VOL. 53 NO. 5, MAY 2000 THE JOURNAL OF ANTIBIOTICS pp. 502 — 508

Cloning, Characterization and Heterologous Expression of a Polyketide Synthase and P-450 Oxidase Involved in the Biosynthesis of the Antibiotic Oleandomycin

SANJAY SHAH, QUN XUE, LI TANG, JOHN R. CARNEY, MARY BETLACH and ROBERT MCDANIEL*

Kosan Biosciences, Inc.
3832 Bay Center Place, Hayward, CA 94545, U.S.A.

(Received for publication December 24, 1999)

The gene cluster encoding the deoxyoleandolide polyketide synthase (OlePKS) was isolated from the oleandomycin producing strain *Streptomyces antibioticus*. Sequencing of the first two genes encoding OlePKS, together with the previously identified third gene revealed an overall genetic and protein architecture similar to that of the erythromycin gene cluster encoding the 6-deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea*. When the entire OlePKS (10,487 amino acids) was expressed in the heterologous host *Streptomyces lividans*, it produced 8,8a-deoxyoleandolide, an aglycone precursor of oleandomycin. The role of the P-450 monooxygenase, OleP, in oleandomycin biosynthesis was also examined *in vivo* by co-expression with DEBS in *S. lividans*. The production of 8,8a-dihydroxy-6-deoxyerythronolide B and other derivatives indicates that OleP is involved in the epoxidation pathway of oleandomycin biosynthesis. Since there are currently no genetic systems available for manipulation of the natural oleandomycin producing strain, the heterologous expression system reported here provides a useful tool for studying this important macrolide antibiotic.

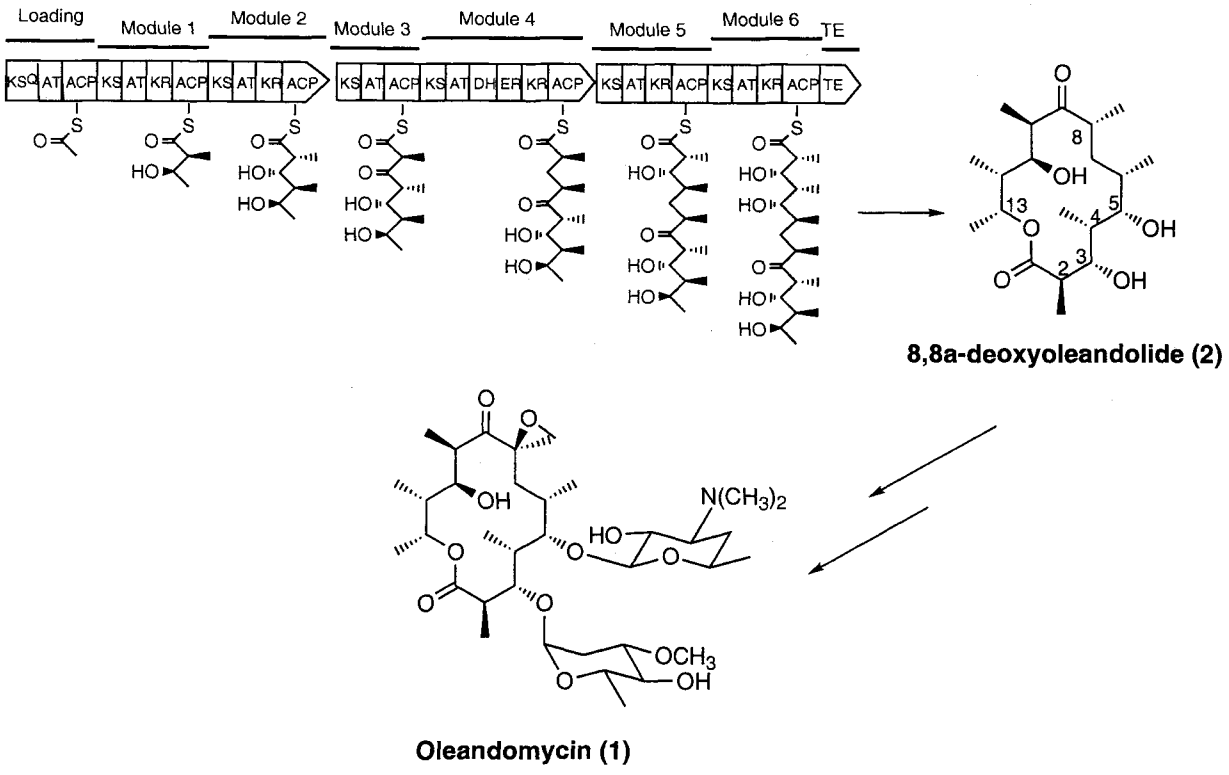
Oleandomycin (**1**) is a member of the macrolide class of antibiotics. Macrolides are a large family of polyketide natural products which include erythromycin, spiramycin, FK506, and avermectin[1,2]. The macrolactone core of oleandomycin, 8,8a-deoxyoleandolide (**2**), like those of other macrolides, is synthesized through the actions of a modular polyketide synthase (PKS)[3] (Fig. 1). It is structurally identical to the macrolactone precursor of erythromycin, 6-deoxyerythronolide B (6-dEB, **3**), with the exception of a C-13 methyl compared to the C-13 ethyl group of 6-dEB. Thus, whereas 6-dEB is derived from condensations between a propionate starter unit and six methylmalonate extender units, 8,8a-deoxyoleandolide begins with an acetate unit.

