The Novel Hybrid Antitumor Compound Premithramycinone H Provides Indirect Evidence for a Tricyclic Intermediate of the Biosynthesis of the Aureolic Acid Antibiotic Mithramycin**

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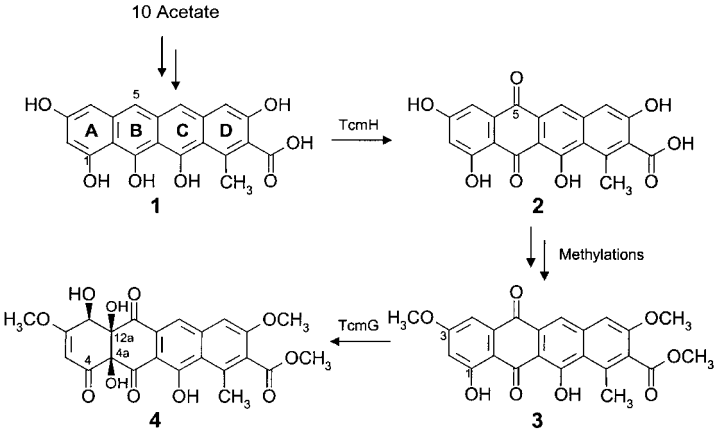
Dedicated to Professor Axel Zeeck on the occasion of his 60th birthday

Since the pioneering work of Hopwood et al. in 1985[1] the use of recombinant DNA technology has offered interesting perspectives for the generation of novel "unnatural" or "hybrid" natural products through the manipulation of biosynthetic gene clusters (combinatorial biosynthesis). Combinatorial biosynthesis is now emerging as a potential tool for the production of structural biodiversity. Gene disruption and recombination experiments are also suitable for gaining insight into biosynthetic pathways.[2]

Our work has been focused on post-polyketide synthase (PKS) tailoring steps, mainly oxidoreductase and group transfer steps, which occur late in the biosynthetic pathways and are often responsible for the conversion of biologically inactive intermediates into active products.[3]

We describe here a recombination of the mithramycin PKS genes with oxygenase-encoding genes from the tetracenomycin gene cluster. The experiments were not only aimed at the generation of novel biologically active hybrid natural products, but also were designed to intercept a labile, early intermediate of the mithramycin biosynthesis and to provide evidence for its structure.

The oxygenases TcmG and TcmH are responsible for the introduction of four oxygen atoms in the biosynthesis of tetracenomycin C **4**. TcmH catalyzes the oxidation of the first tetracyclic anthrone intermediate tetracenomycin F1 **1** into the naphthacenequinone tetracenomycin D3 **2**. TcmG is responsible for the very last step of this biosynthesis, the conversion of tetracenomycin A2 **3** into tetracenomycin C **4** (Scheme 1), through which three further oxygen atoms are introduced.[4]



Scheme 1. Oxygenation reactions of the tetracenomycin biosynthetic pathway.

Mithramycin **8** is the most important member of the aureolic acid antitumor drugs.[5] During the past three years, we have increased the knowledge regarding the biosynthesis of this class of antibiotics tremendously. A large number of genes of the mithramycin gene cluster have been characterized and various intermediates have been identified.[6–9] The earliest known intermediates are premithramycinone **6** and its 4-demethylated derivative **5**,[7] which is converted into premithramycin B **7** through several methyl and glycosyl transfer steps. The latter is finally converted into mithramycin **8** by an oxidative opening and a ketoreduction step (Scheme 2).[6a, 8]

The premithramycinones **5** and **6** resemble tetracenomycin F1 **1** in that they are also tetracyclic biosynthetic intermediates and rings C and D of **5** and **6** are identical to rings A and B of **1**. Thus, it should be possible to intercept the mithramycin biosynthesis through the influence of one or both oxygenases from the tetracenomycin C gene cluster in order to produce premithramycin or mithramycin analogues, in which the normally aromatic rings are highly functionalized, as in **4**. If successful, this "interception experiment" should also provide further insight into the mithramycin biosynthetic pathway.

