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Premithramycinone G, an Early Shunt Product of the Mithramycin Biosynthetic Pathway Accumulated upon Inactivation of Oxygenase MtmOII

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Mithramycin (**1**, MTM, Scheme 1, also known as mithramycin A, mithracin, and plicamycin) is an aureolic acid anti-cancer agent produced by various soil bacteria of the genus *Streptomyces*, including *Streptomyces argillaceus* (ATCC12956). It inhibits the growth of cancer cells by cross-linking GC-rich DNA, thereby shutting down specificity protein (Sp1 or Sp3) dependent pathways towards proto-oncogenes, such as *c-myc*,^[1] *APC*,^[2] and *c-src*.^[3] The last gene is also associated with the unique hypocalcemic activity of mithramycin.^[3] MTM has become a popular biochemical tool to study Sp-dependent signal-transduction pathways, but because of its toxic side effects is rarely used as an anticancer agent, except for the treatment of tumor hypercalcemia refractory to other chemotherapy.^[4–8] However, MTM was recently identified as a potential lead drug against neurological diseases,^[9,10] arthritis,^[11] and for the treatment of hematologic disorders.^[12] All these new applications require only very small, less-toxic concentrations of the drug, although the mode-of-action in these contexts remains obscure.

The biosynthesis of MTM has been studied intensively during the past couple of years,^[13–18] and combinatorial biosynthetic efforts have already revealed various new MTM analogues, some with apparently advantageous biological activity profiles.^[19–21] The biosynthesis of MTM proceeds through a type II polyketide synthase (PKS) mediated condensation of multiple acyl-CoA units to the formation of the first isolable tetracyclic intermediates demethylpremithramycinone and premithramycinone (**2**), which in turn is glycosylated and C-methylated to give premithramycin B (**3**). The final steps in mithramycin biosynthesis are an oxidative cleavage of the fourth ring of **3**, followed by decarboxylation and reduction of the 4'-keto group, catalyzed by oxygenase MtmOIV and ketoreductase MtmW, respectively (Scheme 1).^[20,22,23] However, virtually nothing is known about the post-PKS steps prior to the formation of **2**; particularly unclear is the role of the products of the three oxygenase encoding genes *mtmOI*, *mtmOII*, and *mtmOIII*.^[13] Here, we describe the

isolation, structure elucidation, and putative biosynthetic impact of a novel early shunt product of mithramycin biosynthesis, premithramycinone G (**4**, Figure 1 and Scheme 2), which is accumulated by the MtmOII⁻ mutant *S. argillaceus* M7OII.^[13]

Gene inactivation of post-PKS enzymes does not only present a very useful tool for the elucidation of biosynthetic pathways by analysis of accumulated intermediates or shunt products, but also has the potential to generate new “non-natural” natural products with better or altered biological activities.^[24] However, this approach only gave one clear result in regard to the oxygenases of the mithramycin pathway. Four oxygenase genes (*mtmOI*, *mtmOII*, *mtmOIII*, and *mtmOIV*) were identified in the MTM gene cluster and inactivated by insertion of resistance cassettes. While the inactivation of *mtmOIV* resulted in clear evidence of the action of the corresponding enzyme MtmOIV,^[22,23] the inactivation of neither *mtmOI* nor *mtmOIII* appear to have any consequence for mithramycin biosynthesis.^[13] Inactivation of *mtmOII* resulted in the “non-producing” mutant strain M7OII, which generated an unstable compound.^[13,14]

After several attempts to isolate this unstable, putative early intermediate of the mithramycin biosynthesis failed, we designed a gene complementation experiment, in which TcmH, an early acting oxygenase from the tetracenomycin gene cluster, intercepted the intermediate and led to the formation of premithramycinone H (**5**). These experiments provided vague, indirect conclusions on the role of MtmOII in MTM biosynthesis.^[25] We were now able to isolate a novel shunt product directly from the M7OII mutant which gives unexpected new evidence of the role of MtmOII in mithramycin biosynthesis.

Analysis of the crude extract of the *S. argillaceus* M7OII mutant strain by HPLC/MS (Figure 1) showed the presence of a major product (which we later named premithramycinone G) with a UV spectrum significantly different from any known intermediate of the MTM biosynthesis. It is produced in amounts of 2.5 mgL⁻¹ and has a molecular weight of $M_r = 452 \text{ g mol}^{-1}$, based on the deprotonated molecular ion peak at $m/z = 451$ in the negative mode APCI-MS spectrum. Its molecular weight was also confirmed by ESI-MS operated in the positive mode (m/z 475 [$M+\text{Na}$]⁺ and 453 [$M+\text{H}$]⁺), and high-resolution EIMS (m/z 452.3667, 8%; calcd 452.3671) revealed its molecular formula unambiguously as C₂₃H₁₆O₁₀.