Over the past decade, progress in the study of oleandomycin biosynthesis has been due largely to the identification and sequencing of several biosynthetic and related genes by SALAS and coworkers. Analysis of these gene sequences has revealed enzymes putatively involved in synthesis and attachment of the two deoxysugars, regulatory and antibiotic resistance genes, and a P-450 monooxygenase[4~6]. A single open reading frame (ORF) encoding a type I polyketide synthase was also identified and, based on comparison to 6-deoxyerythronolide B synthase (DEBS), was most likely to encode the last two modules of the oleandomycin PKS (OlePKS)[3]. Despite these efforts, difficulties in the development of efficient genetic techniques for the oleandomycin producing organism, *Streptomyces antibioticus*, has hindered a more in-depth analysis of the pathway. In addition, the remaining PKS gene sequence has not been reported.

We report here the cloning, sequence, and analysis of the

Abbreviations: PKS, polyketide synthase; AT, acyltransferase; KR, ketoreductase; DH, dehydratase, ER, enoylreductase; ACP, acyl carrier protein; DEBS, 6-deoxyerythronolide B synthase; 6-dEB, 6-deoxyerythronolide B.

Fig. 1. The biosynthesis of 8,8a-deoxyoleandolide (**2**), the macrolactone precursor of oleandomycin (**1**).



The oleandomycin PKS consists of six modules, a loading domain and a thioesterase (TE) on three separate polypeptides. KS, ketosynthase; AT, acyltransferase; KR, ketoreductase; DH, dehydratase; ER, enoylreductase; ACP, acyl carrier protein; KS^Q, a KS-like domain which contains an active-site cysteine to glutamine mutation.

remaining OlePKS genes and the heterologous expression of the complete OlePKS in *Streptomyces lividans*. Not surprisingly, the gross genetic architecture of the OlePKS closely resembles that of DEBS, and together with other macrolide PKS gene clusters that have been identified should be useful in the construction of hybrid PKSs which produce novel macrolide antibiotics[7]. We also present evidence that OleP, a cytochrome P-450 monooxygenase previously identified in the gene cluster, is involved in formation of the oleandomycin epoxide.

