Urdamycin L: A Novel Metabolic Shunt Product that Provides Evidence for the Role of the *urd*M Gene in the Urdamycin A Biosynthetic Pathway of *Streptomyces fradiae* TÜ 2717

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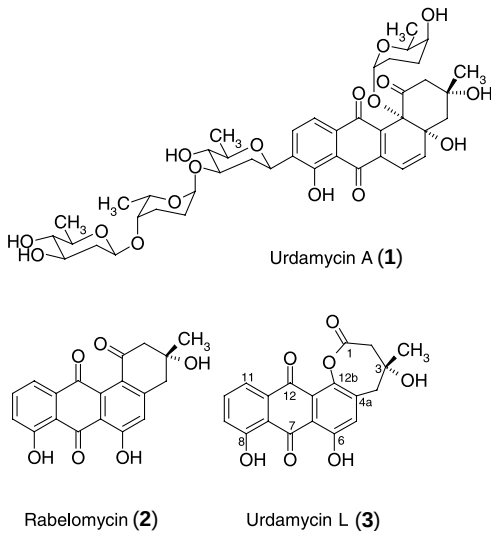
The antitumor antibiotic urdamycin A (**1**; Scheme 1) produced by the soil bacteria *Streptomyces fradiae* TÜ 2717 is one of the most prominent members of the angucycline family. Over the past few years, significant progress has been made in elucidation of the biosynthetic pathway of this polyketide compound.[1–3] However, some obscurity remained with regard to the early post-polyketide-synthase tailoring steps, which involve *p*-quinone formation, *C*-glycosylation, and a 12b-hydroxylation step, and their sequence of events. Recently, we described the characterization and inactivation of the *urd*GT2 and *urd*M genes from the urdamycin gene cluster,[4] which led to the identification of UrdGT2 as a glycosyltransferase responsible for the *C*-glycosidic attachment of D-olivose to the aglycon.[5] It was also found that *urd*M encodes a bifunctional enzyme with a C-terminal reductase domain and an N-terminal flavin-dependent oxygenase domain that is responsible for the 12b-hydroxylation in the biosynthetic pathway of urdamycin A (**1**).[4] Here, we provide further evidence for the mechanism of this unusual hydroxylation step.



Scheme 1. *Structure and numbering of the minor metabolite urdamycin L (**3**) of mutant strain* Streptomyces fradiae *TÜ 2717 ΔM, the major metabolite rabelomycin (**2**), and the principle product of the wild-type strain* Streptomyces fradiae *TÜ 2717, urdamycin A (**1**).*

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The original investigations showed rabelomycin (**2**), a well-known angucyclinone,[6, 7] to be the single main (shunt) product of the *urd*M-inactivated mutant. We here report the finding and structure elucidation of a second compound, urdamycin L (**3**), which allows some interesting conclusions to be made about the mechanism of this 12b-hydroxylation step. This new shunt product is formed in trace amounts ($<1\,\mathrm{mg\,L^{-1}}$) and was therefore initially overlooked in the ΔM mutant strain. Reinvestigations led to the identification of **3** in the *S. fradiae* ΔM mutant, but it was neither detectable in the *S. fradiae* wild-type nor in any of the other *S. fradiae* mutant strains.

The structure of urdamycin L (**3**) shows significant similarities to that of rabelomycin (**2**); in fact, the only difference is the presence of a seven-membered lactone moiety in **3** (ring A) instead of a six-membered carbocycle as in **2**. In order to depict the differences in the NMR spectra more clearly, the complete set of data for rabelomycin was acquired and all ^{13}C NMR signals for this compound were assigned for the first time (Table 1). Most signals are essentially the same for both compounds, however, two major differences in the ^{13}C NMR data were found: the C-1 carbonyl carbon atom of **3** shows, compared to that of **2**, an upfield shift from $\delta = 196.2$ to $\delta = 170.7$ ppm, which indicates an ester (or lactone) carbonyl group instead of a ketone. Also, the C-12b signal of **3** is shifted significantly downfield to $\delta = 154.7$ (from $\delta = 129.0$), which indicates the presence of a directly bound O atom. While the hydroxy groups in the 3-, 6-, and 8-positions were visible, additional D_2O exchangeable downfield ^{1}H NMR signals were absent. MS data showed a molecular ion peak for **3** ($m/z = 354$) that is 16 amu higher than that of **2**. These NMR spectroscopy and MS results prove ring A to be a seven-membered lactone and not an open-chain carboxylic acid analogue as found, for example, in fridamycin E[8] or vineomycin B_2.[9] Structure **3** is also consistent with all other observed NMR data (Table 1).

