Communication

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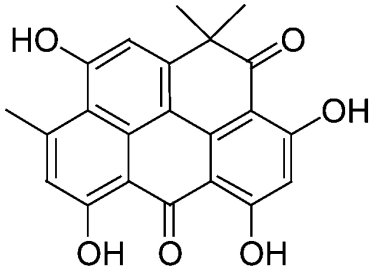
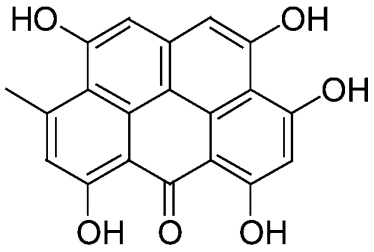


Resistomycin



Bis-nor-resistomycin

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Geminal Tandem *C*-Methylation in the Discoid Resistomycin Pathway

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Bacterial aromatic polyketides represent a large group of structurally diverse natural products many of which are medicinally relevant.[1] It is remarkable that virtually all members of this group feature linear or angular ring topologies. A clear exception, however, is the discoid naphthanthracene derivative resistomycin (*rem*, **1**) from *Streptomyces resistomycificus*.[2] The orange fluorescent compound exhibits antibiotic activities against gram positive bacteria and mycobacteria, inhibits HIV-1-protease as well as DNA- and RNA-polymerases, and modulates apoptosis.[3,4]Besides the cumulated pentacyclic ring system **1** features a rare geminal bismethyl substitution pattern that is derived from two methionine carbons.[5] According to the pathway model (Figure 1), **1** likely shares early biosynthetic steps with linear decaketides of the tetracenomycin (*tcm*) family, but both pathways diverge owing to varying U- and S-shape cyclization of the putative decaketide. Considering the S-shape cyclization pattern it is conceivable that bismethylation is the key to discoid cyclization. If the geminal bismethylation took place prior to the final cyclization, the condensation of C2 and C19 in the *tcm* pathway would be blocked. Conversely, in the discoid *rem* pathway a geminal bisalkylated C2 position would not only favor a C1−C10 cyclization, but would also hamper the reverse reaction owing to angle compression (Thorpe−Ingold effect).

To gain an insight into the mechanisms of discoid polyketide formation we have cloned and sequenced the entire resistomycin/resistoflavin biosynthetic gene cluster[6,7] (Figure 2). According to database searches enzymes involved in the unusual bismethylation could be encoded by *remG* and/or *remH*. The deduced gene products show moderate overall sequence similarity to SAM-dependent methyl transferases (MT). To investigate the function of *remG* and *remH* we performed gene inactivation and complementation. Mutants lacking *remG* and *remH* as well as both genes were constructed by PCR-targeted recombination using cosmid pKJ05, which harbors the entire *rem* gene cluster,[6] and appropriate primers (Figure S1). MT gene deficient plasmids were introduced into *S. lividans* TK23 by protoplast transformation, and the resulting mutants were cultivated. HPLC−MS monitoring of the metabolic profiles revealed that both the Δ*remGH* double mutant and the Δ*remG* mutant are incapable of producing resistomycin. In contrast, we were surprised to find resistomycin biosynthesis was not affected in the Δ*remH* mutant. In all cases polar effects could be excluded by successful complementation. To achieve this goal, *remG*, *remH*, and *remGH* were PCR-amplified together with their native ribosome binding sites, sequenced and cloned downstream of the constitutive *ermE* promotor of a pSET152-based vector, pKJ55 (Figure S2). The Δ*remGH* mutant could be complemented through simultaneous expression of *remGH*. Furthermore, resistomycin biosynthesis could also be restored through coexpression of *remG* alone but not by *remH*. This clearly demonstrates that only a single MT, RemG, is required for introducing both methyl groups into **1**.

