Biosynthesis

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Geminal Bismethylation Prevents Polyketide Oxidation and Dimerization in the Benastatin Pathway**

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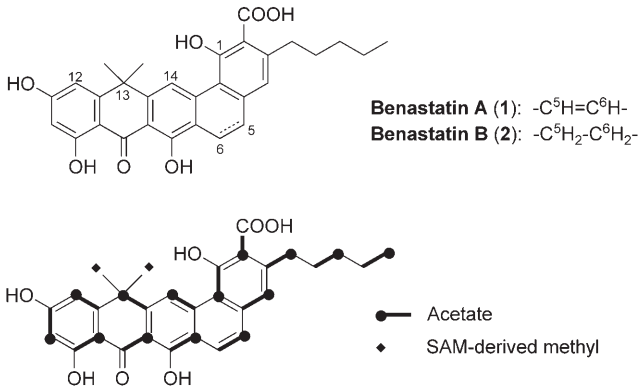
Aromatic polyketides comprise a structurally diverse group of polyphenolic compounds that differ largely in the number of carbocycles, their topology, and ring substitutions.[1–4] One of the most frequently found substructures is the quinone moiety,[4] which results from either spontaneous[5] or enzymatic[6] oxidation at benzylic positions of the polyketide. A clear exception is the small family of benastatins (Scheme 1),[7,8] which have attracted considerable interest as potent glutathione *S*-transferase (GST) inhibitors[9] and inducers of apoptosis.[10] Instead of the quinone moiety, these unusual fatty acid/polyketide hybrid metabolites produced by *Streptomyces* species bear highly unusual geminal methyl substituents. Isotope labeling studies have revealed that both methyl carbon atoms are derived from *S*-adenosylmethionine (SAM).[11] Considering the large number of aromatic polyketides, it is remarkable that similar ring substitutions are only known for two other polyphenols: resistomycin[12,13] and the antibiotic L-755,805.[14] It should be noted that quinone analogues of benastatins, named bequinostatins, have been isolated from another *Streptomyces* sp.[15]



Scheme 1. Structures of benastatins and their isotope labeling pattern.

To elucidate the pathway to the pentangular[16] benastatin and to engineer novel antitumor agents, we recently cloned and sequenced the entire benastatin biosynthesis gene cluster (*ben*) and proved its identity through heterologous expression (Figure 1).[17] Here we report the first functional analysis of a gene that encodes a novel *C*-methyltransferase (BenF) that is unique in catalyzing a geminal bismethylation. In the absence of BenF, alternative reaction channels take place involving quinone formation and dimerization.

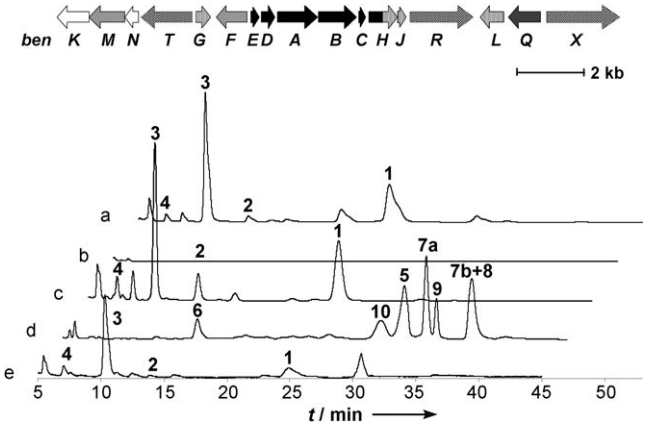


Figure 1. Organization of the *ben* biosynthesis gene cluster and chromatographic profiles of extracts from a) *Streptomyces* sp. A2991200 (wild-type producer), b) *S. lividans* TK23 (heterologous host), c) *S. lividans* TK23/p5H09 (entire *ben* gene cluster), d) *S. lividans* TK23/pXU-B02 (Δ*benF* mutant), and e) *S. lividans* TK23/pXU-B02 + pXU109 (complemented mutant).

