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Biosynthesis of the apoptolidins in *Nocardiopsis* sp. FU 40

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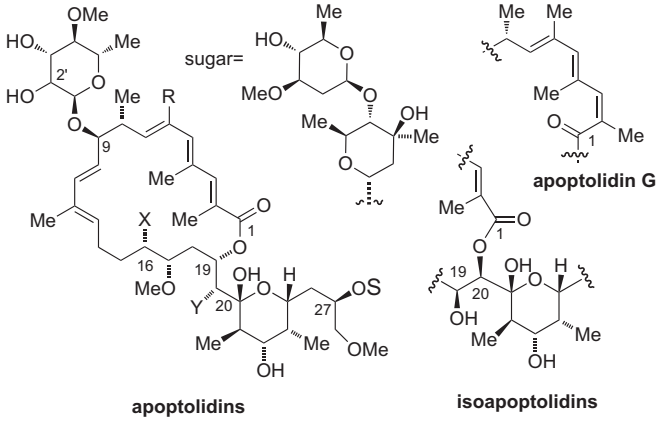
ABSTRACT

The apoptolidins are 20/21-membered macrolides produced by *Nocardiopsis* sp. FU40. Several members of this family are potent and remarkably selective inducers of apoptosis in cancer cell lines, likely via a distinct mitochondria associated target. To investigate the biosynthesis of this natural product, the complete genome of the apoptolidin producer *Nocardiopsis* sp. FU40 was sequenced and a 116 kb region was identified containing a putative apoptolidin biosynthetic gene cluster. The apoptolidin gene cluster comprises a type I polyketide synthase, with 13 homologating modules, apparently initiated in an unprecedented fashion via transfer from a methoxymalonyl-acyl carrier protein loading module. Spanning approximately 39 open reading frames, the gene cluster was cloned into a series of overlapping cosmids and functionally validated by targeted gene disruption experiments in the producing organism. Disruption of putative PKS and P_{450} genes delineated the roles of these genes in apoptolidin biosynthesis and chemical complementation studies demonstrated intact biosynthesis peripheral to the disrupted genes. This work provides insight into details of the biosynthesis of this biologically significant natural product and provides a basis for future mutasynthetic methods for the generation of non-natural apoptolidins.

1. Introduction

Glycosylated polyketide macrolides from actinomycetes comprise a diverse class of natural products that demonstrate a variety of therapeutic potentials as evidenced by their antibiotic, antifungal, anticancer, and antiparistic properties.[1–3] Access to structural variants of macrolide natural products is limited due to their structural and functional complexity that features a stereochemically rich macrolide core and often one or more decorating deoxysugar moieties. In complement to synthetic chemical approaches, classical methods of semisynthesis, precursor-directed biosynthesis, and chemical degradation provide access to analogs of parent metabolites. However, recent efforts based on combining chemical approaches with genetic and metabolic engineering promise to significantly broaden the scope of structural modifications and potential for developing therapeutic lead compounds.[4–8]

The apoptolidin macrolides (Fig. 1) have drawn attention for their reported ability to selectively induce apoptosis in several cancer cell lines.[9–11] Produced by *Nocardiopsis* sp. FU40 apoptolidins are 20/21-membered macrolides, appended variously with 6-deoxysugars at C9 and with or without an invariant disaccharide



apoptolidin A (**1**,R = Me; X = Y = OH; 2' = β-OH; S = sugar)
apoptolidin B (**2**, R = Me; X = H; Y = OH; 2' = β-OH; S = sugar)
apoptolidin C (**3**, R = Me; X = Y = H; 2' = β-OH; S = sugar)
apoptolidin D (**4**, R = H; X = Y = OH; 2' = β-OH; S = sugar)
apoptolidin E (**5**, R = Me; X = Y = H; 2' = α-OH; S = sugar)
apoptolidin F (**6**, R = Me; X = Y = H; 2' = α-OH; S = H)
apoptolidin G (**7**, R = Me; X = Y = OH; 2' = β-OH; S = sugar)
isoapoptolidin A (**8**, R = Me; X = Y = OH; S = sugar)
isoapoptolidin B (**9**. R = Me; X = H; Y = OH; S = sugar)
isoapoptolidin D (**10**, R= H; X = Y = OH; S = sugar)

Fig. 1. Chemical structures of apoptolidins and isoapoptolidins produced by *Nocardiopsis* sp. FU40.