The fact that a compound with an oxygenated 12b-position was found in the *urd*M inactivation mutant at all was at first surprising, since all previous data strongly suggested that UrdM is responsible for this oxygenation step.[4] However, the structure of urdamycin L (**3**) reveals that **3** cannot be an intermediate of urdamycin A biosynthesis, but could be a shunt product, which is likely to derive from compound **5** by spontaneous oxidation to the quinone and loss of water (Scheme 2). The structure of lactone **5** in turn suggests it is an intermediate of the biosynthesis of **1**, generated from UWM 6 (**4**)[10] through a

Table 1. *^{1}H NMR and ^{13}C NMR data for rabelomycin and urdamycin L*[a]

	^{1}H NMR		^{13}C NMR		HMBC	
	2[b]	**3**[c]	**2**[b]	**3**[c]	**2**[b]	**3**[c]
1			196.2	170.7		
2	2.95 d (15) 3.04 d (15)	2.98 m	53.9	44.1	1, 3, 4, 12b	1, 3, 3-CH_3, 4
3			72.3	89.4		
3-CH_3	1.49 s	1.66 s	30.0	26.6	1, 2, 3, 4, 4a	2, 3, 4
3-OH	1.88 s(br)[d]	4.05 s[d]				
4	3.09 s	3.24 d (19, 2) 3.66 d (19, 2)	44.2	40.4	2, 3, 3-CH_3, 4a, 5,6, 6a, 12a, 12b	2, 3, 3-CH_3, 4a, 5, 12b
4a			150.6	145.2		
5	7.00 s	7.29 s	122.1	122.0	4, 6, 6a, 7, 12a, 12b	4, 6, 6a, 12b
6			163.8	159.4		
6-OH	12.30 s[c]	12.60 s[c]			5, 6, 6a	
6a			116.9	112.1		
7			192.6	192.5		
7a			115.1	116.6		
8			162.0	162.3		
8-OH	11.66 s[d]	12.15 s[d]			7a, 8, 9	
9	7.26 dd (8, 2)	7.31 dd (8, 1)	124.0	123.2	7a, 8, 10	7a, 8, 11
10	7.68 dd (8, 8)	7.81 dd (8, 8)	137.7	137.5	8, 9, 11a, 12	8, 11a
11	7.65 dd (8, 2)	7.74 dd (8, 1)	120.1	119.1	7a, 9, 12	7a, 9, 12
11a			135.5	135.1		
12			183.3	179.6		
12a			138.0	114.3		
12b			129.0	154.7		

[a] ^{1}H NMR measurements were made at 300 MHz and 500 MHz for rabelomycin and urdamycin L, respectively, and ^{13}C NMR data were taken at 75.5 MHz and 125 MHz, respectively. NMR data are relative to internal tetramethylsilane and *J* values are given in Hz. Assignments are based on HSQC, HMBC, and H,H-COSY experiments. [b] In $CDCl_3$. [c] In [d_6]acetone. [d] Exchangeable with D_2O.

Baeyer–Villiger oxygenation reaction, which, however, is only an incomplete process with regard to the overall 12b-hydroxylation step. We assume that the native UrdM protein catalyzes two distinct steps: the Baeyer–Villiger oxygenation to form an ε lactone in ring A and a subsequent, base-assisted rearrangement of ring A that re-establishes the six-membered carbocycle to yield alcoholate **6**. This rearrangement happens either in a concerted fashion (as indicated in Scheme 2) or stepwise with a carbanion intermediate, which is mesomerically stabilized by the aromatic system and the 6-carbonyl group. This second catalytic step involves the formal reduction of an ester carbonyl group to a keto carbonyl group. Alcoholate **6** is finally protonated to form 12b-hydroxy-UWM 6 (**7**), the likely natural substrate for either the UrdGT2 glycosyltransferase or the UrdE oxygenase (responsible for *p*-quinone formation).[5] Although we cannot completely rule out other enzymes/routes with this "whole organism" approach, we believe that the production of **3** can be best explained by residual activity of a modified UrdM enzyme, which can still catalyze the Baeyer–Villiger reaction but is unable to perform the rearrangement step.[11] This hypothesis is consistent with the chosen inactivation method of the *urd*M gene, which involves an in-frame deletion of a large part of the C-terminal reductase domain but does not affect the N-terminal oxygenase-encoding domain of *urd*M.[4] This process apparently led to a truncated and most likely partially misfolded protein with greatly reduced Baeyer–Villiger oxygenase activity and no activity regarding the second rearrangement step, possibly because of the absence of the hypothesized general base in the active site.

