Elucidation of the Glycosylation Sequence of Mithramycin Biosynthesis: Isolation of 3A-Deolivosylpremithramycin B and Its Conversion to Premithramycin B by Glycosyltransferase MtmGII

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Dedicated to H. G. Floss on the occasion of his 70th birthday.

Mithramycin (MTM) is an aureolic acid-type polyketide produced by various soil bacteria of the genus *Streptomyces* including *Streptomyces argillaceus* (ATCC 12956).[1–3] MTM has been used clinically to treat Paget's disease and testicular carcinoma,[4–7] and MTM's hypocalcemic effect has been used to manage hypercalcemia in patients with malignancy-associated bone lesions.[8] Mithramycin has also been shown to act as neuroprotective drug.[9]

All aureolic acid-group drugs (i.e. MTM, chromomycin A_3, olivomycin A, UCH9, and durhamycin A)[1,10,11] contain the same tricyclic core moiety, but differ mainly with regard to their saccharide moieties, which consist of various 2,6-dideoxysugar chains that are linked at the 2- and 6-positions of the aglycon moiety. The structural variations in the glycosidic moieties are responsible for subtle differences in the DNA binding and activity profiles amongst the members of the aureolic acid group.[1,12–15] While extensive DNA–antibiotic interaction studies clearly revealed that the intact C-D-E trisaccharide moiety is essential for dimer formation as well as optimal DNA binding of MTM and chromomycin,[14–18] the role of the disaccharide chain at C-6 is less well characterized. However, the X-ray structure of the MTM–DNA complex revealed that this disaccharide interacts with the phosphate backbone of the DNA, and suggested that modifications of this disaccharide chain may have a profound impact on the biological activity. Unfortunately, derivatives, which differ from their parent drug with respect to the sugar units of the disaccharide chain, were not available at that point, but are badly needed to further investigate the structure–activity relationships (SAR) of the aureolic acids in general, and of mithramycin in particular. Biosynthetic-pathway engineering attempts were hampered by the fact that it was not then possible to determine exactly which glycosyltransferases were responsible for the attachments of the two olivose moieties of the disaccharide chain. Here we describe experiments that led to a clear assignment of the two glycosyltransferases involved in the formation of the mithramycin disaccharide chain.

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It has been shown that MTM biosynthesis[3,19–28] proceeds through tetracyclic intermediates (premithramycins) with glycosylation steps occurring on these tetracyclic biosynthetic intermediates. Initially the C-D-E-trisaccharide chain, consisting of a D-olivose, a D-oliose, and a D-mycarose, is attached at the 12a-position of premithramycinone (which later becomes the 2-position in mithramycin), while the disaccharide moiety is attached afterwards. Generation of GT-minus mutants suggested that MtmGIV and MtmGIII are involved in the trisaccharide chain formation, while MtmGI and MtmGII share the responsibility of the attachment of the two D-olivoses that form the disaccharide at C-6. Surprisingly, the GI$^-$ and the GII$^-$ mutants accumulate exactly the same pattern of metabolites, including premithramycins A_1, A_2, $A_{2'}$, A_3, and $A_{3'}$ (see Scheme 1), the last two possessing the complete C-D-E trisaccharide chain. Although these results made it clear that both MtmGI and MtmGII take part in disaccharide formation, it was unclear which GT catalyzes which exact step, since neither the GI$^-$ nor the GII$^-$ mutant accumulated a premithramycin with four sugars including the first D-olivose unit of the disaccharide, as one could have expected. To explain the results, it was speculated that one of the two GTs (GI or GII) might catalyze the formation of an NDP-activated diolivoside, while the other one attaches the diolivoside to the aglycon. Evidence for this view came from model experiments with the sugar-cosubstrate flexible glycosyltransferase ElmGT, which generated inter alia a diolivosyltetracenomycin upon its heterologous expression in *S. argillaceus*,[29] and from experiments in which *S. argillaceus* M3ΔMG, a mutant in which all GTs of the MTM biosynthesis were inactivated, was transformed with cosmid 16F4 as well as with either the *mtmGI* or *mtmGII* gene. Cosmid 16F4 harbors the entire gene cluster for the 8-demethyltetracenomycin biosynthesis plus ElmGT. Expression of cosmid 16F4 alone yields 8-olivosyltetracenomycin C as well as 8-mycarosyltetracenomycin C, but not 8-diolivosyltetracenomycin C. From the further expression of either the *mtmGI* or *mtmGII* gene, we expected that the 8-diolivosyltetracenomycin C production would be reinstalled, if one of the encoded GTs were responsible for the attachment of the second olivose or for the formation of an NDP-activated diolivoside (assuming that ElmGT can transfer such a diolivoside).