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at C27.[12–14] As with many macrolides containing 20-membered or larger ring sizes, apoptolidins possess a relatively long side chain containing a cyclic hemiketal. To date, over ten apoptolidins have been identified, which vary in substitution,[15–17] double bond geometry at C2/3,[18] and macrocylization regiochemistry[13,14] (Fig. 1).

A mode of action involving the inhibition of mitochondrial ATPase has been proposed for apoptolidin A (**1**) based, in part, in comparison to other polyketide macrolides, such as cytovarisin, ossamycin, and the oligomycins.[10,19] Chemical degradation[11,20–22] and chemical synthesis[23–27] have added to the natural retinue, yielding over twenty-five analogs and congeners of the parent apoptolidin A, and permitting SAR analysis in this series. Notably, apoptolidins B–F[15–17] (**2**–**6**) have been identified by the Wender group as minor metabolites (2–5 mg/L) relative to the parent apoptolidin A (ca. 100 mg/L).[12] To date, SAR studies have shown the parent aglycone (apoptolidinone) is inactive[23,26] and the C27 disaccharide outweighs the C9 monosaccharide in importance to cell cytotoxicity.[17,25,27,28] In considering options to produce quantities of the modified apoptolidins for further biological investigations, studies to date clearly indicate production by fermentation to outpace chemical synthesis as a means to access material.[29] For this reason we turned our attention to the identification and characterization of the apoptolidin gene cluster.

2. Results and discussion

2.1. Cloning and sequencing of the apoptolidin gene cluster from *Nocardiopsis* sp. FU40

Genome sequencing and assembly of *Nocardiopsis* sp. FU40 resulted in a total of 1009 contigs ranging in size from 66 bp to 132,247 bp (264 contigs were larger than 10,000 bp), which formed 53 scaffolds ranging in size from 303 bp to 4,723,578 bp. The largest scaffold contained 801 contigs and corresponded to 50% of the genome, which was estimated at 9,433,896 bp, in agreement with that of other actinomycete genomes previously sequenced.[30–33] The genome was sequenced to 35-fold read coverage. Genome annotation of the scaffolds resulted in the identification of 10,240 apparent coding sequences.

The gene cluster for apoptolidin biosynthesis was identified within the proteome of *Nocardiopsis* sp. FU40 by identifying co-localized genes consistent with a predicted apoptolidin biogenesis using the method as described by Bachmann and Ravel.[33] Correspondingly, the 20/21-membered polyketide core of apoptolidin, variously reduced at C1 position of hypothetical acetate precursors, was predicted to be diagnostic of a non-iterative (type I) polyketide synthase (PKS). Type-1 PKS are multi-domain megasynthases in which the order of the catalytic domains corresponds, with some exceptions,[34,35] to the order of biochemical events entailed in the assembly of encoded polyketides. In these systems, each domain is used only once: acyltransferase (AT) domains mediate the activation of homologating ketide precursors, variously C2 substituted malonyl-CoAs, and their transfer to phosphopantathienylated acyl carrier protein domains (ACP); ketosynthase domains (KS) catalyze the condensation of two tethered acyl-ACP thioesters by transthioesterification of an upstream acyl-ACP onto an active site cysteine followed by decarboxylative (thio)Claisen condensation with the downstream malonyl-ACP; ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains catalyze the reactions for the generation of the appropriate oxidation states along the polyketide chain. A 14-module hypothetical domain sequence (including a loading module) was inferred from this core structure based on the 1,3-oxygenation pattern corresponding to C1 of acetate and propionate precursors. Variable hydroxylation at positions corresponding to C2 of precursors (C16 and C20 of apoptolidins) and the earlier identification[15] of apoptolidins B (2) and C (3) suggested that the polyketide core is further oxidized by oxidases at these positions. Finally, glycosylations at C9 and C27 inform the expectation of multiple glycosyl transferases as well as ancillary predicted deoxysugar biosynthesis enzymes involved in generation of disaccharide precursors NDP-L-olivomycose and NDP-D-oleandrose.

