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Triple Hydroxylation of Tetracenomycin A2 to Tetracenomycin C Involving Two Molecules of O_2 and One Molecule of H_2O

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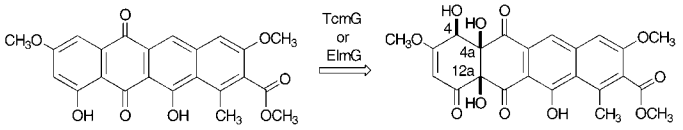
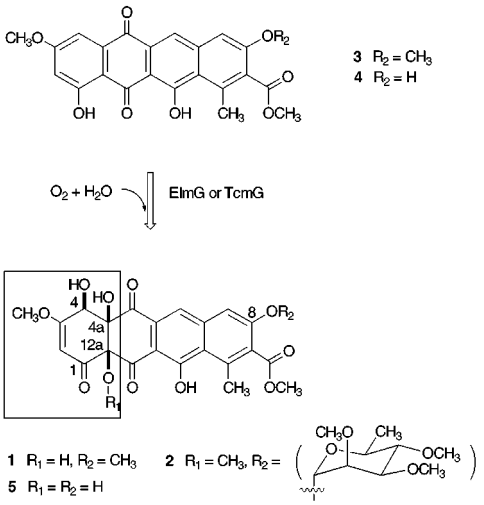
ABSTRACT



The TcmG or ElmG oxygenase-catalyzed triple hydroxylation of tetracenomycin (Tcm) A2 to Tcm C proceeds via a novel monooxygenase–dioxygenase mechanism, deriving the 4- and 12a-OH groups of Tcm C from two molecules of O_2 and the 4a-OH group of Tcm C from a molecule of H_2O. These results suggest a mechanistic analogy among TcmG, ElmG, and the bacterial and fungal hydroquinone epoxidizing dioxygenases, as well as the mammalian vitamin K-dependent γ-glutamyl carboxylase.

Tetracenomycim (Tcm) C (**1**),[1] produced by *Streptomyces glaucescens*, and elloramycin A (**2**),[2] produced by *Streptomyces olivaceus* Tü2353, are polyketide antibiotics characterized by a unique highly hydroxylated cyclohexenone moiety (Scheme 1, boxed). Previous studies have established that the 4- and 12a-OH groups[3] and the 4a-OH group[4] of **1** are derived from molecular O_2 and H_2O, respectively, and that the three hydroxy groups are introduced via an unprecedented triple hydroxylation of Tcm A2 (**3**),[5] catalyzed by the Tcm A2 oxygenase that is encoded by *tcmG*.[5,6] Recent work on the biosynthesis of **2** has led to the identification of ElmG,[7] a homologue of TcmG, which was proposed to catalyze a similar triple hydroxylation of 8-demethyl Tcm A2 (**4**) to 8-demethyl Tcm C (**5**) in vivo (Scheme 1). However, the molecular mechanism for the introduction of

Scheme 1



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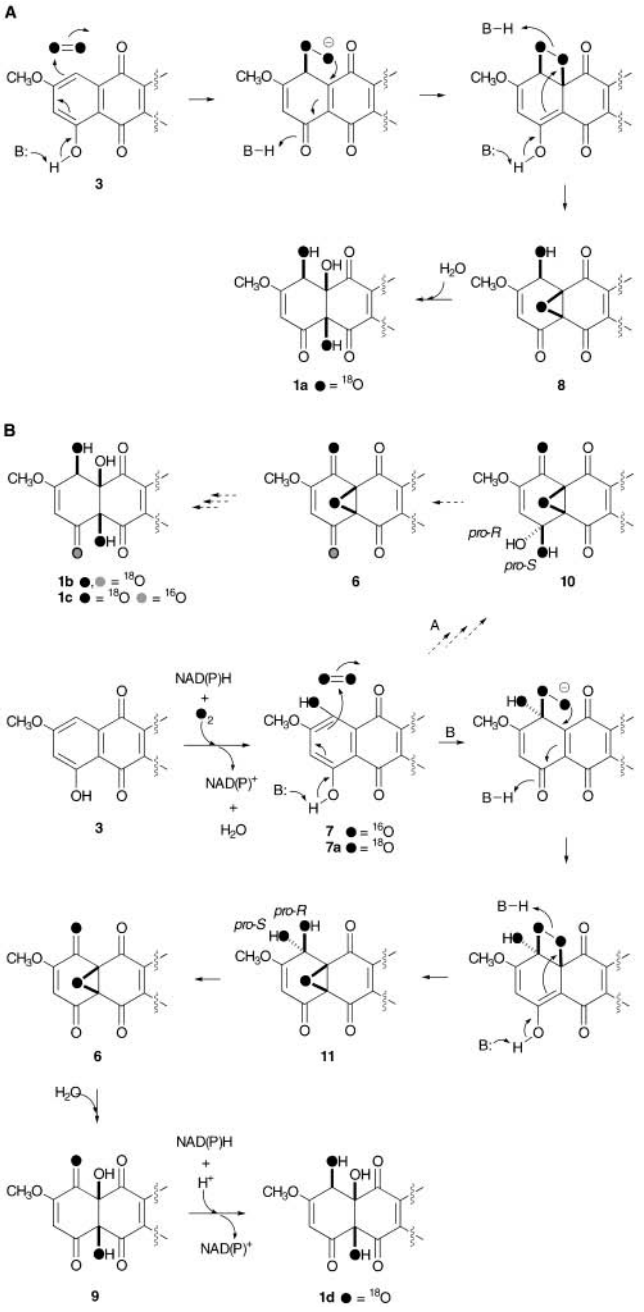
these hydroxy groups into **3** or **4** remains unknown. We now report that hydroxylation of **3** to **1** catalyzed by either TcmG or ElmG under $^{18}O_2$ reveals that the 4- and 12a-OH groups are derived from *two* molecules of O_2 and that this reaction likely involves an epoxyquinone intermediate such as **6** that results from direct oxidation of the hydroquinone precursor, 4-hydroxy Tcm A2 (**7**).