Unexpectedly, neither of the resulting constructs yielded 8-diolivosyltetracenomycin C, and both transformed mutants showed the same product pattern as found previously for the *S. argillaceus* ΔGT mutant expressing cosmid 16F4 alone. Only upon transformation of *S. argillaceus* M3ΔMG(cos16F4) with both GT-encoding genes *mtmGI* and *mtmGII*, diolivosyltetracenomycin production was restored to some extent. Thus, these experiments were totally inconclusive regarding the exact role played by MtmGI and MtmGII in mithramycin biosynthesis.

In this communication, we show strong evidence for the view that these last two glycosylation steps in mithramycin

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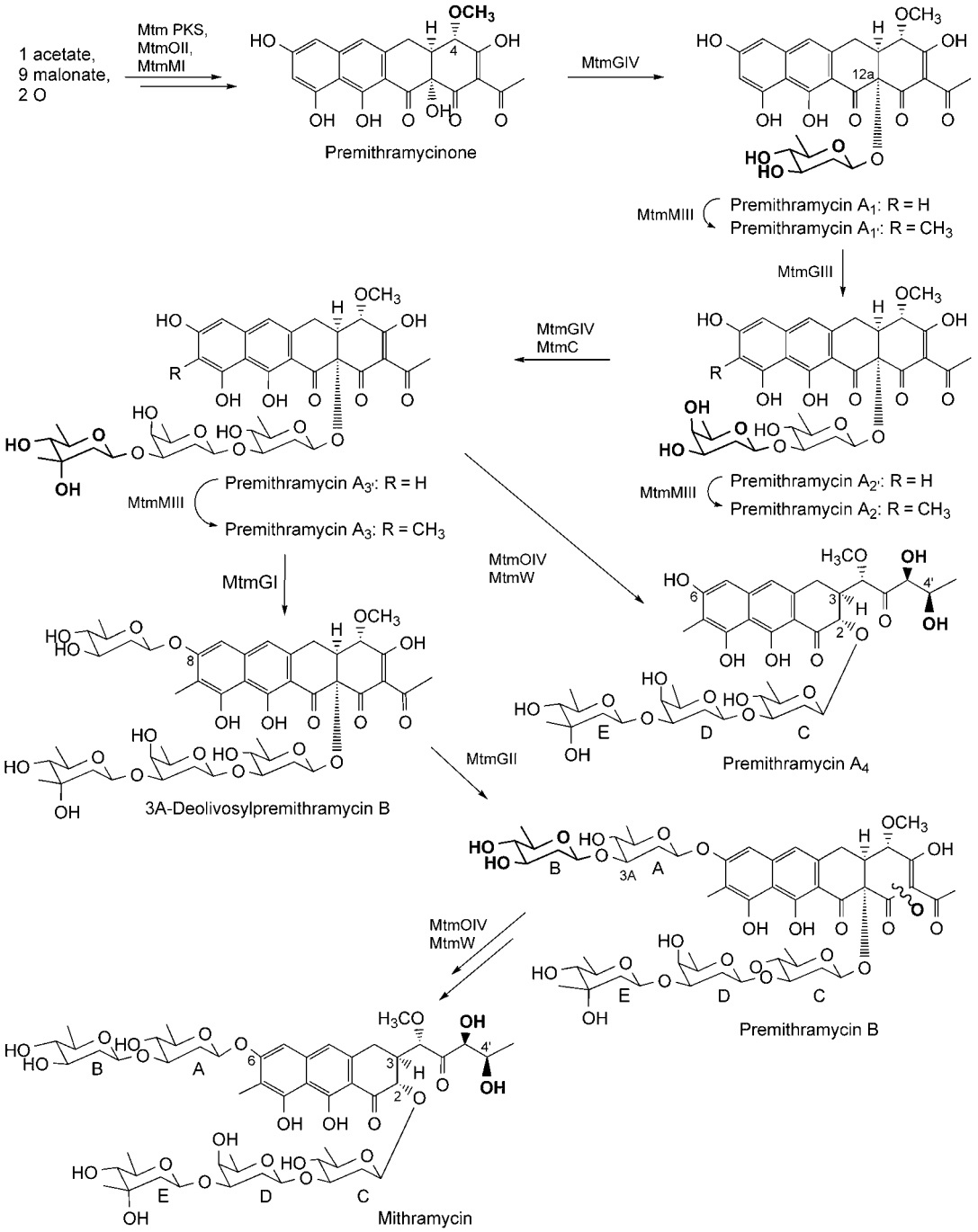