Materials and Methods

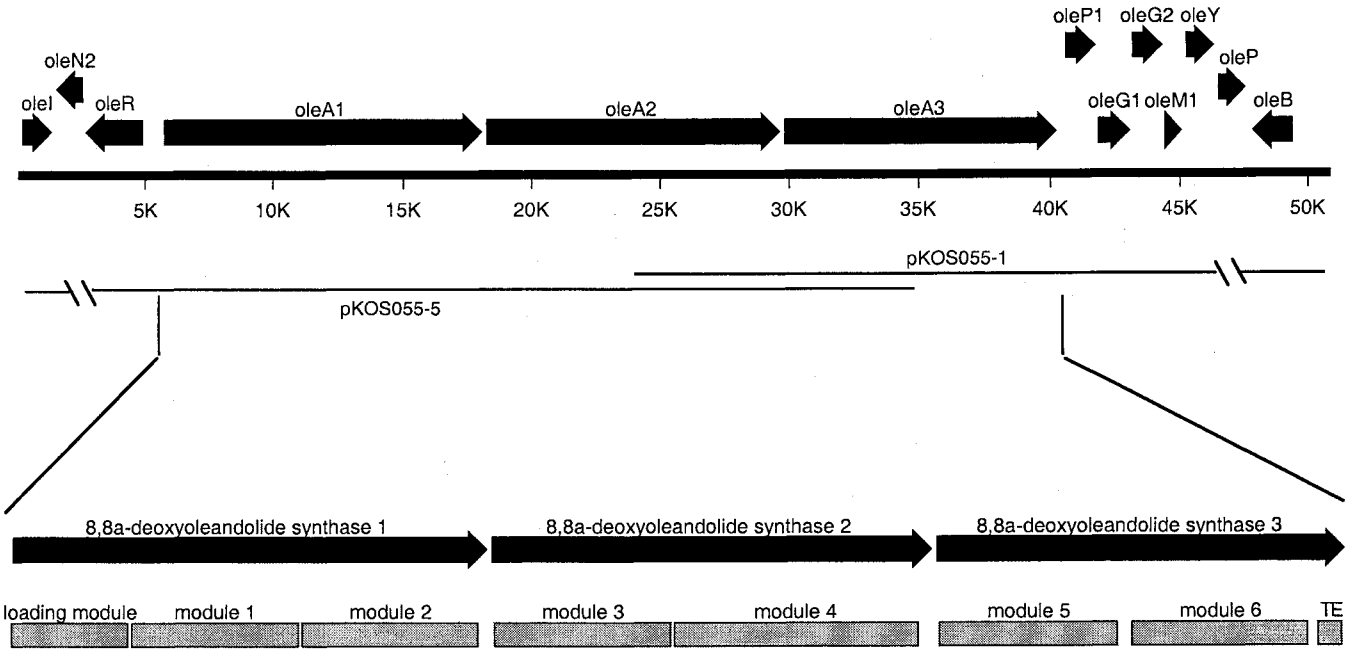
Bacterial Strains and Culture Conditions.

DNA manipulations were performed in *Escherichia coli* XL1-Blue (Stratagene) and DH10B (BRL). *Streptomyces lividans* K4-114 and K4-155, genotypically identical strains which contain deletions of the entire actinorhodin gene cluster, were used as host strains for the production of polyketide compounds[8]. *S. lividans* was transformed according to standard methods[9] and clones were selected with thiostrepton (50 μg/ml) or apramycin (100 μg/ml) overlays on R5 regeneration plates[9].

Cloning of the Oleandomycin Biosynthesis Gene Cluster from *Streptomyces antibioticus*

Genomic DNA was isolated from an oleandomycin producing strain of *S. antiobioticus* (ATCC 11891) using standard procedures[9]. A genomic library was prepared in Supercos (Stratagene) using DNA partially digested with *Sau3*A I following the supplier's protocols. A probe was prepared by PCR amplification of genomic DNA using primers specific to the KS domains of modules 5 and 6 of OlePKS. The genomic library was then probed by colony hybridization with ^{32}P-labeled probe. Cosmids containing the desired DNA inserts were verified by PCR with the same primers and by comparison of restriction digest patterns to known sequences. Two overlapping cosmids, pKOS055-5 and pKOS055-1, were identified which cover

Fig. 2. Organization of the *ole* cluster in *S. antibioticus*.



Cosmid clones are shown as overlapping lines.

approximately 65 kb of DNA (Fig. 2) and likely contain the entire oleandomycin gene cluster.

DNA Sequencing and Analysis

Six fragments ~5 kb in size and containing the desired region of the PKS to be sequenced were subcloned from cosmid pKOS055-5. Shotgun libraries were made from each subclone using *Hin*P1 I partially digested DNA cloned into pUC19. Insert sizes ranged from 500~3000 bp. PCR-based double-stranded DNA sequencing was performed on the shotgun clones using a Beckman CEQ 2000 capillary sequencer. Modules 1~4 of the PKS gene cluster were sequenced to approximately 4× coverage. Sequence was assembled using the Sequencher (Gene Codes Corp.) software package and analyzed with MacVector (Oxford Molecular). The sequence has been deposited in the GenBank database under accession number AF220951.