Based on these assumptions, a ~116 kb region located at 3.39–3.51 Mb was readily identified by the presence of a putative 14-module type I polyketide synthase largely matched the predicted domain organization and contained the appropriate post-PKS elaboration functionality. A second gene cluster putatively encoding a type-I PKS was identified in the genome (data not shown), but not deemed a likely candidate for encoding apoptolidin as it possessed only approximately eight intact modules, a domain sequence consistent with a polyene macrolide, and insufficient evidence of deoxysugar biosynthetic genes consistent with apoptolidin. The putative apoptolidin gene cluster '*apo*', initially possessed eight short sequencing gaps, which were bridged by PCR amplification of gap regions and DNA sequencing. The ends of the *apo* gene cluster were approximately defined by the occurrence of contiguous putative genes with homology to primary metabolic and/or otherwise highly conserved gene sequences and designated as *ApoU1–10* and *ApoD1–10* corresponding to the ten genes upstream and downstream of the apoptolidin gene cluster. With a putative gene locus circumscribed, open reading frames were manually re-annotated by analysis of alternate predicted start codons and by comparison to functional homologs identified using BLAST. The *apo* gene cluster and flanking upstream and downstream region have been deposited into the NCBI with locus accession numbers of JF819834, JF894099, JF894100, and JF894101.

2.2. Analysis of the apoptolidin biosynthetic gene cluster (*apo*)

Fully consistent with the structure of the apoptolidins, the proposed 115.74 kb *apo* gene cluster contains 39 individual open reading frames (ORFs) including nine type I polyketide synthase genes (PKS), six oxidoreductase genes, four methyltranferases, and three glycosyl transferase genes (see gene map in Fig. 2).

Polyketide synthase organization. In *Nocardopsis* sp. FU40, PKS genes *ApoS1–ApoS8*, are proposed to be responsible for the biosynthesis of the apoptolidin polyketide core. A suggested module and domain organization and a proposed model for the PKS templated assembly of the apoptolidin polyketide backbone is shown in Fig. 3. ApoS1 is the putative protein identified as possessing the likely initiating module as it contains the sequence hallmarks of a decarboxylative KS^Q loading module. Specifically, the first KS domain of ApoS1 contains a cysteine to glutamate codon modification mutating the essential active site cysteine (C193Q) involved in the transthioesterification reaction that precedes KS mediated condensation. The next 12 extension modules are proposed to be contained on ApoS1–S7 and were ordered according to the predicted collinear arrangement of required domains. Finally, the terminating module protein was identified in ApoS8 by its terminal punctuation with a thioesterase (TE) domain, required for release from the megasynthase assembly. One additional polyketide containing putative open reading frame ApoS9, contains an incomplete module sequence 'KS-AT-KR' and ApoS10 apparently encodes a free-standing thioesterase protein that may be important in hydrolytic release and/or macrocyclization. Of the 65 domains encoding the biosynthesis of the macrolide core, there are four domains predicted to be inactive (Fig. 3, in black). Analysis of active site consensus residues for these predicted inactive domains reveals nearly universal conservation of all key consensus residues thought to be essential for activity, indicating that the structural determinants of inactivity of these domains may reside distal to these conserved motifs.