Scheme 1. *Chemical formulae of compounds involved in the late steps of mithramycin biosynthesis.*

biosynthesis are achieved by a stepwise attachment of single olivose units catalyzed first by MtmGI (leading to 3A-deolivosylpremithramycin B) and then by MtmGII (leading to premithramycin B).

To look for previously undetected side compounds, we reinvestigated the MtmOIV$^-$ mutant, which accumulates predominantly premithramycin B.[24] Baeyer–Villigerase MtmOIV catalyzes the oxidative cleavage of the fourth ring of premithramycin B to yield the tricyclic immediate precursor of mithramycin (Scheme 1).[24] This is the penultimate[30] and key step of mithramycin biosynthesis (note that only tricyclic but not tetracyclic derivatives/analogues of MTM are biologically active),[30–32] and immediately follows the glycosylation reactions. Since most mutants of the MTM biosynthesis accumulated more than one product, often some earlier intermediates in addition to the major product, which is usually the substrate of the inactivated enzyme, we hoped to find a new premithramycin derivative with an incomplete sugar pattern that might give us further hints about the glycosylation sequence. After cultivating 10 L of the MtmOIV$^-$ mutant and scanning the crude product by HPLC-MS, we indeed found a premithramycin derivative with a molecular mass of 962 g mol^{-1}; this indicates a tetrasac-

charidal compound since it is smaller than premithramycin B (1092 g mol^{-1}) by 130 amu (= 1 olivose unit). This side compound accounts for < 5% of the products, with premithramycin B being the only other product.

About 20 mg of the new premithramycin derivative were isolated from 10 L of crude product of the MtmOIV$^-$ mutant by semipreparative HPLC, and investigated by NMR spectroscopy. The NMR data revealed clearly that both the premithramycin aglycon moiety and the trisaccharidal side chain attached at C-12a (sugars C-D-E) were still present in the molecule. Compared to premithramycin B, the signals of one olivose unit were missing, and compared to premithramycin A_3, an additional D-olivose unit was detected. This single D-olivose unit is β-glycosidically linked at 8-position, as proven by an HMBC coupling between 1A-H and C-8 and the large coupling constant of 9.5 Hz observed for the anomeric 1A-H. Considering that most streptomycete GTs follow mechanisms inverting the anomeric configuration of the NDP-sugar-cosubstrate,[33] and therefore belong to the GT-1 family, this is in agreement with Klyne's rule.[34,35]

Assuming that the new 3A-deolivosylpremithramycin B is the true substrate of either MtmGI or MtmGII, we fed 2 mg of this compound to growing cultures of each the MtmGI$^-$ (= *S. argillaceus* M3G1) and the MtmGII$^-$ (= *S. argillaceus* M3G2) mutants 48 h after inoculation.[22] After another 24 h, the MtmGI$^-$ mutant converted almost all deolivosylpremithramycin B into premithramycin B and partly on to mithramycin, while the MtmGII$^-$ mutant did not produce any premithramycin B or mithramycin under the same conditions and still contained considerable amounts of the exogenously fed deolivosylpremithramycin B as well as apparently several isomerization products of this compound that were not further investigated (Figure 1).