To test this hypothesis, we initially used the premithramycinone/4-demethylpremithramycinone accumulating mutant strain *Streptomyces argillaceus* M7D1[6c, 7] as a host to test whether these compounds can be modified by the foreign oxygenases TcmG and/or TcmH. In a second experiment (see below), we used *S. argillaceus* M7O2[9] as a mutant host strain, in which the oxygenase MtmOII is blocked. This strain accumulates an unknown labile intermediate of the mithramycin biosynthetic pathway which should be intercepted by the foreign oxygenases. Both experiments led unexpectedly to the same hybrid product. Plasmid pWHM1026 contains the

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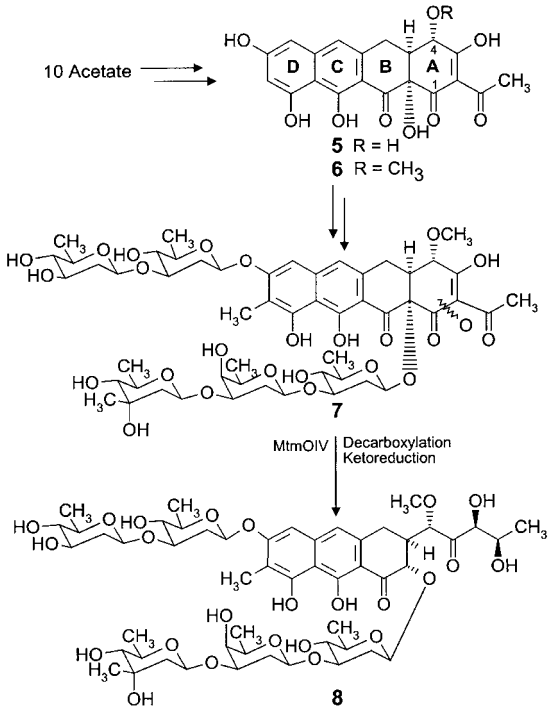


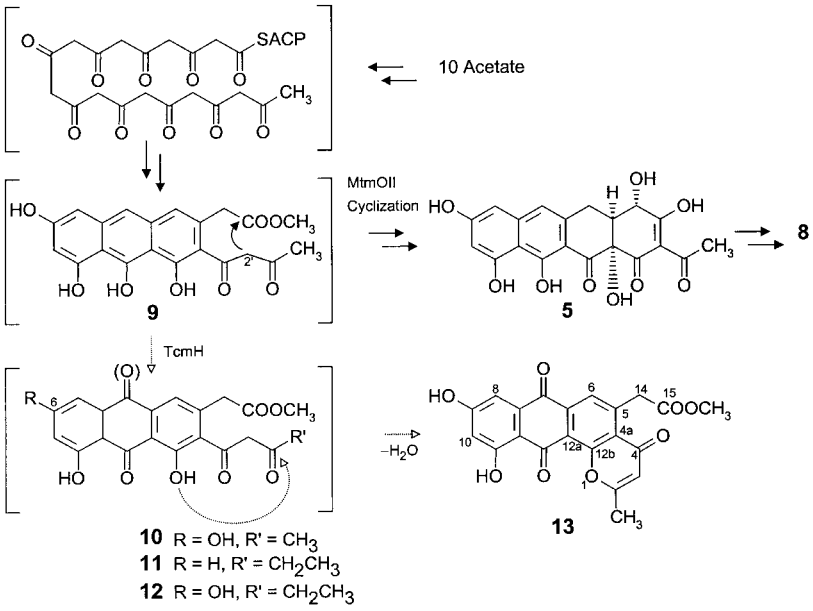
Scheme 2. The biosynthetic pathway of mithramycin **8** and the important intermediates premithramycinone **6** and premithramycin B **7**.

Table 1. NMR Data of Premithramycinone H **13**.[a]

Position	^{1}H	^{13}C	$^nJ_{C,H}$ Observable in HMBC spectrum	^4J Coupling with:[b] ($^4J_{H,H}$ [Hz])
2		167.5		
3	6.31 (d)	112.7	2-C; 4a-C; 13-C	13-H$_3$ (0.5)
4		178.5		
4a		126.6		
5		144.2		
6	7.96 (s)	126.6	4a-C; 7-C; 12a-C; 14-C	14-H$_2$ (nd)[c]
6a		134.5		
7		182.1		
7a		136.5		
8	7.07 (d)	108.5	7-C; 10-C; 11a-C	10-H (2)
9	11.37 (s, OH)	165.4		
10	6.61 (d)	109.4	8-C; 9-C; 11-C; 11a-C	8-H (2)
11	12.85 (s, OH)	165.4	10-C; 11-C; 11a-C	
11a		111.1		
12		185.7		
12a		122.4		
12b		156.0		
13	2.44 (d, 3H)	20.4	2-C; 3-C	3-H (0.5)
14	4.31 (s, 2H)	42.0	4a-C; 5-C; 6-C;15-C	6-H (nd)[c]
15		172.1		
15-OCH$_3$	3.58 (s, 3H)	52.1		

[a] In [D$_6$]DMSO/1% trifluoroacetic acid at 11.74 T. [b] Determined from H,H-COSY spectrum. [c] nd = not determinable in the 1D ^{1}H NMR spectrum.