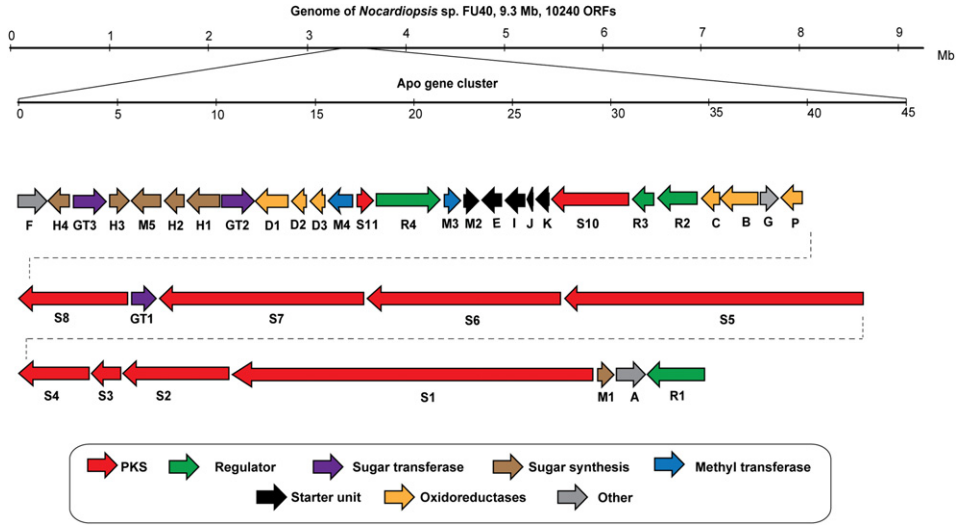

Fig. 2. Genetic organization of the apoptolidin gene cluster in *Nocardiopsis* sp. FU40. The 115.74 Mb gene cluster was identified in the producing organism by searching the sequenced genome for the biogenetically rationalizationed predicted type-I polyketide synthase domain sequence.