Construction of Expression Plasmids for OlePKS and OleP

The OlePKS expression plasmid pKOS098-4 was constructed by replacing the *eryAI-AIII* genes between the *Nde* I and *Eco*R I sites of pKAO127'kan'[8] with the *oleAI-AIII* genes. A 15.2-kb *Nsi* I-*Eco*R I fragment containing *oleAI* and a portion of *oleAII* from cosmid pKOS055-5 was subcloned into a vector containing an *Nde* I site −3 nt from the 5′ terminus of the *Nsi* I site to generate pKOS039-116. The 15.2-kb *Nde* I-*Eco*R I fragment was then subcloned into another vector containing a *Pac* I site −15 nt from the 5′ terminus of the *Nde* I site resulting in pKOS039-110. This generated the following sequence upstream of the *Nsi* I site in *oleAI* (*Pac* I and *Nsi* I sites are underlined, *Nde* I site is in bold): 5′-TTAATTAAGGAGGAC**CATATGCAT**-3′. The 15.2 kb *Pac* I-*Eco*R I fragment from pKOS039-110 was then cloned into the corresponding sites of pKAO127'kan' to yield pKOS038-174.

Next, a 14-kb *Eco*R I-*Eco*R V fragment and a 5.4-kb *Eco*R V-*Pst* I fragment, together containing the remaining portions of the *oleAII* and *oleAIII* genes, were obtained from cosmid pKOS055-1 and cloned successively into pLitmus28 (Stratagene) to give pKOS039-115. A 20-kb *Spe* I-*Xba* I encompassing both of the former fragments was then excised and subcloned into another vector to introduce an *Eco*R I cloning site downstream of the *oleAIII* gene. This allowed a 20-kb *Eco*R I fragment to be extracted from this plasmid and inserted into the *Eco*R I site of pKOS038-174 (see above) to complete construction of the OlePKS expression vector pKOS098-4.

Table 1. Deduced functions of OlePKS domains.

PKS subunit/module	Amino acids, no.	Identified domains					
OleAI	4151						
Loading	~1025	KS^Q	AT	ACP			
Module 1	~1465	KS	AT	KR	ACP		
Module 2	~1520	KS	AT	KR	ACP		
OleAII	3817						
Module3	~1543	KS	AT	KR°	ACP		
Module4	~2117	KS	AT	DH	ER	KR	ACP
OleAIII	3520						
Module5	~1531	KS	AT	KR	ACP		
Module6	~1532	KS	AT	KR	ACP		
TE	~229						

KR°, inactive KR domain

The *oleP* gene was PCR amplified using the following oligonucleotide primers (forward, 5′-TTTCATATGGTG-ACCGATACGCACACCGGA-3′, reverse, 5′-TTTGAATT-CTCACCAGGAGACGATCTGGCG-3′). After subcloning in PCR-Script (Stratagene), the *Nde*I-*Eco*RI fragment containing *oleP* was isolated and cloned into the pSET152-based plasmid pKOS010-153[10], replacing the *Nde*I-*Eco*RI *eryAIII* gene fragment to yield pKOS024-83.

Production and Analysis of Polyketide Analogs

S. lividans transformants were cultured in 5 ml of liquid R5 medium[9] at 30°C for 7 days under the appropriate antibiotic selection (thiostrepton 6 μg/ml, apramycin 50 μg/ml). Samples of fermentation broth were analyzed as previously described[11] by LC/MS on a reversed-phase C-18 column (4.6×150 mm) using a Perkin-Elmer SCIEX API 100 LC mass spectrometer. The polyketides were identified by correspondence of their mass spectrum to the products expected or to known standards. Compound **6** was purified by silica gel chromatography using ethyl acetate/hexane (1 : 1). Fractions were analyzed by mass spectrometry and those containing the desired compound were pooled together. The structure of **6** was determined by NMR and mass spectrometry (details below).