Both dioxygenase and monooxygenase−dioxygenase mechanisms have been proposed for this triple hydroxylation reaction,[4,5] either of which is consistent with all the results from earlier in vivo labeling experiments[3,4] with both $^{18}O_2$ and $H_2{}^{18}O$. As shown in Scheme 2A, the 4- and 12a-OH groups could be introduced in a concerted fashion from one molecule of O_2 in a dioxygenase mechanism to form an epoxysemiquinone intermediate **8**; cis-opening of its oxirane ring by a H_2O molecule could introduce the 4a-OH to yield **1**. (While most known epoxide hydrolases catalyze trans-opening of the oxirane ring,[8] examples of cis-opening have been observed.[9]) Alternatively, as shown in Scheme 2B, the 4- and 12a-OH groups could be introduced stepwise from two molecules of O_2 in a monooxygenase−dioxygenase mechanism to form intermediates **7** and **6**, respectively. Cis-opening of the oxirane ring of **6** by a H_2O molecule could then introduce the 4a-OH to yield dihydroxyquinone **9** that could be finally reduced to **1**.

These mechanisms can be differentiated by carrying out the reaction in vitro under an atmosphere of $^{16}O_2/^{18}O_2$. If the enzyme reaction follows the dioxygenase mechanism (Scheme 2A), both ^{18}O atoms, as opposed to two ^{16}O atoms, from one $^{18}O_2$ molecule will be incorporated into **1** at the C-4 and -12a positions. One would expect an elevated abundance of a molecular ion for **1**, in which the molecular weight is increased by 4 mass units, (M + 4), as appeared in **1a**. If the enzyme reaction follows the monooxygenase−dioxygenase mechanism (Scheme 2B), the two ^{18}O atoms at the C-4 and -12a of **1** will be incorporated from two $^{18}O_2$ molecules. One would then expect the appearance of an elevated abundance of M + 2 in **1** that arises from ^{18}O incorporation exclusively at C-4 by the monooxygenase activity as shown in **7a**. Regiospecific oxygenation of **7** by the dioxygenase activity could subsequently lead to either epoxyquinone hydrate **10** or **11**. Dehydration of **10** to **6** could eliminate diastereospecifically either the *pro-R* ^{16}O- or *pro-S* ^{18}O-hydroxy group at C-1, leading to retention or loss of the ^{18}O label at this site. Consequently, one would expect the appearance of an elevated abundance of M + 2 (^{18}O at C-4), M + 4 (^{18}O at C-1 and −12a), and M + 6 (^{18}O at C-4, -1, and -12a) ions as shown in **1b** if the *pro*-R 1-OH is eliminated, but only that of M + 2 (^{18}O at C-4 or C-12a) and M + 4 (^{18}O at C-4 and −12a) ions as shown in **1c** if the *pro*-S 1-OH is eliminated. In contrast, dehydration of **11** to **6** by eliminating either of the *pro-S* or *pro-R* hydroxy group at C-4 results in the loss of the ^{18}O label at this site. One would then expect the appearance of an elevated abundance of M + 2 (^{18}O at C-4 or C-12a) and M + 4 (^{18}O at C-4 and −12a) ions only as shown in **1d**.

