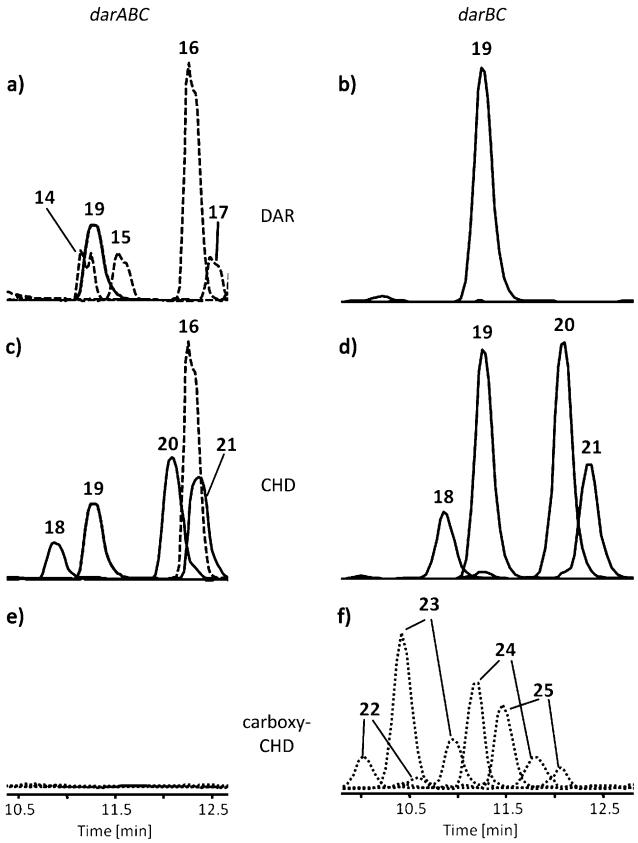
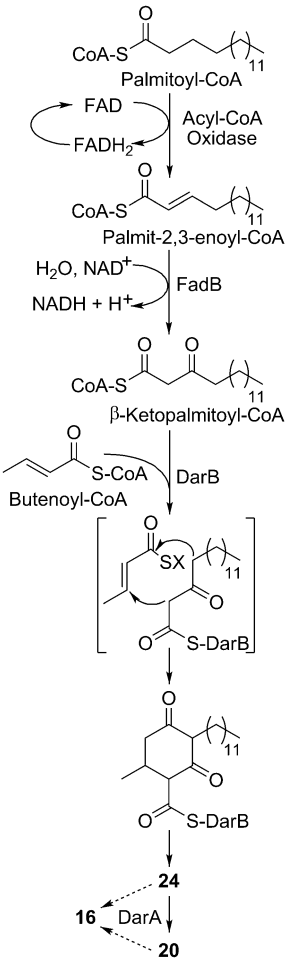


Figure 1. HPLC/MS chromatograms of extracts from *E. coli* Bl21 (DE3) Star expressing *darABC* (a, c, and e) and *darBC* (b, d, and f) from *C. pinensis*. In a and b, c and d, and e and f extracted ion chromatograms (EIC) of the DAR compounds **14**–**17** (-----), the CHD compounds **18**–**21** (——), and the carboxylated CHD compounds **22**–**25** (•••••) are shown, respectively. Chromatogram f is enlarged 10-fold relative to the intensities of b and d. Chromatograms a, c, and e are enlarged 80-fold relative to the intensities of b and d.

The DarAB homologues from *Azoarcus* BH72 (Figure S3), *P. chlororaphis* subsp. *aurantiaca* (Figure S4), and *Photorhabdus asymbiotica* (Figure S5) were also analyzed and led to the identification of additional carboxy-CHD, CHD, and DAR compounds (Table S4). Thus, DarB homologues from different organisms seem to exhibit different specificities for the length of the cyclized fatty acid derived precursors. Interestingly, the simultaneous overexpression of *darA* from *C. pinensis* and *darB* from *P. chlororaphis* subsp. *aurantiaca* also resulted in the conversion of a CHD (**30**; for structure see Figure S4 II) into the corresponding DAR (**32**; Figure S4 I), thus indicating that a cross-reaction between DarA and DarB from different organisms is possible.

To confirm the proposed mechanism for CHD formation by DarB and to show that the in vivo formation of CHD is independent of additional *E. coli* components, an in vitro assay with purified His^6-DarB fusion protein was conducted. The putative substrate β-ketopalmitoyl-coenzyme A (CoA) was generated in situ as recently described.[15] Addition of DarB and the second substrate butenoyl-CoA to β-ketopalmitoyl-CoA resulted in the production of **20** and **24** (Figure S6 I, Table S6) as proposed (Scheme 3). We also tested whether DarB can act as FabF by producing β-ketopalmitoyl-CoA from myristoyl-CoA and malonylated ACP, but here no production of **20** was observed (Figure S6 I). Thus the ketosynthase homologue DarB seems to have lost its ability to catalyze chain-elongation reactions in favor of the ability to catalyze the CHD formation from CoA-bound fatty acid biosynthesis intermediates or degradation intermediates. In vivo experiments (Figure 1) imply an aromatase activity of DarA, which oxidizes CHD to the corresponding DAR. In accordance with our finding, all described DarA homologues