Results and Discussion

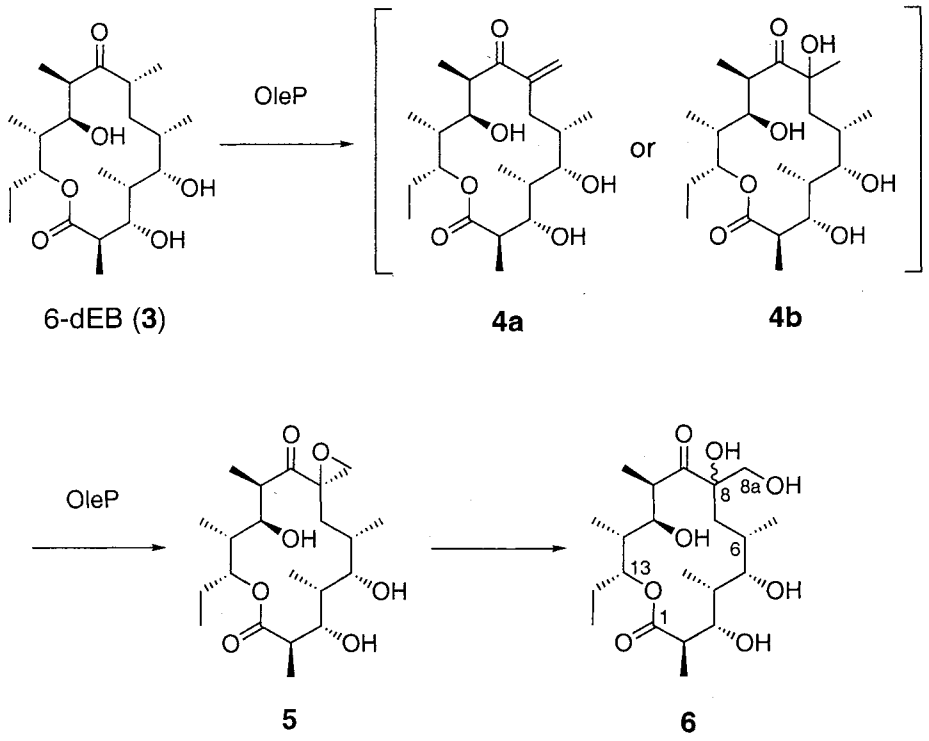
Analysis of the OlePKS Coding Sequence

The OlePKS is encoded by three ORFs – *oleAI*, *oleAII*, and the previously identified ORFB[3] (designated *oleAIII* here) – that span 35-kb of DNA. Each of the ORFs encodes two PKS modules, as in *eryAI-AIII*, and examination of the active site domains within the modules (Table 1) also reveals an organization similar to the active site arrangement of DEBS. This was not unexpected given the structural relationship of the polyketides produced by the enzymes encoded by the two gene clusters. Yet the amino acid sequences of OlePKS and DEBS (45% aa identities) are surprisingly different from one another. The most significant departure was found in the OlePKS loading domain. In contrast with the loading module of DEBS, which consists of an acyl transferase (AT) domain – that loads a propionate starter unit – and an acyl carrier protein (ACP), the OlePKS loading module has an additional KS-like domain (KS^Q) with a glutamine instead of a cysteine at the active site. These domains have recently been shown to decarboxylate acylthioesters within PKSs and the related fatty acid synthases[12,13]. Therefore, it is most likely that OlePKS initiates 8,8a-deoxyoleandolide synthesis by loading the ACP with a malonate unit and performing a decarboxylation to generate acetyl-ACP.

Production of 8,8a-Deoxyoleandolide in *S. lividans*

A vector for heterologous expression of OlePKS was constructed analogous to those developed for DEBS and the picromycin PKS (PicPKS)[14,15]. Plasmid pKOS098-4 is an autonomously replicating SCP2*-based shuttle vector containing the three OlePKS open reading frames downstream of the *Streptomyces coelicolor act*I promoter and *act*II-ORF4 transcriptional activator. Transformation of *S. lividans* K4-155 with pKOS098-4 resulted in a strain that produced ~50 mg/liter of **2**, confirming that 8,8a-

Fig. 3. Proposed hydroxylation pathway of 6-dEB by OleP in *S. lividans*.



deoxyoleandolide is the biosynthetic intermediate produced by the oleandomycin PKS. The mass spectrum and LC retention time of **2** were identical to known standards. The amount of 8,8a-deoxyoleandolide produced is similar to production levels of 6-dEB and narbonolide achieved with DEBS and PicPKS, respectively, using the same host and vector[14,15].