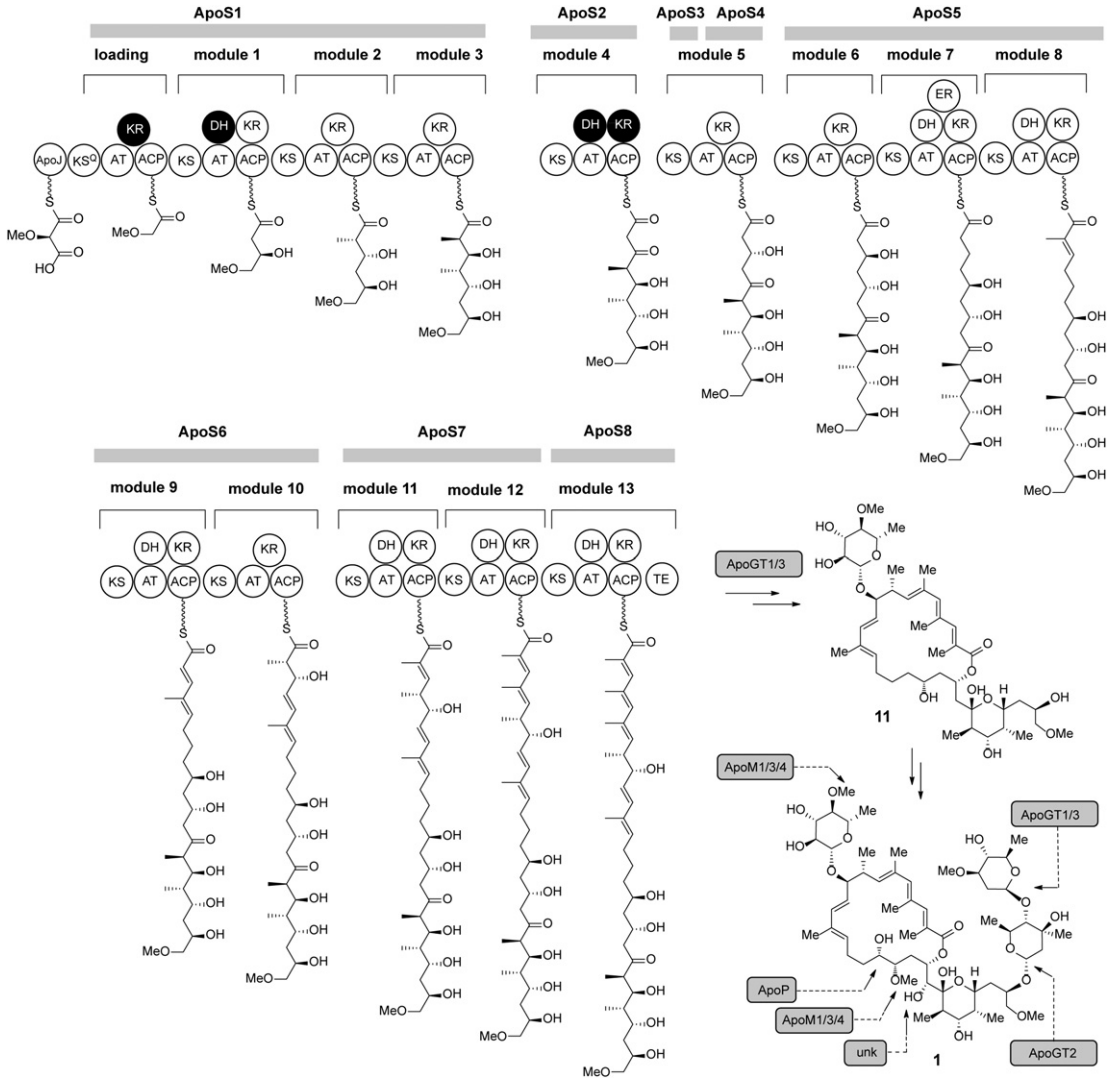

Fig. 3. Proposed pathway overview of apoptolidin assembly. A 14-module polyketide synthase, initiated by an unprecedented methoxymalonyl-ACP loading module, is proposed to assemble the *seco* acid precursor of the apoptolidins. Subsequent tailoring reactions are proposed to oxidize C16/21, methylate C17, and append the C9 monosaccharides and C27 disaccharide resulting in the fully elaborated and biologically active apoptolidin series.

Methoxymalonyl-ACP biosynthesis and initiating module. The proposal of an unusual initiating precursor, (2*R*)-methoxymalonlyl-ACP is based on the observations of (1) the atypical initiating 2-methoxy acetyl group in the apoptolidins,[35b] (2) a decarboxylative initiating KS^{Q} module, and (3) a five gene cassette upstream of the PKS genes, *ApoK–ApoM2*, identified with greater than 50% translated identity to the fkbG–K, proteins from the FK520 ascomycin gene cluster,[36] which are responsible for biosynthesis of (2*R*)-methoxymalonyl-ACP in *S. hygroscopicus* during FK520 production.[36,37] This condensation precursor has previously been observed in various type I polyketide biosyntheses including FK520, soraphen, and ansamitocin pathways.[38] In analogy with FK520 biosynthesis, we propose that the biosynthesis of (2*R*)-methoxymalonyl-ACP in apoptolidins initiates with the enzyme ApoE, which is proposed to dephosphorylate and covalently tether a glycolytic substrate derived from 1,3-bisphosphoglycerate to the 4′-phosphopantatheinyl group of an ACP, forming glyceryl-ApoJ. Next, two enzymes ApoK (NAD^{+}-dependent) and ApoI (FAD-dependent) catalyze the oxidation of glyceryl-ApoJ to the intermediate (2*R*)-hydroxymalonyl-ApoJ. The methyltransferase ApoM2 then catalyzes the O-methylation of (2*R*)-hydroxymalonyl-ApoJ to form (2*R*)-methoxymalonyl-ApoJ (Fig. 4). Unlike FK520, in which (2*R*)-methoxymalonyl-ACP is used in extension chemistry we propose that this precursor is recognized by the loading module of *apo*, which mediates its decarboxylative transfer from ApoJ to the loading module ACP. Particularly intriguing, are the unknown roles of the KS^{Q} and AT domains in the recognition and transfer of (2*R*)-methoxymalonyl-ACP to the loading ACP. Given the mutation of the active site cysteine in the KS^{Q} domain, selection and transfer of methoxymalonate would appear to be mediated by the AT. In any event, to the best of our knowledge, the apoptolidin gene cluster comprises the first example of a gene cluster initiated by priming with methoxylmalonyl-ACP.