Scheme 3. In vitro production of CHD **20** by DarB from *C. pinensis* using an acyl-CoA oxidase and FadB as previously described for the production of β-ketopalmitoyl-CoA.[15] The conversion of **20** or **24** into **16** was shown by heterologous expression of *darABC* in *E. coli* (Figure 1).

exhibit a weak similarity with flavin adenine dinucleotide (FAD)-containing enzymes like flavodoxin and WrpA.[16,17]

Sequence alignments and homology modeling using the crystal structure of FabH from *Aquifex aeolicus* VF5 and *Staphylococcus aureus* resulted in good models for the DarB homologues from *C. pinensis* DSM 2588, *P. chlororaphis* subsp. *aurantiaca*, and *P. asymbiotica* (Figure S7) and allowed the identification of the catalytic triad as C123-H297-N332 in DarB from *C. pinensis* DSM 2588 (Figure S8)[18] as was supported by a loss or decrease in CHD production after production of C123A and N332A DarB variants in *E. coli* (Figure S6 II). As DarB shows a dead-end alkyl-chain tunnel connected to the active site (Figure S9), a flip-flop catalysis mechanism with an open and closed conformation as it has been described for FabH from *M. tuberculosis* can be assumed.[19] In contrast to the FabH catalysis mechanism, the DarB model from *C. pinensis* DSM 2588 exhibits an additional cavity, which allows access of a second CoA-bound precursor to the active site (Figure S10). This observation is in accordance with MALDI-MS data showing that DarB can noncovalently bind two CoA-bound precursors (Figure S11).

A database search for DarAB led to the identification of 89 homologues in other bacteria (Table S10) counting only strains encoding both DarA and DarB in close proximity. A phylogenetic analysis of these DarB homologues together with other ketosynthases revealed that DarB represents a novel clade of ketosynthases (Figure 2, S12, S13). Comparison of the phylogenetic trees of all identified DarA and DarB homologues (Figure S14) suggests their coevolution.

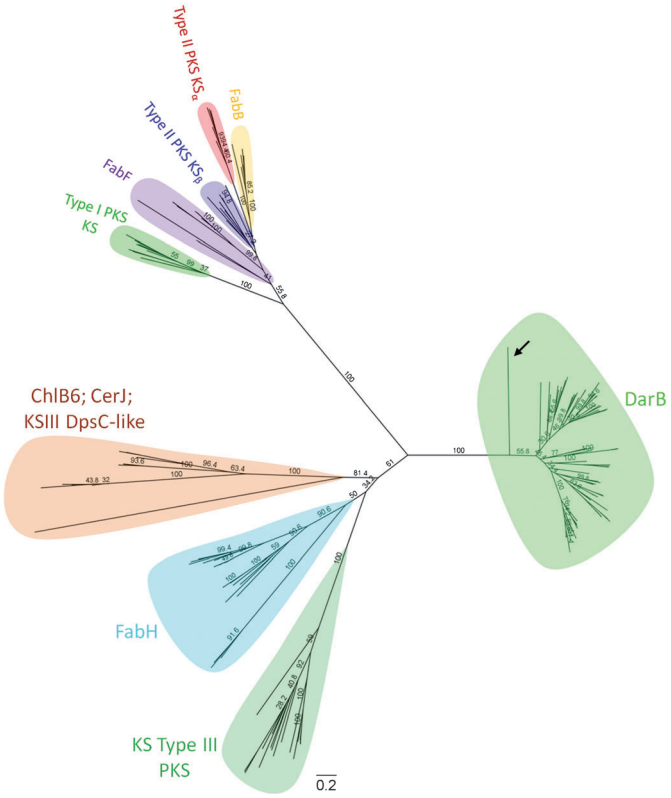


Figure 2. Phylogenetic tree (PHYML) comprising different ketosynthases. Details are listed in Table S11 and a zoomed version of the DarB branch is shown in Figure S14. The arrow points to DarB from the Gram-positive *Nocardia brasiliensis* ATCC 700358. The scale bar indicates the degree of divergence as substitutions per sequence position.

Moreover, *darA* and *darB* are always part of operons (Figure S15) as shown for example by a putative microcarbonin (**4**) biosynthesis gene cluster (Figure S15g) encoding two predicted desaturase genes; a gene cluster in *Methylobacterium* sp. 4–46 (Figure S15j) encoding two type I PKS; the only gene cluster in a Gram-positive bacterium, *Nocardia brasiliensis* ATCC 700358 (Figure S15f); and a putative flexirubin biosynthesis gene cluster[20] in the human pathogen *Myroides odoratus* (Figure S15k).