The ¹H NMR spectrum of premithramycinone G (Table 1) showed two *meta*-coupled aromatic protons at $\delta = 7.66$ and 7.02 ppm as well as one singlet at $\delta = 8.61$ ppm. Two groups of signals at $\delta = 3.38$ and 3.21 ppm for one methylene group as well as two methyl groups at $\delta = 2.80$ and 2.20 ppm were observed in the aliphatic region. The ¹³C NMR spectrum showed all 23 signals, of which 17 resulted from quaternary carbon atoms including 5 carbonyl groups ($\delta = 206.1, 199.1, 196.2, 188.2, \text{ and } 183.3 \text{ ppm}$), while the aliphatic region showed only three methine, one methylene, and two methyl groups. Database searches gave no hits matching these NMR data and molecular weight. 2D NMR studies (HSQC and CIGAR-HMBC,^[26,27] Scheme 2) finally revealed structure **4** for premithramycinone G, a novel molecule with an unprecedented framework derived from 1,3,4,6,8-pentahydroxy-1*H*-cyclopenta[*b*]anthracene-5,10-dione. In the HMBC spectrum, the singlet aromatic proton at $\delta = 8.61 \text{ ppm}$ showed ³J_{C-H} couplings with the carbon atoms at

δ = 183.3, 121.3, 119.7, and 77.4 ppm. The other two aromatic protons in the *meta* positions at δ = 7.66 and 7.02 ppm, respectively, showed $^3J_{C-H}$ couplings with carbon signals at δ = 183.3, 111.8, and 109.9 ppm and with those at δ = 111.8 and 109.0 ppm. The position of the acyl group at C2 was confirmed by the $^3J_{C-H}$ coupling between the methyl protons at δ = 2.80 ppm and the quaternary carbon atom at δ = 112.7 ppm. The long-range correlation in the HMBC spectrum between the methylene group of the butane-2,4-dione side chain and the carbon atom at δ = 77.40 ppm revealed the position of this side chain. Structure **4** and its ^{13}C NMR assignments were further confirmed by incorporation experiments with doubly ^{13}C -labeled acetate.

Premithramycinone G (**4**) contains 23 carbon atoms, that is, 3 more than the decaketide-derived aglycon of MTM. Thus, **4** must be viewed as a dodecaketide-derived compound, which relates it to the group of benanomycin/pradimicin-type antifungal antibiotics.^[28–34] However, an incorporation experiment with [1,2- ^{13}C]acetate showed that only the first 10 acetate units of **4** were ^{13}C -enriched, while 3 carbon atoms of the oxobutyryl side chain did not appear to be labeled (Scheme 3). These results may indicate that the three unlabeled carbon atoms derive either from another biosynthetic pool (for example, acetoacetate from incompletely degraded fatty acids) or are acetate-derived, but come in at a much later stage of the biosynthesis pathway, which was not (or no longer) affected by the exogenously fed ^{13}C -labeled acetate—possibly after the closure of the first three rings of the molecule. Recently, Hertweck et al. also discussed a possible sequence for the biosynthesis of enterocin that envisions a cyclization reaction prior to further chain-elongation steps.^[35]

The fact that this “dodecaketide”-derivative **4** accumulated upon inactivation of oxygenase MtmOII allows the conclusion to be drawn that MtmOII is somewhat involved in controlling the chain length. Similar findings were very recently reported by Hunter and co-workers, who observed the accumulation of polyketide shunt products with altered chain lengths in the oxytetracycline pathway upon inactivation of oxygenase OtcC.^[36] They concluded that OtcC is an essential partner of the polyketide synthase complex. Thus, it is possible that MtmOII contributes to controlling the chain length in MTM biosynthesis by acting as an essential component of the PKS complex. In the absence of MtmOII, the correct regiospecificity in the cyclization step leading to the formation of the fourth ring is also disturbed, which likely occurs normally after α oxidation (possibly through MtmOI) followed by reduction by 2-oxoacyl-ACP reductase MtmTII. A similar role was also proposed by Li and Piel for GrhO2, an enzyme of the griseorhodin A pathway with 50% amino acid identity and 61% similarity to MtmTII.^[37] Furthermore, the missing MtmOII might allow the ACP-bound ester carbonyl group to react with 3-oxobutyrate (in **6**, Scheme 3), which resembles the “lower” oxobutyryl side chain of the biosynthetic intermediate **7**, that is, its normal reaction partner (Scheme 3). Since *mtmTII* is located immediately downstream of *mtmOII*, it is also possible that its encoded ketoreductase was inactivated by a polar effect together with the inactivation of MtmOII. As a consequence, the newly introduced α -keto group can undergo the shunt aldol condensation leading to the five-membered ring found in **4**. This presumably spontaneous cyclization most likely happens after the chain elongation.