Involvement of OleP in Epoxidation

The *oleP* gene[4] is located approximately 6 kb downstream from the end of *oleAIII* (Fig. 2). It encodes a cytochrome P-450 monooxygenase homologous to several macrolide oxidases such as those found in the erythromycin[16~18], picromycin/methymycin[19,20], and tylosin[21] gene clusters. Although it is putatively involved in formation of the oleandomycin epoxide moiety, several experiments have failed to establish its role[4]. Furthermore, neither the biochemical mechanism of epoxidation is known, nor is it clear whether any enzymes in addition to OleP are required. When the epoxidation step occurs during biosynthesis is also not understood[22,23].

Since the OlePKS expression plasmid was not available at the initiation of our study, OleP was expressed in the presence of pKAO127'kan'[8], a DEBS expression plasmid that produces both 6-dEB (**3**) and 8,8a-deoxyoleandolide (**2**) in *S. lividans*, in order to investigate whether OleP catalyzes oxidation of macrolide aglycones and the nature of the resulting structure. The *oleP* gene was integrated into the chromosome of *S. lividans* K4-114/pKAO127'kan' using a ΦC31-based vector, under control of the *act*II-ORF4 activator and *Pact*I promoter. The resulting strain produced a mixture of compounds, of which the predominant components were **2** and **3** (~1 : 7). At least six additional compounds were present at levels between 10~50% of **2**, apparently all derivatives of **3** based on MS analysis. Collectively they amount to approximately a 33% conversion of the 6-dEB produced by the strain. There were no derivatives of **2** detected, presumably because it is produced in such low quantity compared to **3**.

Of the compounds identified, two appeared to be singly hydroxylated species, three appeared to be dehydro derivatives, and one appeared to be dihydroxylated when mass spectra were compared to that of **3**. Since none of the new compounds matched the expected epoxide product, the dihydroxy derivative was selected for structural identification. The structure of this compound, **6** (Fig. 3), was established using mass spectrometry and NMR

Table 2. ^{1}H and ^{13}C NMR data for **6**[a].

No.	^{1}H δ ppm (m, J_{HH})	^{13}C δ ppm (m)	HMBC correlations
1		176.2 (s)	
2	2.66 (dq, 10.5, 6.5)	45.4 (d)	C-1, C-2a, C-3, C-4
2a	1.23 (d, 6.5)	13.7 (q)	C-1, C-2, C-3
3	3.80 (d, 10.5)	72.9 (d)	C-1, C-2, C-2a, C-4, C4a, C-5
4	1.93 (m)	42.8 (d)	C-2, C4a, C-5
4a	1.19 (d, 7.0)	12.0 (q)	C-5
5	3.50 (ovrlp)	90.8 (d)	
6	1.73 (ovrlp)	36.5 (d)	
6a	1.07 (d, 6.0)	17.6 (q)	C-6, C-7, C-8
7	1.56 (dd, 12.5, 12.5)	40.8 (t)	C-8
	2.57 (dd, 12.5, 7.0)		C-5
8		91.6 (s)	
8a	3.45 (d, 11.5)	67.7 (t)	C-7, C-8, C-9
	3.60 (d, 11.5)		C-9
9		219.1 (s)	
10	3.50 (ovrlp)	41.2 (d)	
10a	0.95 (d, 7.5)	7.4 (q)	
11	3.59 (m)	69.2 (d)	
12	1.71 (m)	41.6 (d)	
12a	0.93 (d, 7.5)	9.0 (q)	C-11, C-12
13	5.36 (ddd, 9.5, 7.0, <1)	76.2 (d)	C-1, C-11, C-12, C12a, C-14, C15
14	1.51 (m)	25.7 (t)	
	1.74 (m)		
15	0.90 (t, 7.5)	10.4 (q)	C-13, C-14

[a] Recorded in $CDCl_3$

spectroscopy. HRFABMS was obtained for the $[M-H_2O+H]^+$ ion ($C_{21}H_{37}O_7$, calculated: 401.2539; observed: 401.2534). DEPT spectra indicated the presence of six methyl, three methylene, nine methine, and three quaternary carbons, consistent with the proposal that a methyl and methine from 6-dEB had been replaced by an oxy-methylene (δ 67.7, C-8a) and oxy-quaternary carbon (δ 91.6, C-8). HMQC NMR data allowed the assignment of two protons resonating at δ 3.45 (d, 11.5 Hz) and 3.60 (d, 11.5 Hz) to C-8a. HMBC correlations between H_a-8a and C-8 as well as carbons signals at δ 40.8 (C-7) and 219.1 (C-9) confirmed that the oxidations had occurred at the 8 and 8a positions. Additional NMR data from TOCSY, HMQC, and HMBC (Table 2) experiments fully support the assigned structure of **6**. The absolute configuration at C-8 has not been determined.