Considering the large number of bacterial genomes encoding DarAB homologues, CHD or DAR compounds appear to be a widespread natural product class, most of which remain to be identified. Among the 89 bacterial strains identified, 39 and five are human and animal pathogens, respectively, including 15 *Neisseria* strains. Another nine strains live in association with other organisms. Thus one

might speculate about a conserved function of these compounds as virulence factors as shown for the antibiotic and anticancer-active flexirubins,[13] the pluripotent isopropylstilbene (**6**) or as signaling compounds (e.g. **8**[14]). Additionally, resorstatin (**1**) has been described as a growth stimulator for mammalian cells.[21] The simultaneous production of flexirubin and resorstatin exhibiting antagonistic activities, might indicate that DAR natural products play a role in the modulation of eukaryotic host cells, an idea supported by other known activities of these compounds.[11,13,22,23] Thus the isolation of compounds corresponding to the identified biosynthesis gene clusters must be a future goal in order to assign their biological functions.

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[1] C. Hertweck, *Angew. Chem.* **2009**, *121*, 4782 – 4811; *Angew. Chem. Int. Ed.* **2009**, *48*, 4688 – 4716.
[2] S. W. White, J. Zheng, Y. M. Zhang, C. O. Rock, *Annu. Rev. Biochem.* **2005**, *74*, 791 – 831.
[3] T. Bretschneider, G. Zocher, M. Unger, K. Scherlach, T. Stehle, C. Hertweck, *Nat. Chem. Biol.* **2012**, *8*, 154 – 161.
[4] S. Kato, K. Shindo, H. Kawai, M. Matsuoka, J. Mochizuki, *J. Antibiot.* **1993**, *46*, 1024 – 1026.
[5] B. Nowak-Thompson, P. E. Hammer, D. S. Hill, J. Stafford, N. Torkewitz, T. D. Gaffney, S. T. Lam, I. Molnar, J. M. Ligon, *J. Bacteriol.* **2003**, *185*, 860 – 869.
[6] N. Kanda, N. Ishizaki, N. Inoue, M. Oshima, A. Handa, T. Kitahara, *J. Antibiot.* **1975**, *28*, 935 – 942.
[7] F. H. Stodola, D. Weisleder, R. F. Vesonder, *Phytochemistry* **1973**, *12*, 1797 – 1798.
[8] D. Beresovsky, O. Hadas, A. Livne, A. Sukenik, A. Kaplan, S. Carmeli, *Isr. J. Chem.* **2006**, *46*, 79 – 87.
[9] V. J. Paul, S. Frautschy, W. Fenical, K. H. Nealson, *J. Chem. Ecol.* **1981**, *7*, 589 – 597.
[10] S. A. Joyce, A. O. Brachmann, I. Glazer, L. Lango, G. Schwär, D. J. Clarke, H. B. Bode, *Angew. Chem.* **2008**, *120*, 1968 – 1971; *Angew. Chem. Int. Ed.* **2008**, *47*, 1942 – 1945.
[11] W. H. Richardson, T. M. Schmidt, K. H. Nealson, *Appl. Environ. Microbiol.* **1988**, *54*, 1602 – 1605.
[12] H. Achenbach, W. Kohl, H. Reichenbach, *Rev. Latinoam. Quim.* **1978**, *9*, 111 – 124.
[13] N. Mojib, R. Philpott, J. P. Huang, M. Niederweis, A. K. Bej, *Antonie Van Leeuwenhoek* **2010**, *98*, 531 – 540.
[14] S. Franke, F. Ibarra, C. M. Schulz, R. Twele, J. Poldy, R. A. Barrow, R. Peakall, F. P. Schiestl, W. Francke, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 8877 – 8882.
[15] E. B. Goh, E. E. K. Baidoo, J. D. Keasling, H. R. Beller, *Appl. Environ. Microbiol.* **2012**, *78*, 70 – 80.
[16] J. Sancho, *Cell. Mol. Life Sci.* **2006**, *63*, 855 – 864.
[17] J. Carey, J. Brynda, J. Wolfova, R. Grandori, T. Gustavsson, R. Ettrich, I. K. Smatanova, *Protein Sci.* **2007**, *16*, 2301 – 2305.
[18] X. Qiu, C. A. Janson, A. K. Konstantinidis, S. Nwagwu, C. Silverman, W. W. Smith, S. Khandekar, J. Lonsdale, S. S. Abdel-Meguid, *J. Biol. Chem.* **1999**, *274*, 36465 – 36471.
[19] S. Sachdeva, F. N. Musayev, M. M. Alhamadsheh, J. N. Scarsdale, H. T. Wright, K. A. Reynolds, *Chem. Biol.* **2008**, *15*, 402 – 412.
[20] M. J. McBride, G. Xie, E. C. Martens, A. Lapidus, B. Henrissat, R. G. Rhodes, E. Goltsman, W. Wang, J. Xu, D. W. Hunnicutt, A. M. Staroscik, T. R. Hoover, Y. Q. Cheng, J. L. Stein, *Appl. Environ. Microbiol.* **2009**, *75*, 6864 – 6875.
[21] S. Imai, K. Fujioka, K. Furihata, R. Fudo, S. Yamanaka, H. Seto, *J. Antibiot.* **1993**, *46*, 1319 – 1322.
[22] A. Pohanka, J. Levenfors, A. Broberg, *J. Nat. Prod.* **2006**, *69*, 654 – 657.
[23] A. K. Bej, US 2011/030121A1 (PCT/US10/24823), **2011**.