These results provide clear evidence that MtmGII is capable of attaching a second olivose moiety to the 3A-position of 3A-deolivosylpremithramycin B, thus can use 3A-deolivosylpremithramycin B as its acceptor substrate. Given that MtmGII is responsible for attaching the second olivose unit of the disaccharide chain, MtmGI must be responsible for the attachment of the first olivose to the 8-position of the premithramycin A_3, although no direct proof for this exists. Looking back at the above-mentioned previous expression experiments (*S. argillaceus* M3ΔMG (cos16F4) with *mtmGI* and *mtmGII*), it appears that MtmGII is somewhat specific regarding its acceptor substrate and seems to be unable to use 8-olivosyltetracenomycin C as substrate for the elongation toward diolivosyltetrace-

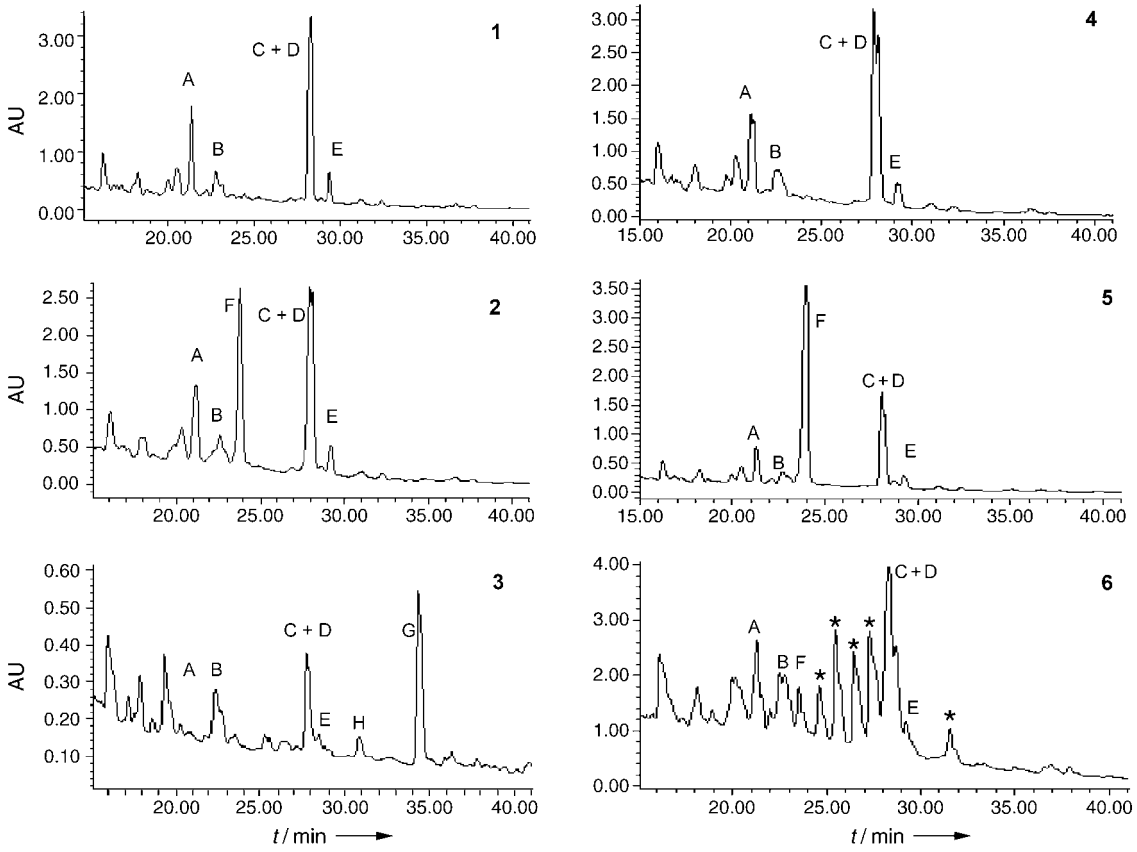

Figure 1. *Conversion of 3A-deolivosylpremithramycin B to premithramycin B in the* S. argillaceus *GI$^-$ mutant M3G1. Chromatograms 1, 2, and 3 refer to the feeding experiment with the GI$^-$ mutant, with peaks A = premithramycin A_1, B = premithramycin A_2, C = premithramycin A_2, D = premithramycin A_3, E = premithramycin A_1. Chromatogram 1 represents the culture of the GI$^-$ mutant after 48 h of growth (no addition); chromatogram 2 immediately after the addition of 3A-deolivosylpremithramycin B (peak F); chromatogram 3 represents the culture 24 h after addition of the precursor, G corresponds to premithramycin B and H to mithramycin.[37] Chromatograms 4, 5, and 6 represent the feeding experiment with the GII$^-$ mutant, *: peaks appear to come from isomers of 3A-deolivosylpremithramycin B (mass always 962 amu), but were not further investigated.*