Only a single candidate gene (*benF*) for a *C*-methyltransferase was detected in the 17 kb benastatin (*ben*) biosynthesis gene cluster. The deduced gene product of *benF* contains a methyltransferase motif (pfam00891) and shows high similarity to a range of methyltransferases that utilize SAM as a methyl group donor. The cosubstrate for BenF is most likely provided by BenM, which is a SAM synthetase homologue. From the co-occurrence of the nor-hydroxy derivatives **3** and **4**, in particular upon increased aeration, we concluded that polyketide oxidation competes with the transfer of a methyl group. It should be noted that similar hydroxymethyl moieties are characteristic for tetracyclines.[1] To establish the function of *benF*, we excised the gene from the *ben* gene cluster in *E. coli* by an in-frame deletion.[18,19] The resulting incomplete

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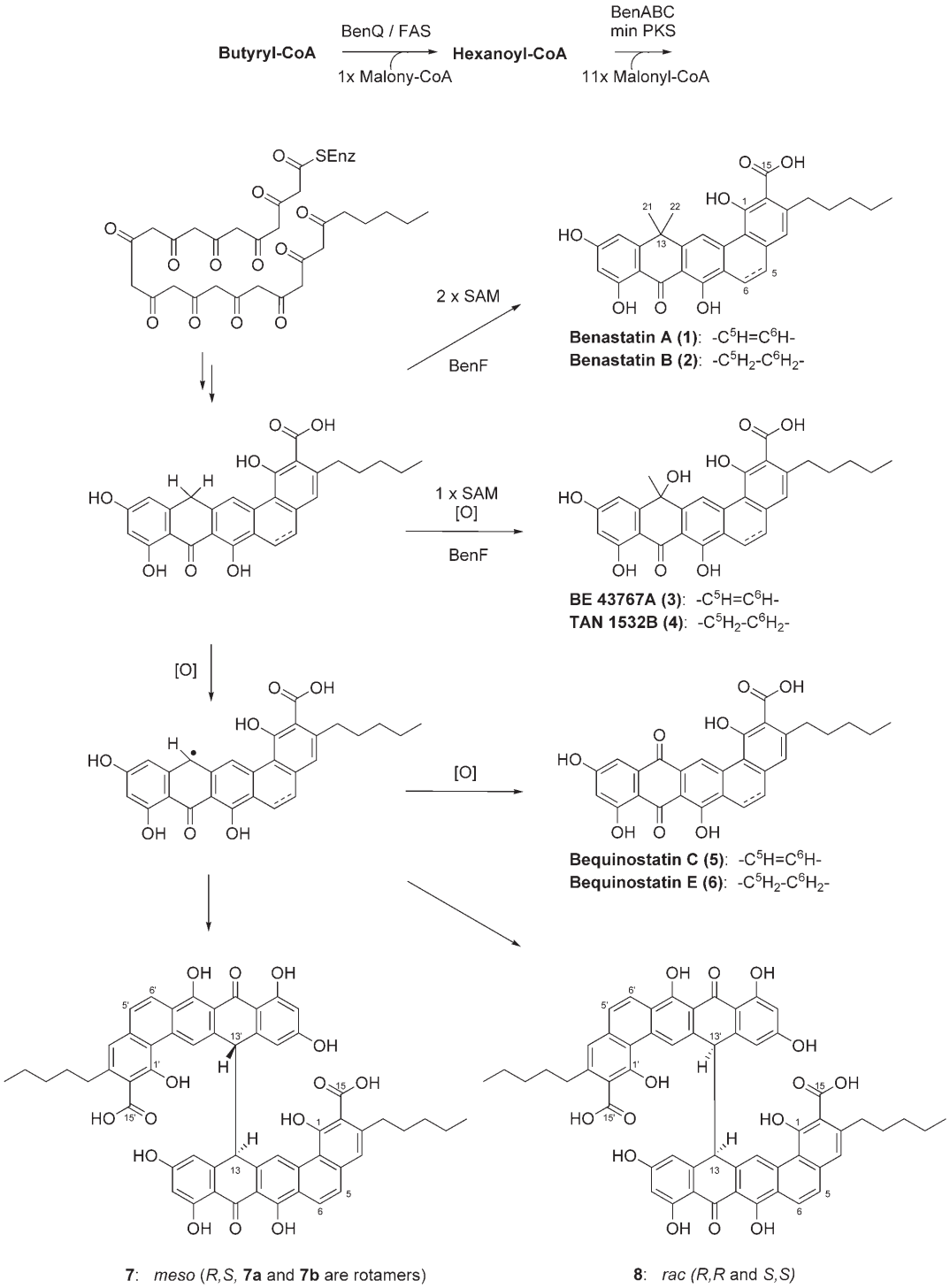
gene set was introduced into the heterologous host *Streptomyces lividans* TK23 for expression, and the metabolic profile was monitored by HPLC-MS. As shown in Figure 1, the mutant (*S. lividans* TK23/pXU-B02) proved to be incapable of benastatin biosynthesis. No benastatins or expected anthrone-like intermediates were detectable. In contrast, several new products (**5**–**10**) were formed (Figure 1). Complementation of the mutant with a coexpressed *benF* gene fully restored the natural benastatin pathway, clearly ruling out any polar effects. The major metabolites (**5**–**8**) were isolated from a scaled-up *S. lividans* TK23/pXU-B02 fermentation (20 L). Comparison of spectroscopic data revealed that **5** is identical with the known bequinostatin C,[20] which bears a benzo[*a*]naphthacenylquinone chromophore. Compound **6** appeared to be similar to **5** (Scheme 2), but has a molecular formula of $C_{28}H_{24}O_8$, as deduced from HRMS and ^{13}C NMR spectroscopic analysis. Data from both compounds only differ in the chemical shifts of C-5 and C-6, which correspond to