Alternative mechanisms, such as epoxidation or dioxygenation of an enolated 1-carbonyl species, would lead to the formation of a hydrated 1-carbonyl molecule, followed by the loss of water. Therefore, one would expect the carbonyl O atom to derive partly (up to 50%) from aerial oxygen and partly from acetate. However, previous labeling experiments with $^{18}O_2$ showed the incorporation of an ^{18}O atom only at the 12b-position and not in the 1-position. Feeding of ^{18}O-labeled acetate, on the other hand, gave a particularly strong enrichment at the 1-position.[12] Thus, the incorporation experiments[12] offer further support for the Baeyer–Villiger initiated two-step process discussed above. Also, enzymatic epoxidation reactions are typically carried out by cytochrome P-450 oxygenases (CYP450s). Amino acid sequence investigations of the *urd*M oxygenase domain,[4] however, reveal no resemblance of this domain to CYP450s and only weak homologies with dioxygenases, but strong homologies with flavin-dependent monooxygenases. Moreover, a closer look at the amino acid sequence reveals a characteristically invariant aspartic acid residue five amino acids upstream of the N-terminal flavin adenine dinucleotide (FAD) binding site motif, which has been hypothesized to be indicative of type-1 Baeyer–Villiger monooxygenases (FAD and reduced nicotinamide adenine dinucleotide phosphate dependent).[13]

In summary, we here describe the characterization of the novel shunt product urdamycin L (**3**), whose lactone ring structure provides evidence for the hypothesis that the 12b-hydroxylation step catalyzed by the UrdM enzyme during urdamycin A (**1**) biosynthesis by *Streptomyces fradiae* TÜ 2717

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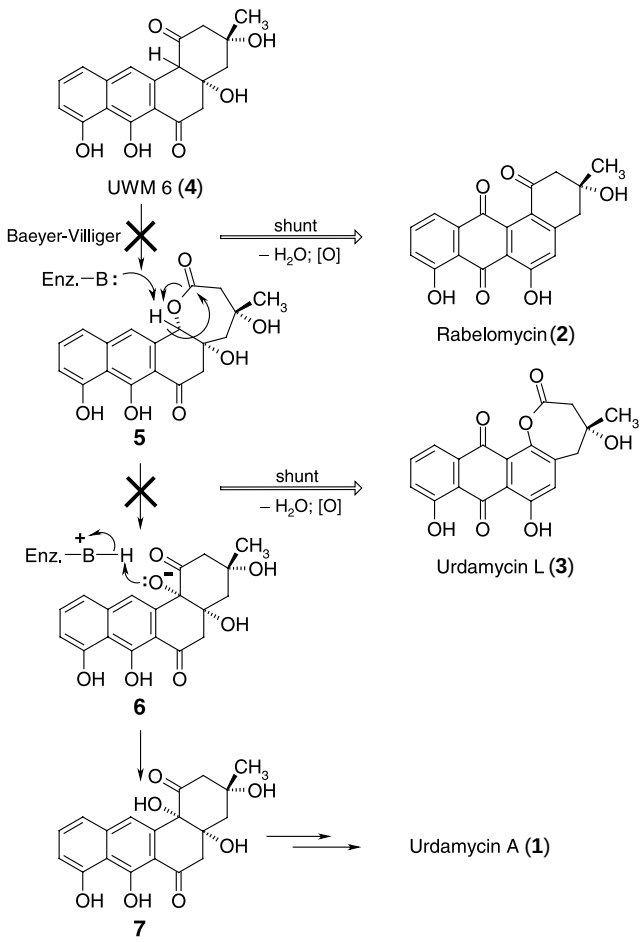

Scheme 2. *Mechanistic suggestion for the biosynthetic pathway to urdamycin A: 12b-hydroxylation catalyzed by oxygenase UrdM. Enz. = enzyme.*

involves a Baeyer–Villiger monooxygenation reaction. This hypothesis is further supported by the results of earlier labeling experiments and gene sequence alignment studies. Although similar Baeyer–Villiger oxygenations have been discussed for several other polyketides,[8, 9, 14–16] this study represents to our knowledge the first case (in the context of polyketide biosynthesis) in which a lactone has been isolated to provide direct evidence for a Baeyer–Villiger mechanism. As a consequence of this Baeyer–Villiger mechanism, we postulate an unusual subsequent rearrangement of the seven-membered lactone ring, which leads to the reestablishment of the six-membered carbocycle and the generation of the 12b-hydroxy group, both of which are observed in the biosynthetic pathways of many angucycline-group antibiotics,[7] such as urdamycin A.

Experimental Section

The generation of the *S. fradiae* TÜ 2717 ΔM mutant strain has been described elsewhere.[4] A spore suspension of a freshly sporulated culture on an M_2CaO (M = malt extract) agar plate was used to inoculate the main culture of the same media, which was grown for 72 h at 30 °C. The agar was ground and extracted with ethyl acetate. The extracts were evaporated, redissolved in methanol and analyzed on silica gel plates (Merck) by TLC (chloroform/methanol = 9:1). The observed R_f values for **2** (yellow) and **3** (orange) are 0.56 and 0.28, respectively, and **3** showed a characteristic yellow fluorescence under UV light ($\lambda = 366$ nm). Compounds were subsequently purified by column chromatography on silica gel (chloroform/methanol = 9:1) and Sephadex LH20 (methanol; methylene chloride), and submitted to NMR and MS analysis.