entire **4** gene cluster.[10] A 2.5-kB *NcoI-BglII* DNA fragment (both sites blunt-ended) from pWHM1026, which contained the *tcmG* and *tcmH* genes, was subcloned into the *Bam*HI site (blunt-ended) of the plasmid pIAGO[11] under the control of the erythromycin resistance promoter, *ermE*. This construct (pFLOGH) was used to transform protoplasts of mutant M7D1. The resulting new hybrid strain *S. argillaceus* M7D1 (pFLOGH) initially accumulated mainly premithramycinone **6** when grown on R5 solid medium but, after 7 days of incubation, a new yellow compound was visible which differed from the known M7D1-products **5** and **6**. This compound was subsequently called premithramycinone H. The bathochrome shift of its UV spectrum (λ_{max} = 290, 442 nm; **6**: λ_{max} = 280, 418 nm) indicates a quinone moiety. This was confirmed by the typical decoloration reaction with sodium hydrosulfite and the NMR data (Table 1). The structure elucidation was complicated, since the ^{1}H NMR data show almost only singlets (few long-range couplings) and the ^{13}C NMR spectrum consists of a large number of quarternary signals. The FAB mass spectrum resolved the molecular formula of $C_{21}H_{14}O_8$. This and the few detectable H,H and C,H long-range couplings allow the structure for premithramycinone H to be determined unambiguously as **13** (Table 1).



Scheme 3. Normal biosynthetic pathway of mithramycin **8** (solid arrows) and the new shunt pathway to premithramycinone H **13** (dotted arrows) mediated by foreign oxygenase TcmH.

Premithramycinone H **13** is a shunt product formed by spontaneous cyclization of anthraquinone **10**, which is probably derived from tricyclic intermediate **9** (Scheme 3). The oxygenase TcmH oxidizes anthrone **9** (shown as the enol in Scheme 3) into the anthraquinone **10**. The anthraquinone formation stabilizes **9** and changes its properties. This oxidation reaction is accompanied by the attack of the phenolic 1-OH group at the 3′-keto group (dotted arrow), while in the normal mithramycin biosynthetic pathway the interchain cyclization between C-2′ and the ester carbonyl (solid arrow), which is catalyzed by an Mtm cyclase, presumably MtmX, dominates.[6a, 12] Intermediate **10** closely resembles **11**[12b] and other early intermediates (such as compound 58B **12**) of the biosynthetic pathways of anthracyclines

(for example doxorubicin).[13] Compound **9** resembles 12-deoxyaklanonic acid, a proposed intermediate of the doxorubicin biosynthesis,[13a] and also tetracenomycin F2.[14a] Like the latter in the tetracenomycin pathway, **9** represents the earliest discovered intermediate of the mithramycin biosynthetic pathway for which evidence now exists. Premithramycinone H **13** is a hybrid natural product which is formed under the influence of the foreign oxygenase TcmH; the other heterologously expressed oxygenase TcmG has no effect.[14b] TcmG fails to react on intermediate **10**, although this resembles its natural substrate tetracenomycin A2 **3** in respect of two rings (see above). Thus, for an interaction of TcmG with compound **10**, a methylated 6-OH group is probably necessary (this is the equivalent of the 3-OCH_3 group of **3**).

Since premithramycinone H **13** does not contain the hydroxyl groups that are supposed to be introduced by the MtmOII oxygenase to form 4-demethylpremithramycinone **5**, it might be expected that TcmH could act on a nonhydroxylated mithramycin intermediate prior to **5**. M7D1 (pFLOGH) was able to either hydroxylate this hypothetical intermediate **9** through the action of MtmOII oxygenase generating **5** and **6** or to generate premithramycinone H **13** by the action of TcmH. For this reason we tested a possible action of TcmH on the intermediate accumulated by mutant M7O2.[9] The M7O2 mutant was transformed with pFLOGH and, interestingly, **13** was also accumulated by this recombinant strain. This supports the hypothesis of a tricyclic intermediate **9** since the oxygenase MtmOII, which is supposed to convert **9** or a corresponding tetracyclic intermediate,[9] cannot act once anthraquinone **10** is formed through the action of TcmH. In contrast to mutant M7D1 (pFLOGH) which also produces the premithramycinones **5** and **6**, mutant M7O2 (pFLOGH) solely accumulates premithramycinone H **13**. This suggests that the proposed tricyclic intermediate **9** is likely to be the true substrate of oxygenase MtmOII and in the normal mithramycin biosynthetic pathway the oxygenation step catalyzed by MtmOII occurs prior to the fourth cyclization to 4-demethylpremithramycinone **5**. This is in contrast to our earlier assumptions, in which we favored a tetracyclic compound to be the substrate of MtmOII.[9] Since it was not yet possible to isolate the unstable compound accumulated by the M7O2 mutant, this interception experiment provides the first indirect information regarding the MtmOII substrate. The pyranone formation of **13** from **10** seems unlikely due to the poor nucleophilicity of the *peri*-hydroxy groups in anthraquinones.[14c] Thus, quinone **10** might not be a true intermediate of the shunt pathway to **13**, and TcmH may interact simultaneously with or even after the pyranone formation. This also means for the normal pathway to mithramycin **8** that cyclization to **5** can only occur after the action of MtmOII on the tricyclic intermediate **9**. The M7O2 mutant is not affected in its deoxysugar biosynthesis or glycosyl transfer (in contrast to the M7D1 mutant) and it has the full capability to synthesize and transfer the mithramycin sugars. However, no glycosylated derivatives of **13** could be detected, probably due to a lack of substrate flexibility of the Mtm glycosyl transferases.