nomycin C. However, MtmGII appears to be able to attach a second olivose to 8-olivosyltetracenomycin C in the presence of MtmGI; this might indicate that MtmGI and MtmGII assist each other in their activity and are compromised when expressed alone.[36a] Monitoring the secondary-metabolite production of the MtmOIV mutant also gives some evidence that MtmGII is somewhat slowed down when out of context of the normal MTM-biosynthetic machinery. The relative production curves of deolivosylpremithramycin B versus premithramycin B show an almost 50:50 relation 12 h after inoculation, while premithramycin B dominates (>95%) at harvesting time approximately 100 h after inoculation (Figure 2). This indicates that MtmGII lags behind somewhat in its activity compared to all the other enzymes contributing to the formation of premithramycin B.[36b]

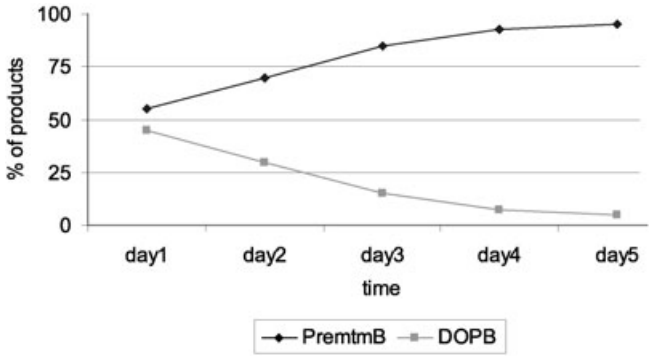

Figure 2. *Monitoring of the* S. argillaceus *MtmOIV⁻ mutant M3ΔO4 by HPLC: PremtmB = premithramycin B; DOPB = 3A-deolivosyl-premithramycin B.*

Experimental Section

Strains and cultures: All *S. argillaceus* mutant strains mentioned in this publication were previously described, that is, the MtmOIV⁻ mutant M3ΔO4,[24] the MtmGI⁻ mutant M3G1,[22] the MtmGII⁻ mutant M3G2,[22] and the GT-deletion mutant M3ΔGM.[24]

Isolation and physicochemical properties of 3A-deolivosylpremithramycin B: A seed culture was prepared by using tryptone soya broth media inoculated with spores of the *S. argillaceus* MtmOIV⁻ mutant and incubated in an orbital shaker (24 h, 30°C, 250 rpm). This seed culture was used to inoculate (at 2.5%, *v/v*) one hundred Erlenmeyer flasks (250 mL, each containing 100 mL of modified R5 medium) for 5 days. Product formation was monitored every 24 h by HPLC-MS. After 5 days, the solid culture media were centrifuged (4000 rpm, 30 min), and the combined, concentrated supernatants were separated on a chromatography column (5×10 cm^2, RP C_{18}, irregular; acetonitrile/water 35:65). Further purification was achieved by HPLC (1–2 min: 100% water, then 3–30 min: 70% water/30% acetonitrile to 30% water/70% acetonitrile, followed by a recalibration of the column by 7 min of 100% water. Flow rate: 10 mL min^{-1}, Waters symmetry-semiprep RP-18, 7 μm, 19×150 mm) to yield two compounds, 3A-deolivosylpremithramycin B (8.2 mg, 962 g mol^{-1}) and premithramycin B (162.8 mg, 1092 g mol^{-1}).

Physicochemical properties of 3A-deolivosylpremithramycin B: ^{1}H NMR (400 MHz, [D_6]acetone/[D_4]methanol, 9:1) and ^{13}C NMR (100 MHz, [D_6]acetone/[D_4]methanol, 9:1): see Table 1; IR (KBr): ν = 3423 (OH), 2971 (CH), 2931 (CH), 2882 (CH), 1716 (C=O), 1701 (C=O), 1598 (C=O), 1519 (C=C), 1361, 1157, 1063 cm^{-1}; UV/Vis (methanol): λ_{max} (°) = 433 (9100), 316 (6100), 282 nm (54200); positive-mode ESI-MS: *m/z* = 985.3 [*M*+Na], negative mode ESI-MS: *m/z* = 961.3 [*M*−H]; the molecular formula of $C_{47}H_{62}O_{21}$ (962 g mol^{-1}) was proven by HRMS: calcd 962.37837, found 961.37786.