HPLC−MS analyses of the fermentation broths pointed out that the mutants lacking *remG* produce a highly unstable new compound

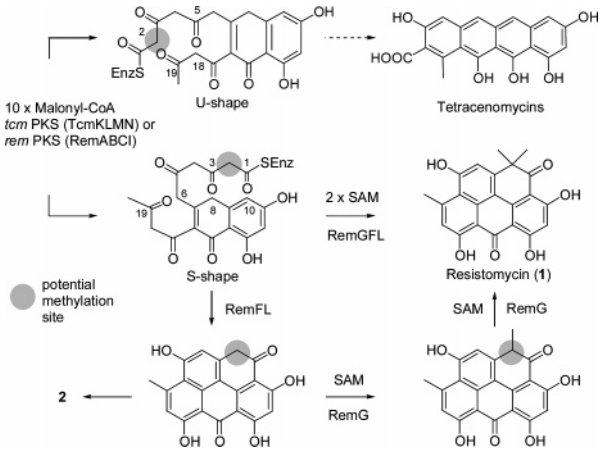

Figure 1. Model for the biosynthesis of tetracenomycins versus discoid resistomycin and potential role of alkylation in the mode of cyclization.

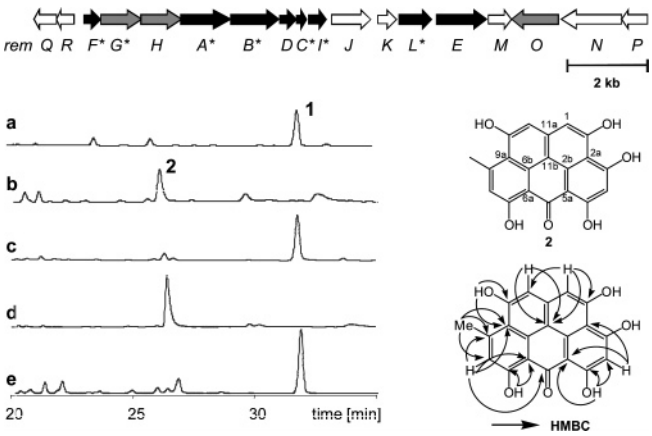

Figure 2. Organization of the *rem* gene cluster. Genes essential for resistomycin biosynthesis are marked with an asterisk (top). Selected results from gene inactivation and complementation experiments: HPLC profiles of extracts from (a) host harboring entire *rem* cluster, (b) Δ*remGH* mutant, (c) Δ*remH* mutant, (d) Δ*remG* mutant, (e) complemented Δ*remG* mutant (bottom left). Structure of **2** and HMBC correlation (bottom right).

(**2**, *m/z*: 347 [M−H]$^-$) related to resistomycin. For a full structure elucidation the crude extract from an upscaled fermentation of the mutant was subjected to open column chromatography on silica and subsequent preparative RP-HPLC. The fraction containing **2** was further purified on Sephadex LH-20. Because of the instability of the metabolite, all steps needed to be performed with exclusion of air, otherwise complex mixtures of inseparable polymers were formed. The high-resolution ESI-MS (*m/z* 347.0548 [M−H]$^-$) indicated that **2** had the molecular formula of $C_{20}H_{12}O_6$ and pointed toward a bis-nor-precursor of resistomycin. ^{1}H and ^{13}C NMR data confirmed the absence of aliphatic methyl groups. The pentacyclic structure of **2** was fully elucidated by the detailed analyses of HMBC and HMQC spectra (Figures S4−S7). Notably, **2** was

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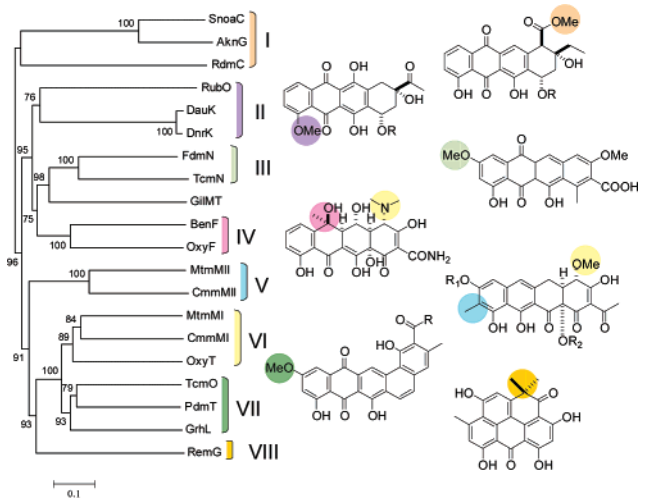