Like mithramycin **8**, premithramycinone H **13** showed antibacterial activity against *Micrococcus luteus* and *Streptomyces albus*. The anticancer activity (see Table 2)[15] is in almost the same range as that of mithramycin, in contrast to premithramycinone **6** which shows no antibacterial and only very weak antitumor activities.

Table 2. Proliferation inhibition assays with four different cancer cell lines (IC_{50} [$\mu g\,mL^{-1}$]).[15]

	13	**8**	**6**
Squamous cell carcinoma KB	11.7	10	29.3
Melanoma SK-Mel 30	15.3	9.6	–[a]
Non-small cell lung carcinoma A549	19.1	12.9	25.2
Breast carcinoma MDA-MB 231	15.2	12.3	37.7

[a] No inhibition ($> 40\,\mu g\,mL^{-1}$).

Experimental Section

Microorganisms and culture conditions: *S. argillaceus* M7D1[6c] and M7O2[9] were used as hosts for the transformations. *Escherichia coli* XL1-Blue[16] was used as the host for subcloning and was grown at 37 °C in TSB medium (trypticase soya broth, Oxoid). Growth of the different clones in solid medium was in modified R5 agar medium (R5A).[6d]

DNA manipulation: Plasmid-DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations, and other DNA manipulations were according to standard procedures for *E. coli*[16d] and *Streptomyces*.[16c] Transformation of protoplasts of the two mutants followed standard procedures[16c, d] and transformants were selected for resistance to thiostrepton ($25\,mg\,L^{-1}$).

Production and isolation of premithramycinone H: Strain M7D1 (pFLOGH) was grown on R5 agar plates for 17 days at 28 °C. Strain M7O2 (pFLOGH) was grown on modified R5 liquid medium[6d] for 3 days at 30 °C. In both cases, $50\,mg\,L^{-1}$ thiostrepton was added to the medium. The cultures were extracted four times with EtOAc/0.1 % HCO_2H, concentrated in vacuo until the volume was approximately 20 mL and clarified by centrifugation. HPLC analysis of the extract revealed a main product that was purified by preparative HPLC (Column: μBondapak C18 radial compression cartridge, PrepPak Cartridge, 25×100 mm, Waters; eluent: MeOH (Gradient from 50 to 80 % methanol in 30 min)/0.1 % trifluoroacetic acid (TFA) in H_2O; flow rate: $10\,mL\,min^{-1}$). The purified material was adjusted to pH 4.0 with 0.2 M potassium phosphate buffer (pH 7.0) and diluted fourfold with water. This solution was applied to a solid-phase extraction column (Lichrolut RP-18, Merck), washed with water to eliminate salts, eluted with methanol and dried in vacuo. A final purification was achieved with Sephadex LH-20 (MeOH/TFA 99:1).

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A Metalloid Al_{14} Cluster with the Structure of a "Nano-Wheel"**

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Dedicated to Professor Heinrich Vahrenkamp on the occasion of his 60th birthday

Solutions of metastable aluminum(I) halides[1] can be prepared by condensation of the high-temperature molecules AlX (X = Cl, Br, I) with suitable donor-containing solvents. The thermodynamically favored disproportionation of these compounds to aluminum metal and the trihalide can be controlled kinetically by the choice of halide, donor, and temperature. Thus, with NEt_3 as donor a planar Al_4 species (for example, $Al_4Br_4 \cdot 4NEt_3$[2]) is obtained, whereas with THF the polyhedral subhalide $Al_{12}(AlBr_2 \cdot THF)_{10} \cdot 2THF$ forms as a result of "internal" disproportionation.[3]

An additional variation of the disproportionation is acheived by replacement of the halide with suitable bulky substituents. Use of the $N(SiMe_3)_2$ group proved to be especially successful: Reaction of $LiN(SiMe_3)_2$ with a solution of AlI provided a $Al_{77}R_{20}^{2-}$ compound with the largest *metalloid* cluster yet characterized by diffraction methods. This compound can be viewed as an intermediate on the way

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