Scheme 2



The *tcmG* and *elmG* genes were overexpressed in *Streptomyces lividans* 1326 using pWHM68[5] and pBS4006,[7b] respectively, and the resultant TcmG and ElmG proteins were

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Table 1. Incorporation of ^{18}O into **1** by TcmG- or ElmG-Catalyzed Oxygenation of **3** under $^{18}O_2$ ($^{18}O_2/^{16}O_2$ = 61/39) As Determined by EI-MS

		[molecular ion]			
		M	M +2	M + 4	M + 6
calculated:	natural abundance	100	5.4	0.1	0
	dioxygenase (Scheme 2A)	100	5.4	156	0
	monooxygenase−diooxygenase (Scheme 2B)				
	pathway A: loss of *pro-R* 1-OH	100	161	156	245
	loss of *pro-S* 1-OH	100	318	245	0
	pathway B: loss of *pro-R* 4-OH	100	318	245	0
	loss of *pro-S* 4-OH	100	5.4	156	0
found:	control (air, $^{16}O_2$)	100	12 ± 6	0[a]	0[a]
	TcmG	100	230 ± 50	146 ± 32	0[a]
	ElmG	100	221 ± 10	117 ± 5	0[a]

[a] Too low to be determined meaningfully.

purified to homogeneity.[5,7b] Incubations[5,7b] (60 mL) of either TcmG (2.5 mg) or ElmG (2.5 mg) with 50 μM **3**, 250 μM NADPH, 1 mM DTT, and 10% DMSO, in 50 mM Tris-HCl, pH 9.0, under an atmosphere of $^{18}O_2$ ($^{18}O_2/^{16}O_2$ = 61/39) for 1 h at 30 °C, resulted in the formation of ^{18}O-labeled **1**. The latter was purified by HPLC[5,7b] and subjected to electron-spray mass spectrometry (EI-MS) analysis to determine the incorporation of the ^{18}O label; under the negative mode of EI-MS **1** yields a molecular ion at *m*/*e* 471 as a base peak. Table 1 summarizes the calculated and observed relative abundance of M, M + 2, M + 4, and M + 6 ions for the ^{18}O-labeled **1**. The found data were the average of two runs each for TcmG and ElmG, both of which yield **1** with similar levels (within the standard deviation) of ^{18}O incorporation.

The following conclusions can be drawn based on these results. (1) The observed abundance of the M + 2 ion—230 ± 50% from TcmG and 221 ± 10% from ElmG—conclusively excludes the dioxygenase mechanism (Scheme 2A) that predicts the M + 2 ion with only 5.4% intensity resulting from natural abundance. (It has been demonstrated previously that the oxygen atoms at C-1, -4, and -12a do not undergo exchange with $H_2{}^{18}O$ during the biosynthesis,[4] precluding the loss of ^{18}O label by exchange.) (2) The elevated abundance of the M + 2 ion also establishes that the 4- and 12a-OH groups are derived from two molecules of O_2, supporting the monooxygenase−dioxygenase mechanism (Scheme 2B). (3) The involvement of **7** as an intermediate is consistent with the amino acid sequence homology between TcmG and ElmG and other bacterial hydroxylases.[6,7] Direct epoxidation of **7** to **6** is mechanistically analogous to the dihydroxyacetanilide epoxidase-I (DHAE-I),[10] a member of the recently identified hydroquinone epoxidizing dioxygenase family involved in the biosynthesis of numerous bacterial and fungal epoxyquinones or epoxysemiquinones,[10,11] as well as to the mammalian vitamin K-dependent γ-glutamyl carboxylase.[12] Both DHAE-I[10c] and vitamin K-dependent γ-glutamyl carboxylase[12a,b] have been found to operate as dioxygenases via an epoxyquinone hydrate intermediate similar to **10** and **11**. On the assumption that dehydration of **10** and **11** proceed similarly in a nondiastereospecifically fashion,[10c,12a,b] the absence of the M + 6 ion suggests the lack of ^{18}O incorporation at C-1, as shown in **1c**, arguing against the involvement of pathway A (Scheme 2b). We therefore favor pathway B (Scheme 2B), which is supported by the observed abundance of M + 2 and M + 4. Recently, we have reported the crystallization of the TcmG protein and its preliminary X-ray data.[13] We envisage that TcmG or ElmG could serve as an excellent model to investigate the molecular mechanism of this family of intriguing enzymes.

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