Figure 3. Phylogenetic tree of representative *O*-, *C*-, and *N*-MTs in type II PKS systems and selected methylated products. All relevant bootstrapping values are shown (nodes).

isolated exclusively in the enol form and exhibits a pseudosymmetric pentacyclic structure. We next aimed at characterizing the unusual MT that is capable of a tandem *C*-methylation. Soluble RemG was produced in *E. coli* as MalE fusion protein using the pMal c2x vector. The identity of the protein was confirmed by MALDI-TOF fingerprint analysis yielding a sequence coverage of 41.9% (Figure S8). Unfortunately, all attempts to transform **2** by RemG in vivo or in vitro were apparently hampered by the lability of the substrate and its low solubility in aqueous buffers. Nonetheless, by gene inactivation and complementation experiments we could unequivocally demonstrate that RemG is responsible for the introduction of both methyl groups. While bismethylation is well-known for dimethylamino moieties,[8,9] the only other known bis-*C*-MT has been implicated in the context of benastatin biosynthesis.[10−12] Furthermore, the structure of **2** indicates that methylation occurs after, and not during, chain elongation and cyclization. This was further corroborated by coexpression of *remG* with incomplete sets of *rem* pathway genes. Deletion of each structural gene of the *rem* cluster and investigation of the metabolic profiles of the mutants did not result in the formation of any methylated shunt products. Taken together, these results give strong evidence that methylation does not take place on an ACP-bound intermediate prior to the final cyclization. Because of the unique substrate and mechanism we reasoned that RemG represents a novel type of MT and therefore compared its amino acid sequence with related MT sequences in a phylogenetic analysis based on the neighbor-joining method (see experimental). Carboxy-*O*-MTs[13] and hydrolases served as an outgroup (clade I).

The cladogram (Figure 3) shows that the site of methyl transfer correlates well with the types of MTs. Clade II represents *O*-MTs that transfer the methyl group to chelated hydroxyls on the A-ring of anthracyclic and pentangular polyphenols. Clades III and VII comprise A- (or terminal) ring *O*-MTs involved in the biosyntheses of pentangular polyphenols[14,15] and tetracenomycins,[16] respectively. The gilvocarcin MT[17] seems related but does not fit into any particular group. Conversely, B-ring *C*-MTs in tetracycline[18] and benastatin[12] biosynthesis form a well supported clade IV, and A-ring *C*-MTs from the mithramycin[19] and coumermycin pathways[20] can be found in clade V. In clade VI both D-ring *N*- and *O*-MTs from tetracyclic polyphenols are assembled. Finally, the cladogram indicates that RemG from the *rem* pathway is the only representative of a novel group of *C*-MTs (clade VIII). Another interesting observation is that the MTs cluster according to the site of methylation not according to their function (mono-*C*-MT OxyF vs bis-*C*-MT BenF, *O*-MT MtmMI vs bis-*N*-MT OxyT). Thus, the phylogeny may be used as a map to estimate the site of methylation based on the sequence.

In summary, gene inactivation, complementation, and reconstruction of the biosynthetic pathway gave a surprising result. It revealed that only one of the two methyltransferases encoded in the *rem* gene cluster is required for the introduction of two geminal methyl groups of resistomycin. We succeeded in the isolation and full structure elucidation of the highly labile pathway intermediate, bis-nor-resistomycin (**2**), from a mutant lacking *remG*. The structure of **2** and further mutational studies indicated that bismethylation is the last step in resistomycin biosynthesis and not essential for discoid cyclization. The successful complementation of the mutant proved that RemG is a rare bismethyltransferase. Its identity was confirmed by isolation and purification of the protein and MALDI-TOF fingerprinting. Our phylogenetic studies using MT sequences demonstrated for the first time that the amino acid sequence correlates with the site of methylation in polyphenols. The cladogram also highlighted RemG as a novel type of bis-*C*-MT that employs a unique discoid substrate. Future structural studies will aim at elucidating the binding of the pseudosymmetric substrate and the mechanism of tandem methyl transfer.

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Supporting Information Available: Experimental details, sequence data, MALDI fingerprinting, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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