In the normal MTM biosynthesis it is likely that MtmOII catalyzes an epoxidation reaction either simultaneously with or shortly after the correct fourth cyclization to give the tetracyclic premithramycin framework. This reaction introduces the oxygen atom in the 2-position of **1** (=12a-position of **2**); this oxygen atom is not found in either shunt pathway resulting from the inactivation of MtmOII. A possible mechanism for this is epoxidation followed by a reductive opening of the epoxide. A similar sequence of events was discussed in the context of the reaction cascade catalyzed by oxygenase TcmG in the biosynthesis of tetracenomyacin C,^[38] and can be proposed for the introduction of the tertiary alcohol function during the biosynthesis of tetracycline. The latter reaction may be catalyzed by oxygenase OxyL,^[39] which shows 48% amino acid identity and 63% similarity to MtmOII. Finally, if MtmOII is a component of a multienzyme complex it may also prevent spontaneous cyclizations and the spontaneous or MtmOIII-catalyzed anthrone oxidation to give the anthraquinone shunt-products premithramycinone G (**4**) and H (**5**). These reactions only occur in the absence of MtmOII.

As mentioned previously, the oxidation pattern of **4** along with the role suggested here of MtmOII in the mithramycin biosynthetic pathway also allows first conclusions to be drawn regarding the roles of oxygenases MtmOI and MtmOIII, whose involvements in MTM biosynthesis have so far remained unclear. As shown in Scheme 3, it is necessary that one of these two enzymes, presumably MtmOI, oxidizes the carbon atom in the α -position to the acyl-ACP ester carbonyl group, which eventually becomes the O atom in the 1'-position of **1** (=4-position of **2** and **3**, =1-position of **4**). MtmOIII normally does not participate in MTM biosynthesis, but might however be responsible for the anthrone oxidations observed in the shunt pathways to **4** and **5** since MtmOIII shows high similarities to anthrone oxygenases, such as AknX (40% amino acid identity, 55% similarity to MtmOIII), involved in akalavinone biosynthesis^[40] or HedQ (36% amino acid identity, 51% similarity), the anthrone oxygenase of the hedamycin biosynthesis.^[41]

In summary, the inactivation of the *mtmOII* gene resulted in an unexpected metabolite, premithramycinone G (**4**), which we assume to be a shunt product of the biosynthetic pathway. The structure of the accumulated product suggests that **4** is derived from 10 acetate units, with 3 extra carbon atoms that are introduced either in the form of two extra malonyl-CoA extender units at a later biosynthetic stage (which is no longer affected by the feeding experiments with ¹³C-labeled acetate) or from a different source, for example, 3-oxobutyrate from incomplete fatty acid degradation. These shunt reactions are probably only possible because in the absence of MtmOII, control over both the chain length and the fourth cyclization reaction is lacking, in addition to a possibly impaired 2-oxoacyl-ACP reductase MtmTII. It can be suggested that in the biosynthesis of mithramycin the oxygen atom at the 1'-position of **1** (=4-position in **2** and **3**) is likely introduced by MtmOI, while MtmOII—when present in a multienzyme complex—may: 1) help to control the correct chain length, 2) protect positions of tricyclic aromatic intermediates, such as **6** (Scheme 3), from unwanted anthrone oxidations, and 3) ensure correct regiochemical control of the fourth cyclization, besides its main function, namely, the introduction of the oxygen atom which ends up in the 2-position of MTM (=12a-position in **2** and **3**).

Experimental Section

The inactivation of *mtmOII* was achieved through a frameshift mutation, see Ref. [13]. For the instruments/NMR methods used, see Ref. [42]. The labeled sodium [1,2-¹³C₂]acetate used in the incorporation experiment was obtained from Isotec (Miamisburg, OH, USA).