Based on the structure of **6** we conclude that OleP is partly or wholly responsible for introduction of the oleandomycin epoxide. The presence of the diol in our experiment could be explained by formation of the epoxide (**5**) and subsequent hydrolysis by an endogenous enzyme in *S. lividans* to **6**. Two different intermediates, **4a** and **4b**, preceding the epoxide can be proposed (Fig. 3). However, both dehydro and singly hydroxylated derivatives were putatively identified in the fermentation mixture. Since precedent exists for both epoxidation routes[24~26], it is not possible to say which mechanism might be used by OleP. Characterization of the remaining compounds may provide further insight. Alternatively, it is possible that an additional enzyme(s) present in *S. antibioticus* is required for formation of the epoxide and in its absence OleP performs the double hydroxylation. In either case, our result strongly suggests that epoxide formation occurs prior to attachment of the two sugars, which was heretofore unknown.

Acknowledgements

We thank WILLIAM GERWICK and R. THOMAS WILLIAMSON at Oregon State University for FABMS data and DAVID HOPWOOD and LEONARD KATZ helpful comments on the manuscript.

References

1) KATZ, L. & S. DONADIO: Polyketide synthesis: Prospects for hybrid antibiotics. Ann. Rev. Microbiol. 47: 875~912, 1993
2) HOPWOOD, D.: Genetic contributions to understanding polyketide synthases. Chem. Rev. 97: 2465~2497, 1997
3) SWAN, D. G.; A. M. RODRIGUEZ, C. VILCHES, C. MENDEZ & J. A. SALAS: Characterisation of a *Streptomyces antibioticus* gene encoding a type I polyketide synthase which has an unusual coding sequence. Mol. Gen. Genet. 242: 358~362, 1994
4) RODRIGUEZ, A. M.; C. OLANO, C. MENDEZ & C. R. HUTCHINSON: A cytochrome P450-like gene possibly involved in oleandomycin biosynthesis by *Streptomyces antibioticus*. FEMS Microbiol. Lett. 127: 117~120, 1995
5) OLANO, C.; A. M. RODRIGUEZ, J. M. MICHEL, C. MENDEZ, M. C. RAYNAL & J. A. SALAS: Analysis of a *Streptomyces antibioticus* chromosomal region involved in oleandomycin biosynthesis, which encodes two glycosyltransferases responsible for glycosylation of the macrolactone ring. Mol. Gen. Genet. 259: 299~308, 1998
6) QUIROS, L. M.; I. AGUIRREZABALAGA, C. OLANO, C. MENDEZ & J. A. SALAS: Two glycosyltransferases and a glycosidase are involved in oleandomycin modification during its biosynthesis by *Streptomyces antibioticus*. Mol. Microbiol. 28: 1177~1185, 1998
7) TANG, L.; H. FU & R. MCDANIEL: Formation of functional heterologous complexes using subunits from the picromycin, erythromycin, and oleandomycin polyketide synthases. Chem. & Biol. 7: 77~84, 2000
8) ZIERMANN, R. & M. C. BETLACH: Recombinant polyketide synthesis in *Streptomyces*: Engineering of improved host strains. Biotechniques 26: 106~110, 1999
9) HOPWOOD, D. A.; M. J. BIBB, K. F. CHATER, T. KIESER, C. J. BRUTON, H. M. KIESER, D. J. LYDIATE, C. P. SMITH, J. M. WARD & H. SCHREMPF: Genetic Manipulation of Streptomyces: A Laboratory Manual. The John Innes Foundation, Norwich, UK, 1985
10) XUE, Q.; G. ASHLEY, C. R. HUTCHINSON & D. V. SANTI: A multi-plasmid approach to preparing large libraries of polyketides. Proc. Natl. Acad. Sci. USA 96: 11740~11745, 1999
11) MCDANIEL, R.; A. THAMCHAIPENET, C. GUSTAFSSON, H. FU, M. BETLACH, M. BETLACH & G. ASHLEY: Multiple genetic modifications of the erythromycin gene cluster to produce a library of novel "unnatural" natural products. Proc. Natl. Acad. Sci. USA 96: 1846~1851, 1999
