Biosynthesis

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Cyanobacterial *ent*-Sterol-Like Natural Products from a Deviated Ubiquinone Pathway

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Abstract: *Natural products from marine animals show high potential for the development of new medicines, but drug development based on these compounds is commonly hampered by their low natural abundance. Since many of these metabolites are suspected or known to be produced by uncultivated bacterial symbionts, the rapidly growing diversity of sequenced prokaryotic genomes offers the opportunity to identify alternative, culturable sources of natural products computationally. In this work, we investigated the potential of using this sequenced resource to facilitate the production of meroterpenoid-like compounds related to those from marine sources. This genome-mining strategy revealed a biosynthetic gene cluster for highly modified cytotoxic meroterpenoids related to pelorol and other compounds isolated from sponges. Functional characterization of the terpene cyclase MstE showed that it generates an ent-sterol-like skeleton fused to an aryl moiety from an open-chain precursor and is therefore a promising tool for the chemoenzymatic preparation of synthetically challenging chemical scaffolds.*

Marine animals, such as sponges and tunicates, represent a vast resource of specialized metabolites with diverse pharmacological activities.[1] Several of these compounds form the basis of established drugs or are currently undergoing clinical evaluation. Commonly, however, their limited supply from slow-growing organisms represents a challenging bottleneck in drug development.[2] For an increasing number of cases, it has been shown or proposed that the true producers are symbiotic bacteria,[3] which could potentially provide access to renewable production in various ways. These include the cultivation of symbionts, heterologous pathway expression, and the identification of alternative producers with related pathways.[4] The latter strategy has been validated by us and others for several different polyketides and nonribosomal peptides.[3i–k,4c] Considering the extensive horizontal transfer of biosynthetic gene clusters in nature,[5] it is reasonable to assume that the rapidly increasing number of sequenced bacterial genomes will become an important resource for the identification of alternative producers.

To date, no biosynthetic enzymes are known for the meroterpenes that have been identified in various marine animals.[6] These compounds (e.g., **1**–**3**,[7] Figure 1) commonly contain a *p*-hydroxybenzoate (pHB, **4**) or (hydro)quinone moiety. In contrast, terrestrial meroterpenes usually carry a polyketide, amino acid derived, or other non-terpenoid portion.[8] Retrobiosynthetic analysis suggests that the marine compounds are generated through prenylation of pHB or a similar moiety, decarboxylation in case of (hydro)quinones, and terpene-cyclase-catalyzed formation of the polycyclic skeleton. To search for bacterial genes that might encode similar pathways, we examined genomes in the GenBank database or those sequenced in our laboratories for clustered genes for pHB biosynthesis, prenylation, and cyclization. This revealed a gene cluster in the cyanobacterium *Scytonema* sp. PCC 10023 with candidate genes for all three features (Figure 2 and Table S4 in the Supporting Information).

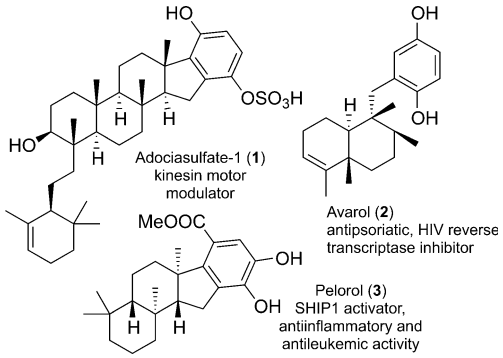

Figure 1. Selected natural products of suspected meroterpene biosynthetic origin isolated from marine sponges.

The genes encode homologues of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase (MstT) and pHB synthetase (MstD), pHB nonaprenyltransferase (MstC), and squalene-hopene cyclase (MstE). In addition, we identified further genes for terpene biosynthesis, oxygenation, and

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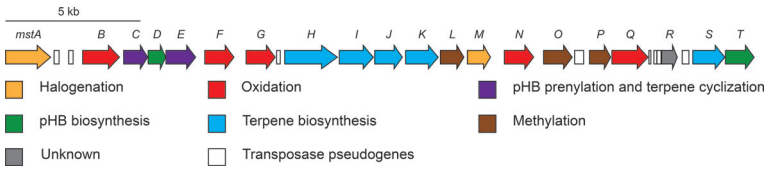

Figure 2. Proposed gene cluster for merosterol biosynthesis. Putative enzyme functions are color coded.