Production and isolation of 4: A seed culture was prepared by using tryptone soya broth media inoculated with spores of *S. argillaceus* M7OII and incubated in an orbital shaker (24 h, 30°C, 250 rpm). This seed culture was used to inoculate 50 Erlenmeyer flasks each containing 100 mL of R5A medium and was cultivated for 6 days. The culture broth was centrifuged (4200 rpm for 30 min), and then the solution adjusted to pH 5.0 with acetic acid and extracted repeatedly with ethyl acetate. The mycelia were extracted with acetone (4 0 0.5 L), and the extract was concentrated under reduced pressure. The resulting aqueous solution was extracted with ethyl acetate. The combined ethyl acetate extracts from the supernatants and mycelia were analyzed by HPLC-MS and concentrated under reduced pressure. The resulting crude extract was subjected to purification on Sephadex LH-20 (MeOH) to afford three fractions. All attempts to purify **4** by column chromatography on silica gel and preparative TLC failed because it decomposed. The middle fraction containing **4** was purified by preparative HPLC (column: μ Bondapak C₁₈ radial compression cartridge, PrepPak cartridge, 19 0 150 mm, Waters; eluent: acetonitrile and water (gradient from 35 to 100% in 43 min); flow rate: 10 mLmin⁻¹).

Feeding experiment: A seed culture was prepared using TSB inoculated with spores of *S. argillaceus* M7OII. The culture was incubated in an orbital shaker at 30°C for 24 h and 250 rpm. The seed culture was used to inoculate (at 2.5% v/v) 16 250-mL Erlenmeyer flasks, each containing 100 mL of modified R5 medium. After 24 h, the pulse feeding of [1, 2-¹³C]sodium acetate was started and continued for 48 h at 12 h intervals (4 feedings for a total of 1 g of sodium acetate per liter of culture). The culture was then left for an additional 72 h before extraction. ¹³C-Labeled **4** was isolated as described above for the unlabeled compound.

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Figure 1.
HPLC/MS analysis of the crude extract of the M7OII mutant.

Scheme 1.

Biosynthetic pathway of mithramycin (MTM, **1**) showing intermediates premithramycinone (**2**) and premithramycin B (**3**), as well as the late oxidative rearrangement catalyzed by MtmOIV.

Scheme 2.

Top: Structures of premithramycinone G (**4**) and premithramycinone H (**5**); bottom: $^2,3J_{C-H}$ couplings observed in the HMBC spectrum of **4**.

Scheme 3.

Hypothetical early pathway (thick black arrows) to MTM (**1**) and shunt pathways (open and dotted arrows) to **4** and **5** with the concluded hypothetical new roles of oxygenases MtmOI, MtmOII, and MtmOIII, as well as ketoreductase MtmTII. The structural formula of **4** shows the carbon enrichments after feeding MtmOII⁻ mutant *S. argillaceus* M7OII with [1,2-¹³C₂]acetate.

Table 1¹H and ¹³C NMR data as well as incorporation of [1,2-¹³C₂]acetate data (* = enriched C atoms) of **4**.^[a]

Position	Premithramycinone G (4)	
	δ_{H}	δ_{C}
1*	—	77.4 (27.5)
2*	—	112.7 (30.1)
3*	—	186.4 (30.1)
3a*	—	121.3 (36.0)
4*	—	166.3 (36.0)
4a*	—	119.7 (28.0)
5*	—	188.2 (28.0)
5a*	—	111.8 (33.4)
6*	—	166.4 (33.4)
7*	7.02 (d, 1.80 Hz, 1 H)	109.9 (32.6)
8*	—	166.5 (32.6)
9*	7.66 (d, 1.80 Hz, 1 H)	109.0 (30.1)
9a*	—	137.5 (30.1)
10*	—	183.3 (35.2)
10a*	—	124.7 (35.2)
11*	8.61 (s, 1 H)	114.9 (31.4)
11a*	—	152.1 (31.4)
1'*	—	196.2 (27.5)
2'	3.38, 3.21 (dd, 12.1 Hz, 2 H)	59.2
3'	—	206.1
4'	2.20 (s, 3 H)	31.2
1''*	—	199.1 (29.3)
2''*	2.80 (s, 3 H)	32.9 (29.3)

^[a] 9.4 T, [D₅] pyridine; chemical shifts in ppm (multiplicity; *J* in Hz; *J*_{C-C} from the incorporation experiment with [1,2-¹³C₂]acetate).