Scheme 2. Structures of benastatins (**1** and **2**) and their derivatives **3**—**8**, as well as a proposed biosynthetic pathway.

saturated carbon atoms in the case of **6**. An assignment of all the NMR signals by DEPT135, COSY, HMQC, and HMBC experiments fully established the structure of **6** as a quinone analogue of **2**, and was thus named bequinostatin E (Scheme 2).

LC-MS indicated that compounds **7a**, **7b**, and **8** share the same molecular mass (*m*/*z* 942). Compound **7a** was first separated from a mixture of **7b** and **8** by preparative HPLC; the compounds being partially separated by using a different gradient and by collecting the flanks of the overlapping peaks. While **8** proved to be relatively stable, we noted that **7a** and **7b** are interchangeable. Therefore, **7a** and **7b** could only be obtained in small amounts, as they reestablish their equilibrium within one day. Consequently, their identification proved to be challenging.

For all three compounds (**7a**, **7b**, **8**) a molecular formula of $C_{56}H_{46}O_{14}$ was deduced from the HRMS data, and all the NMR spectra were reminiscent of that of **1**. However, the spectra were lacking signals for the C-13 methyl groups and **7a** and **7b** show additional ^{1}H signals (H-13) at $\delta = 4.84$ ppm and 4.50 ppm, respectively. Furthermore, the H-12 signals are shifted to higher field at $\delta = 6.17$ ppm (**7a**) and 6.04 ppm (**7b**). H-13 of **8** resonates at $\delta = 4.91$ ppm. Interestingly, the H-12 signal ($\delta =$ 5.14 ppm) in **8** is shifted to an unusually high field, while the H-14 signal ($\delta = 10.34$ ppm) is shifted to very low field. From the MS data and observed cross-couplings of the H-13 signals with the C-13 signal in the HMBC spectra we concluded that **7a**, **7b**, and **8** represent 13,13′-fused dimers. This finding was also corroborated by the cross-peaks of H-13 with C-14 and C-12. All the data from DEPT135, COSY, HMQC, and HMBC experiments clearly indicated that **7a**, **7b**, and **8** represent 13,13′-fused bis-nor-benastatin A dimers. However, this data did not lend support to any reliable assignment of the possible *meso* (*R*,*S*) and racemic (*R*,*R*/*S*,*S*) configurations. To solve this issue, we first carried out NOESY and ROESY experiments, but these techniques did not provide any relevant insights.

We next performed extensive theoretical calculations, including the simulation of the NMR spectra for various possible benastatin dimers and their conformers. Despite overall good agreement, a comparison with the NMR data did not lead to any satisfactory conclusion. To learn more about the possible conformers of the *meso* and *rac* dimers, we established an ensemble of lowest energy conformers by a Monte–Carlo search using the program package "Spartan".[21] The eight lowest energy conformers from each set where chosen and a local energy minimization was performed at a semiempirical level (AM1, 6-31G*). These calculations revealed a single stable conformer for the *rac* dimer and various rotamers of very similar energies for the *meso* dimer. The *rac* dimer exists in a preferred conformation as a result of strong intramolecular hydrogen-bonding interactions of both the C-15 and C-15′ carboxy groups (Figure 2). In contrast, the *meso* dimer (**7**) shows no clear preference for a single conformation and several almost isoenergetic conformers exist.