methylation, as well as a flavin-dependent halogenase and an Fe^{II}/α-ketoglutarate-dependent halogenase. The presence of halogenases suggests at least two halogen atoms in the metabolite, which provide a convenient handle for MS-guided isolation based on isotope patterns. To identify the unknown compound, the strain was grown for 3 to 4 months, and methanolic cell extracts were analyzed by liquid chromatography/high-resolution MS (LC/HRMS). Two peaks in the chromatogram corresponded to substances with isotopic patterns consistent with dichlorination. The *m*/*z* of the larger peak indicated a sum formula of $C_{28}H_{32}Cl_2O_8$. Purification and characterization by NMR (Figure S2–S13 in the Supporting Information) suggested the two possible regioisomers **5** and **6** (Figure 3), which could not be distinguished on the basis of the spectral data. Therefore, ^{13}C chemical shifts were calculated by quantum mechanical predictions and compared to the NMR data.[9] Calculations for **5** showed an excellent fit in contrast to the data for isomer **6** (Figure S14). Structure **5** is further supported by predictions obtained by the DP4 NMR method.[10] The relative configuration was determined by NOESY experiments and coupling constants, while the absolute configuration was determined by comparing the CD spectrum to calculated theoretical CD spectra of both enantiomers (Figure S15). This analysis suggested the final structure of **5**, which belongs to a new natural product named merosterol A. As predicted from the genomic data, the structure suggests a meroterpene biosynthetic origin with a diterpenoid moiety attached to an aromatic ring. Diverse modifications by oxygen, methyl(ene), and halogen functions match predicted gene functions, but no candidates were found for pHB decarboxylation. We were also looking for homologues of Bmp5, but could not identify any candidates.[11]

The *m*/*z* of the less abundant peak suggested a formula of $C_{29}H_{34}Cl_2O_8$. The structure was elucidated in a similar fashion as for **5** (Figure S16–S24), resulting in merosterol B (**7**, Figure 3), which differs from **5** by the absence of a lactone, the presence of a ketone, and a different position for one of the chlorine moieties.

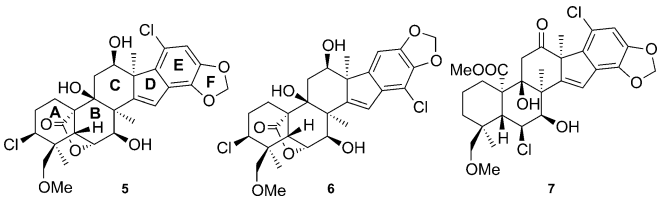

Figure 3. Structures of merosterols: Merosterol A (**5**), alternative hypothetical regioisomer of merosterol A (**6**), and merosterol B (**7**).

Merosterols contain an interesting steroid-like carbon skeleton annelated to the aromatic E moiety, but they have the opposite stereochemistry to common natural steroids. Another example of steroid-like meroterpenes are andrastins generated from a distinct polyketide synthase (PKS)/terpene biosynthesis pathway.[12] Areno-annelated and *ent*-steroids both attract interest as pharmacophores and cellular tools, but access to these substances relies on limited natural material or complex synthesis.[13] To explore enzymatic routes towards such skeletons and to test our pathway hypothesis (Figure 4 A), we investigated the initial steps of merosterol formation. Phylogenetic analysis of MstC places this enzyme into a clade containing UbiA-type enzymes that prenylate pHB in the ubi- and plastoquinone pathways (Figure 4 B and Figure S34).[14] This position and the close similarity of MstDT to enzymes participating in such routes (Table S4) suggest that the *mst* pathway has diverged from ubi/plastoquinone biosynthesis to specialized metabolism through the acquisition a terpene cyclase and diverse other modifying enzymes.