Rabelomycin (**2**): $C_{19}H_{14}O_6$ ($M_w = 338$); ^{1}H NMR and ^{13}C NMR: see Table 1.

Urdamycin L (**3**): $C_{19}H_{14}O_7$ ($M_w = 354$); ^{1}H NMR and ^{13}C NMR: see Table 1; IR (Methanol): $\tilde{\nu} = 3453$ (br; OH, H-bonded), 2923 (alkyl C—H), 2854 (alkyl C—H), 1781 (lactone C=O), 1674 (*p*-quinone C=O), 1432 (CH_3), 1175 (ε-lactone C=O), 1120 (C—OH) cm^{-1}; UV/Vis (Methanol): $\lambda_{max}(\varepsilon) = 487$ (5110), 287 (5790), 249 (10810), 232 (22380) nm; positive mode EI MS: *m/z*: 354 (10; high resolution: calc. for $C_{19}H_{14}O_7$: 354.0740, found: 354.0746) [M^+], 336 (6) [$M^+ - H_2O$], 310 (100) [$M^+ - CO_2$], 308 (10) [$M^+ - H_2O - CO$], 292 (70) [$M^+ - CO_2 - H_2O$].

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[1] A. Trefzer, D. Hoffmeister, E. Künzel, S. Stockert, G. Weitnauer, L. Westrich, U. Rix, J. Fuchser, K. U. Bindseil, J. Rohr, A. Bechthold, *Chem. Biol.* **2000**, *7*, 133–142.
[2] D. Hoffmeister, K. Ichinose, S. Domann, B. Faust, A. Trefzer, G. Dräger, A. Kirschning, C. Fischer, E. Künzel, D. W. Bearden, J. Rohr, A. Bechthold, *Chem. Biol.* **2000**, *7*, 821–831.
[3] D. Hoffmeister, K. Ichinose, A. Bechthold, *Chem. Biol.* **2001**, *8*, 557–567.
[4] B. Faust, D. Hoffmeister, G. Weitnauer, L. Westrich, S. Haag, P. Schneider, H. Decker, E. Künzel, J. Rohr, A. Bechthold, *Microbiology* **2000**, *146*, 147–154.
[5] E. Künzel, B. Faust, C. Oelkers, U. Weißbach, D. W. Bearden, G. Weitnauer, L. Westrich, A. Bechthold, J. Rohr, *J. Am. Chem. Soc.* **1999**, *121*, 11058–11062.
[6] W.-C. Liu, W. L. Parker, D. S. Slusarchyk, G. L. Greenwood, S. F. Graham, E. Meyers, *J. Antibiot.* **1970**, *23*, 437–441.
[7] a) J. Rohr, R. Thiericke, *Nat. Prod. Rep.* **1992**, *9*, 103–137; b) K. Krohn, J. Rohr, *Top. Curr. Chem.* **1997**, *188*, 27–195.
[8] S.-T. Hong, J. R. Carney, S. J. Gould, *J. Bacteriol.* **1997**, *179*, 470–476.
[9] N. Imamura, K. Kakinuma, N. Ikekawa, *J. Antibiot.* **1982**, *35*, 602–608.
[10] K. Kulowski, E. Wendt-Pienkowski, L. Han, K. Yang, L. C. Vining, C. R. Hutchinson, *J. Am. Chem. Soc.* **1999**, *121*, 1786–1794.
[11] Only the ΔM mutant produces this new metabolite, which we could detect neither in the wild-type strain nor in any other *S. fradiae* mutant. Thus, neither the other oxygenase of the urdamycin gene cluster, UrdE, which is responsible for the introduction of the 12-oxygen atom,[17] nor any unidentified oxygenase of *S. fradiae*, is likely to be responsible for this lactone formation.
[12] G. Udvarnoki, T. Henkel, R. Machinek, J. Rohr, *J. Org. Chem.* **1992**, *57*, 1274–1276.
[13] A. Willetts, *TrendsBiotech.* **1997**, *15*, 55–62.
[14] L. Prado, E. Fernández, U. Weißbach, G. Blanco, L. M. Quirós, A. F. Braña, C. Méndez, J. Rohr, J. A. Salas, *Chem. Biol.* **1998**, *6*, 19–30.
[15] I. Fujii, Y. Ebizuka, U. Sankawa, *J. Biochem.* **1988**, *103*, 878–883.
[16] M. Gerlitz, G. Udvarnoki, J. Rohr, *Angew. Chem.* **1995**, *107*, 1757–1761; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1617–1621.
[17] U. Rix, C. Fischer, L. L. Remsing, J. Rohr, *Nat. Prod. Rep.* **2002**, *19*, 542–580.

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