Table 1. *^{1}H and ^{13}C NMR data of 3A-deolivosyl-premithramycin B.*

Position	δ ^{1}H [ppm]	Multiplicity (*J* [Hz])	δ ^{13}C [ppm]	Important $^3J_{C\text{-}H}$ couplings (HMBC)
Aglycon				
1	–	–	196.7	–
2	–	–	114.4	–
3	–	–	189.0	
4	4.13	d (2.8)	77.4	2, 4-OCH$_3$, 5
4-OCH$_3$	3.43	s	60.7	4
4a	3.12	m	42.4	1, 3, 5a
5	4.09	dd (16, 12)	28.5	4, 6
	3.34	dd (16, 3)		
5a	–		134.5	–
6	6.38	br s	118.4	7, 11a, 12a
6a	–	–	139.4	–
7	6.66	s	101.6	6, 9, 10a
8	–	–	160.2	–
9	–	–	111.2	–
9-CH$_3$	2.08	s	7.7	8, 9, 10
10	–	–	155.8	–
10a	–	–	107.6	–
11	–	–	165.5	–
11a	–	–	107.5	–
12	–		193.2	–
12a			81.5	2, 12a
1′	–	–	204.4	–
2′	2.86	s	26.0	1′, 2
Sugar A (β-D-olivose)				
1A	5.23	dd (10, 2)	96.9	8
2A$_{ax}$	1.53	ddd (12, 12, 10)	39.7	
2A$_{eq}$	2.55	m (overlap)		
3A	3.55	ddd (12, 9, 5)	70.3	
4A	3.35	dd (9, 9)	76.4	
5A	4.25	m (overlap)	72.5	
6A	1.36	d (6)	18.0	
Sugar C (β-D-olivose)				
1C	4.73	dd (10, 2)	100.1	12a
2C$_{ax}$	1.55	ddd (12, 12, 10)	39.2	
2C$_{eq}$	2.67	ddd (12, 5, 2)		
3C	3.78	m (overlap)	81.5	1D
4C	3.57	dd (9, 9)	75.0	
5C	3.70	dq (9, 6)	72.2	
6C	1.38	d (6)	17.8	
Sugar D (β-D-oliose)				
1D	4.76	dd (10, 2)	97.6	3C
2D$_{ax}$	1.11	ddd (12, 12, 10)	32.2	
2D$_{eq}$	2.14	ddd (12, 5, 2)		
3D	3.52	ddd (12, 5, 3)	77.4	1E
4D	3.68	br s	68.5	
5D	3.78	m (overlap)	70.7	
6D	1.23	d (6)	16.3	
Sugar E (β-D-mycarose)				
1E	5.19	dd (9.5, 2)	97.6	3D
2E$_{ax}$	2.31	dd (13, 9.5)	44.1	
2E$_{eq}$	2.67	dd (13, 2)		
3E	–	–	70.3	
3E-CH$_3$	1.48	s	27.0	2E, 3E, 4E
4E	3.01	d (9)	76.5	
5E	3.72	m (overlap)	71.0	
6E	1.20	d (6)	17.7	

Feeding of 3A-deolivosylpremithramycin B to *mtmΔ GI* and *mtmΔ GII* mutants: The culture was prepared by using modified R5 medium inoculated with spores of *S. argillaceus* mutants M3G1 and M3G2 in two different Erlenmeyer flasks (250 mL, each containing 100 mL of media) and incubated in an orbital shaker (72 h, 30 °C, 250 rpm). 3A-Deolivosylpremithramycin B (2 mg each) was fed to the growing cultures after 48 h of inoculation of both *S. argillaceus* M3G1 and M3G2 (i.e., the *mtmΔGI* and *mtmΔGII* mutants). The cultures were grown for another 24 h. HPLC control samples were taken before and just after the addition of 3A-deolivosylpremithramycin B as well as 24 h after the feeding, Figure 1). The following HPLC gradient solvent system was used: 1–2 min 100% water, 3–45 min 75% water/25% acetonitrile to 20% water/80% acetonitrile, then recalibration of the column with 100% water for another 10 min. All the peaks of the chromatograms have been identified through HPLC-UV, HPLC-MS, and authentic samples.