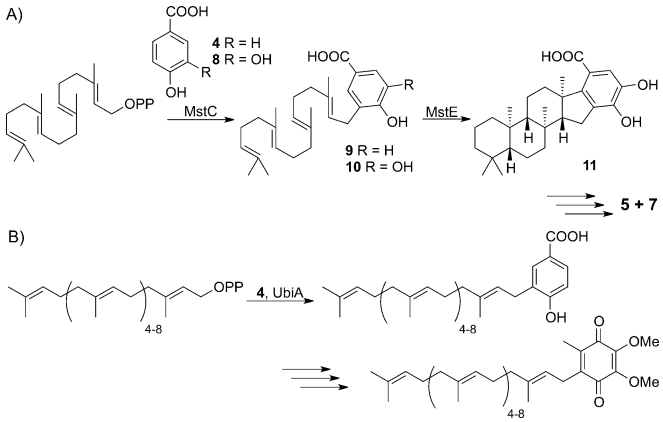

Figure 4. Proposed early steps of merosterol formation and its relationship to prenylated quinone biosynthesis in central metabolism. A) In the proposed merosterol route, MstC attaches a geranylgeranyl unit to pHB (**4**) or 3,4-dihydroxy benzoate (**8**), which is then cyclized by MstE. Further modifications likely happen after cyclization. It is still unclear whether **4** or **8** is prenylated. B) Role of the prenyltransferase UbiA in ubiquinone biosynthesis.

To investigate formation of the polycyclic core structure, we expressed the putative terpene cyclase gene *mstE* as a His-tagged protein in *E. coli* (Figure S35). Suspecting a ubiquinone-like biosynthesis, we synthesized 3-geranylgeranyl-(GG)-pHB (**9**) according to a published procedure.[15] However, assays with the purified terpene cyclase MstE did not show conversion (Figure 5 A,B).

To test for alternative substrates, we performed a protein assay based on intrinsic tryptophan fluorescence.[16] In this assay, substrate binding changes the protein conformation, which affects the fluorescence if a change in the environment of tryptophan residues occurs. This change can be expected

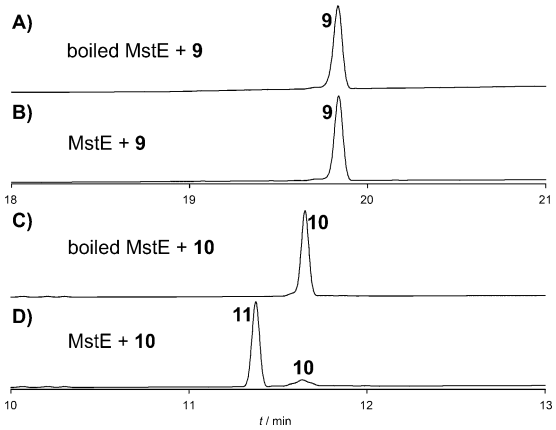

Figure 5. HPLC traces (at 254 nm) of MstE assay products. A) 3-GG-pHB (**9**) with boiled MstE. B) **9** with active MstE. C) 3-GG-4,5-dihydroxy benzoic acid (**10**) with boiled MstE. D) **10** with active MstE. Only for the dihydroxylated substrate **10** does the enzyme produce a new product.

for tryptophans in the active site of class II cyclases, to which MstE belongs.[17] Assaying pHB (the core moiety of **9**) along with six variants, we observed that 3,4-dihydroxy benzoate (**8**) and other tested 3,4-oxygen-substituted variants showed a distinct fluorescence change, while pHB was virtually inactive (Figure S36). Based on this result, we synthesized 3-GG-4,5-dihydroxy benzoate (**10**) by following a similar procedure as for **9**. Reactions with MstE resulted in conversion of **10** into a product with the same *m*/*z* but a different HPLC retention time as the substrate (Figure 5C,D, Figure S25). NMR and CD analysis of the purified product along with computational studies (Figure S26–S33) confirmed the cyclized product as **11** (Figure 4A). To test the possibility of this being a side reaction of a standard squalene-hopene cyclase, squalene was also tested with MstE, but no product was detected (Figure S42).

In bioassays, the merosterols were inactive against Gram-positive (*Arthrobacter crystallopoietes*) or Gram-negative (*Pseudomonas syringae*) bacteria up to 100 $\mu g\,mL^{-1}$, but they were cytotoxic against HeLa cancer cells with an IC_{50} of 1.8 μM for **5** and 11.6 μM for **7** (Table S3). With its benzo-fused structure, quasi-enantiomeric stereochemistry compared to standard steroids, and ease of chemoenzymatic preparation, the merosterol skeleton could be the basis of interesting pharmacological applications, especially considering the wide range of activities displayed by the structurally and possibly also biosynthetically related compounds from marine animals (Figure 1).[7,18] The identification of the merosterols provides further evidence that Cyanobacteria are a rich source of natural product types previously associated with marine animals.[3a,i,4c,19]

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Conflict of interest

The authors declare no conflict of interest.

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