12) WITKOWSKI, A.; A. K. JOSHI, Y. LINDQVIST & S. SMITH: Conversion of a β-ketoacyl synthase to a malonyl decarboxylase by replacement of the active-site cysteine with glutamine. Biochemistry 38: 11643~11650, 1999
13) BISANG, C.; P. F. LONG, J. CORTÉS, J. WESTCOTT, J. CROSBY, A.-L. MATHARU, R. J. COX, T. J. SIMPSON, J. STAUNTON & P. F. LEADLAY: A chain initiation factor common to both modular and aromatic polyketide synthases. Nature 401: 502~505, 1999
14) KAO, C. M.; L. KATZ & C. KHOSLA: Engineered biosynthesis of a complete macrolactone in a heterologous host. Science 265: 509~512, 1994
15) TANG, L.; H. FU, M. C. BETLACH & R. MCDANIEL: Elucidating the mechanism of chain termination switching in the picromycin/methymycin polyketide synthase. Chem. & Biol. 6: 553~558, 1999
16) WEBER, J. M.; J. O. LEUNG, S. J. SWANSON, K. B. IDLER & J. B. MCALPINE: An erythromycin derivative produced by targeted gene disruption in *Saccharopolyspora erythraea*. Science 252: 114~117, 1991
17) HAYDOCK, S. F.; J. A. DOWSON, N. DHILLON, G. A. ROBERTS, J. CORTES & P. F. LEADLAY: Cloning and sequence analysis of the genes involved in erythromycin biosynthesis in *Saccharopolyspora erythraea*; sequence similarities between EryG and a family of S-adenosylmethionine-dependent methyltransferases. Mol. Gen. Genet. 230: 120~128, 1991
18) STASSI, D.; S. DONADIO, M. J. STAVER & L. KATZ: Identification of a *Saccharopolyspora erythraea* gene required for the final hydroxylation step in erythromycin biosynthesis. J. Bacteriol. 175: 182~189, 1993
19) BETLACH, M. C.; J. T. KEALEY, M. C. BETLACH, G. A. ASHLEY & R. MCDANIEL: Characterization of the macrolide P-450 hydroxylase from *Streptomyces venezuelae* which converts narbomycin to picromycin. Biochemistry 37: 14937~14942, 1998
20) XUE, Y.; D. WILSON, L. ZHAO, H.-W. LIU & D. H. SHERMAN: Hydroxylation of macrolactones YC-17 and narbomycin is mediated by the *pikC*-encoded cytochrome P450 in *Streptomyces venezuelae*. Chem. & Biol. 5: 661~667, 1998
21) MERSON-DAVIES, L. A. & E. CUNDLIFFE: Analysis of five tylosin biosynthetic genes from the *tylIBA* region of the *Streptomyces fradiae* genome. Mol. Microbiol. 13: 349~355, 1994
22) SPAGNOLI, R. & L. CAPPELLETTI: Biological conversion of erythronolide B, an intermediate of erythromycin biogenesis, into new "hybrid" macrolide antibiotics. J. Antibiotics 36: 365~375, 1983
23) TATSUTA, K.; H. GUNJI, S. TAJIMA, T. ISHIYAMA, S. IMAI, S. OKUYAMA & S. FUKATSU: Biosynthetic studies on oleandomycin by incorporation of the chemically synthesized aglycones. J. Antibiotics 43: 909~911, 1990
24) INOUYE, M.; Y. TAKADA, N. MUTO, T. BEPPU & S. HORINOUCHI: Characterization and expression of a P-450-like mycinamicin biosynthesis gene using a novel *Micromonospora-Escherichia coli* shuttle cosmid vector. Mol. Gen. Genet. 245: 456~464, 1994
25) HASHIMOTO, T.; J. MATSUDA & Y. YAMADA: Two-step epoxidation of hyoscyamine to scopolamine is catalyzed by bifunctional hyoscyamine 6 beta-hydroxylase. FEBS Lett. 329: 35~39, 1993
26) HASHIMOTO, T. & Y. YAMADA: Purification and characterization of hyoscyamine 6 beta-hydroxylase from root cultures of *Hyoscyamine niger* L. Hydroxylase and epoxidase activities in the enzyme preparation. Eur. J. Biochem. 164: 277~285, 1987