HPLC-MS: HPLC/MS was performed on a Waters Alliance 2695 system, equipped with a Waters 2996 photodiode array detector and a Micromass ZQ2000 mass spectrometer by using an APCI probe (solvent A: 0.1% formic acid in H_2O; solvent B: acetonitrile; flow rate = 0.5 mL min^{-1}; 0–10 min 75% A and 25% B to 100% B, linear gradient, 10–12 min 100% B, 12–14 min 100% B to 75% A and 25% B (linear gradient), 14–15 min 75% A and 25% B). We used column Waters Symmetry C-18, 4.6×50 mm, particle size 5 μm. The column temperature was 23 °C, and the UV detection wavelength was 452 nm.

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[1] J. D. Skarbek, M. K. Speedie in *Antitumor Compounds of Natural Origin: Chemistry and Biochemistry, Vol.* 1 (Ed.: A. Aszalos), CRC Press, Boca Raton, **1981**, pp. 191–235.
[2] S. E. Wohlert, E. Kunzel, R. Machinek, C. Mendez, C. J. A. Salas, J. Rohr, *J. Nat. Prod.* **1999**, *62*, 119–121.
[3] J. Rohr, C. Mendez, J. A. Salas, *Bioorg. Chem.* **1999**, *27*, 41–54.
[4] S. Majee, S. D. Dasgupta, A. Chakrabarti, *Eur. J. Biochem.* **1999**, *260*, 619–626.
[5] E. G. Elias, J. T. Evans, *J. Bone Jt. Surg. Am. Vol.* **1972**, *54A*, 1730–1736.
[6] J. H. Brown, B. J. Kennedy, *N. Engl. J. Med.* **1965**, *272*, 111–118.
[7] W. G. Ryan, T. B. Schwartz, G. Northrop, *JAMA J. Am. Med. Assoc.* **1970**, *213*, 1153–1157.
[8] P. R. Robins, J. Jowsey, *J. Lab. Clin. Med.* **1973**, *82*, 576–586.
[9] S. Chatterjee, K. Zaman, H. Ryu, A. Conforto, R. R. Ratan, *Ann. Neurol.* **2001**, *49*, 345–354.
[10] R. Katahira, Y. Uosaki, H. Ogawa, Y. Yamashita, H. Nakano, M. Yoshida, *J. Antibiot.* **1998**, *51*, 267–274.
[11] H. Jayasuriya, R. B. Lingham, P. Graham, D. Quamina, L. Herranz, O. Genilloud, M. Gagliardi, R. Danzeisen, J. E. Tomassini, D. L. Zink, Z. Q. Guan, S. B. Singh, *J. Nat. Prod.* **2002**, *65*, 1091–1095.
[12] G. F. Gause in *Antibiotics III—Mechanism of Action of Antimicrobial Antitumor Agents*, *Vol.* 3 (Eds.: J. W. Corcoran, F. E. Hahn), Springer, Berlin, **1975**, pp. 197–202.
[13] K. R. Fox, N. R. Howarth, *Nucleic Acids Res.* **1985**, *13*, 8695–8714.
[14] M. Sastry, D. J. Patel, *Biochemistry* **1993**, *32*, 6588–6604.
[15] M. Sastry, R. Fiala, R. , D. J. Patel, *J. Mol. Biol.* **1995**, *251*, 674–689.
[16] D. J. Silva, D. E Kahne, *J. Am. Chem. Soc.* **1993**, *115*, 7962–7970.
[17] D. J. Silva, R. Goodnow, Jr., D. Kahne, *Biochemistry* **1993**, *32*, 463–471.
[18] X. L. Gao, P. Mirau, D. J. Patel, *J. Mol. Biol.* **1992**, *223*, 259–279.
[19] F. Lombó, G. Blanco, E. Fernandez, C. Méndez, J. A. Salas, *Gene* **1996**, *172*, 87–91.
[20] F. Lombó, F. , K. Siems, A. F. Braña, C. Méndez, K. Bindseil, J. A. Salas, *J. Bacteriol.* **1997**, *179*, 3354–3357.
[21] F. Lombó, A. F. Braña, C. Méndez, J. A. Salas, *J. Bacteriol.* **1999**, *181*, 642–647.