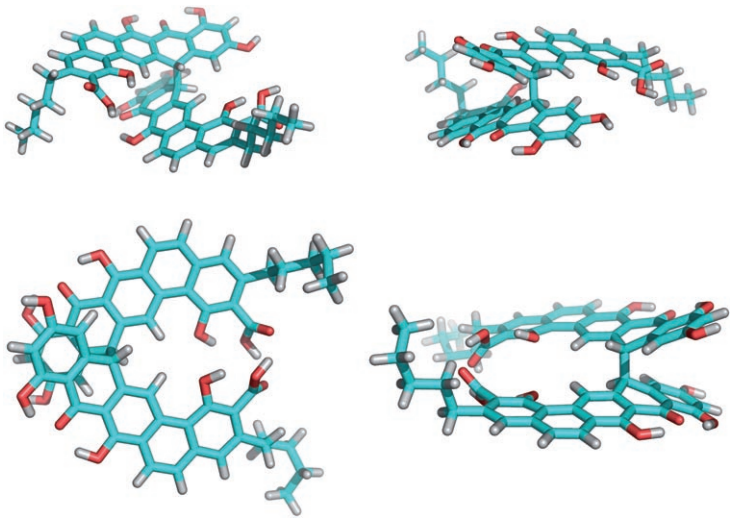


Figure 2. Three-dimensional structures of selected lowest energy conformers of benastatin derivatives **7** (top) and **8** (bottom), identified by a Monte–Carlo conformational search using the program package Spartan.[21]

From these results we could deduce that **8** represents the stabilized *rac* dimer with an *R*,*R* or *S*,*S* configuration, which gives a single NMR spectrum. The *meso* dimer is represented by **7**, which exists in conformers (**7a** and **7b**) that can be separated by HPLC but rapidly equilibrate.

Finally, LC-MS data suggested that compounds **9** and **10** represent "mixed dimers" resulting from the cross-coupling of C5–C6 saturated and unsaturated anthrone intermediates (see the Supporting Information).

The unusual benastatin dimers **7**–**10** are somewhat reminiscent of various plant-derived bisanthrones.[22] An important example is emodin bianthrone, the proposed key intermediate in hypericin biosynthesis.[23] It is known that nonsubstituted anthrones are highly unstable and readily oxidized to yield quinones and dimeric products via benzyl radicals.[5,23–26] In contrast to enzymatic aryl coupling reactions,[27,28] these reactions do not necessarily require biocatalysts. Thus, geminal bismethylation of the benastatin anthrone precursor may be regarded as a means to block these alternative oxidative reaction channels. In fact, as opposed to the parent benastatins, the dimers proved to be inactive in all cytotoxicity assays. Both our inactivation and complementation experiments revealed that both C-methylation steps are catalyzed by BenF. Partial methylation with subsequent oxygenation leads to hydroxymethyl moieties that are known from tetracyclines. In the future it will be interesting to learn why BenF catalyzes two methylations, while an as yet uncharacterized *C*-methyltransferase from the oxytetracycline pathway transfers only a single methyl group to a dihydroanthrone. It should also be noted that N-dimethylating enzymes are known from aminosugar biosynthesis,[29,30] and the *C*- and *N*-dimethyltransferases likely share the same underlying mechanisms.

In summary, we have shown by heterologous expression of complete and incomplete sets of benastatin biosynthesis genes that the unusual geminal bismethylation is catalyzed by a single *C*-methyltransferase (BenF). Although numerous

methyl transferases are known from all sorts of organisms, *benF* codes for a rare methyltransferase that can introduce two methyl groups at the same carbon atom. However, methylation at the highly reactive benzylic position competes with oxygenation, as evidenced by the formation of hydroxymethyl derivatives when fermenting the wild type with increased aeriation. In the absence of BenF, the unstable anthrone-like intermediate is subject to oxidation to yield the quinone derivative bequinostatin C, as well as the previously unknown bequinostatin E. In addition, various structurally intriguing bis-nor-benastatin dimers are formed in the mutant broth. Their configurations (*meso*/*rac*) could only be solved in conjunction with computational simulation, thus highlighting the synergistic effects of experimental and theoretical approaches. Finally, geminal bismethylation catalyzed by BenF represents a novel polyketide modification, and is an unprecedented example of a natural protection strategy to block alternative reaction channels.

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