[22] E. Fernandez, U. Weißbach, C. Sanchez Reillo, A. F. Braña, C. Méndez, J. Rohr, J. A. Salas, *J. Bacteriol.* **1998**, *180*, 4929–4937.
[23] J. Rohr, U. Weißbach, C. Beninga, E. Künzel, K. Siems, K. Bindseil, L. Prado, F. Lombó, A. F. Braña, C. Méndez, J. A. Salas, *Chem. Commun.* **1998**, 437–438.
[24] L. Prado, E. Fernandez, U. Weißbach, G. Blanco, L. M. Quirós, A. F. Braña, C. Méndez, J. Rohr, J. A. Salas, *Chem. Biol.* **1999**, *6*, 19–30.
[25] G. Blanco, E. Fernandez, M. J. Fernandez, A. F. Braña, U. Weißbach, E. Künzel, J. Rohr, C. Mendez, J. A. Salas, *Mol. Gen. Genet.* **2000**, *262*, 991–1000.
[26] M. J. Fernandez-Lonzano, L. L. Remsing, L. M. Quirós, A. F. Braña, E. Fernandez, C. Sanchez, C. Méndez, J. Rohr, J. A. Salas, *J. Biol. Chem.* **2000**, *275*, 3065–3074.
[27] A. Gonzalez, L. L. Remsing, F. Lombó, M. J. Fernandez, L. Prado, A. F. Braña, E. Künzel, J. Rohr, C. Méndez, J. A. Salas, *Mol. Gen. Genet.* **2001**, *264*, 827–835.
[28] L. L. Remsing, J. Garcia-Bernardo, A. M. Gonzalez, E. Künzel, U. Rix, A. F. Braña, D. W. Bearden, C. Méndez, J. A. Salas, J. Rohr, *J. Am. Chem. Soc.* **2002**, *124*, 1606–1614.
[29] S.-E. Wohlert, G. Blanco, F. Lombó, E. Fernandez, A. F. Braña, S. Reich, G. Udvarnoki, C. Méndez, H. Decker, J. Frevert, J. A. Salas, J. Rohr, *J. Am. Chem. Soc.* **1998**, *120*, 10596–10601.
[30] L. L. Remsing, A. M. Gonzalez, M. Nur-e-Alam, M. J. Fernandez-Lozano, A. F. Braña, U. Rix, M. A. Oliveira, C. Méndez, J. A. Salas, J. Rohr, *J. Am. Chem. Soc.* **2003**, *125*, 5745–5753.
[31] W. R. Roush, N. A. Powell, R. A. James, *Aust. J. Chem.* **2002**, *55*, 113–121.
[32] L. L. Remsing, H. R. Bahadori, G. M. Carbone, E. M. McGuffie, C. V. Catapano, J. Rohr, *Biochemistry* **2003**, *42*, 8313–8324.
[33] L. M. Quirós, R. J. Carbajo, J. A. Salas, *FEBS Lett.* **2000**, *476*, 186 −189.
[34] W. Klyne, *Biochem. J.* **1950**, *47*, 41–42.
[35] U. Rix, C. Fischer, L. L. Remsing, J. Rohr, *Nat. Prod. Rep.* **2002**, *19*, 542–580.
[36] a) This somewhat seems to contradict the fact that the MtmGI-minus mutant converts 3A-deolivosyl-premithramycin B to premithramycin B. However, note that MtmGI was inactivated by insertion of an apramycin-resistance cassette, that is, the enzyme is still present, although catalytically not functional, but might still be able to "support" MtmGII through protein–protein interactions. b) As shown in Figure 2, the activity of MtmGII is somewhat compromised when MtmOIV is inactivated. This might indicate that MtmGII needs the presence of the natural MtmOIV (not the insertionally inactivated bulkier enzyme) to function optimally.
[37] The chromatogram represents a snapshot 24 h after feeding of the intermediate 3A-deolivosyl-premithramycin B. The normal end product mithramycin is better observable much later (after another 48 h).

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