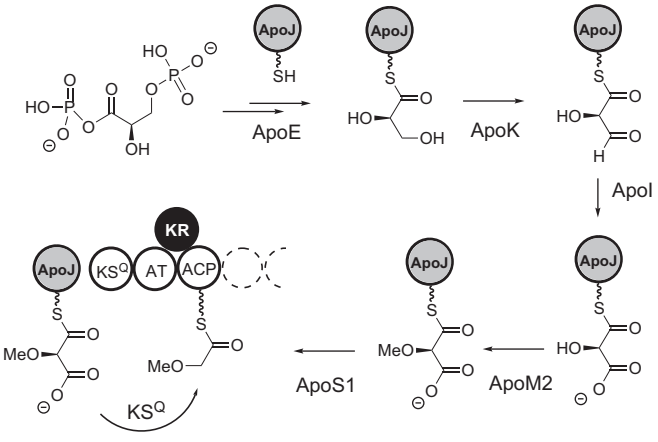

Fig. 4. Proposed biosynthesis and association of methoxymalony-ACP precursor from a potential glycosidic intermediate 1,3-bisphospho-D-glyceric acid. The protein sequences possess >50% identity with the FK520 methoxymalonyl-ACP pathway. Unlike FK520, this precursor serves as the precursor for an apparent glyoxylate starting unit in apoptolidin biosynthesis.

Extender module analysis. The extender module biochemistry of the apoptolidin PKS selects methylmalonyl-CoA or malonyl-CoA precursors via AT domains. Based on the studies of Del Vecchio et al.,[39] the selectivity of AT domains can be predicted by identifying diagnostic 'YASH' and 'HASH/HAFH' sequence motifs. Using this analysis, the AT domains of module 1, module 4, module 5–7, and 9 are predicted to select malonyl-CoA precursors, and the AT domains of module 2, 3, 8, 10, module 11–13 select methylmalonyl-CoA. The predicted AT-domain specificities are in accord with the observed substitution pattern in the apoptolidin polyketide backbone.

Ketoreductase stereoselectivity. The predicted functional ketoreductase (KR) domains in the *apo* gene cluster possess the expected active site consensus motifs[40,41] and the stereochemistry of reduction domains can be estimated by analysis using the Caffrey classification methodology.[42] Correspondingly, this analysis predicts 'a-type' stereochemistry for KR domains in modules 3 and 6, and 'b-type' stereochemistry with modules 1, 2, 5, 7–13. This is generally in accordance with the structure of apoptolidin with one possible exception. Based on the reported structure of apoptolidin, the *R* configuration at C27 should correspond to the a-type stereochemistry of Caffrey, whereas the sequence analysis predicts the opposite configuration (LDN, P, and N motifs at residues 93–95, 144 and 148 in the KR domain of in module 1). Correspondingly, to verify the proposed stereochemistry of C27, we peracetylated apoptolidin A, selectively hydrolyzed the C27 disaccharide and formed (*R*)- and (*S*)-Mosher esters at the revealed C27 hydroxyl.[13] The differences in chemical shift of the C26 ($\Delta\delta^{SR}>0$) and C28 ($\Delta\delta^{SR}<0$) protons[43] confirmed the configuration at this position as originally defined.[7] Due to the likelihood that module 1 is correctly assigned based on its position relative to the KS^{Q}, and the downstream domain organization, this example represents a confirmed exception to the Caffrey analysis of predicting apparent stereochemistry based on sequence analysis. Further experiments are required to determine the biochemical origin of the exception, specifically if the D to N substitution in the LDD motif is a contributor to the unexpected stereochemical outcome.

Analysis of proposed deoxysugar biosynthesis genes. The apoptolidins are decorated by three sugars, which have been demonstrated to be important for selective cytotoxicity.[10] The C9 hydroxyl is appended with 6-deoxy-4-*O*-L-methyl glucose and under some growth conditions, with 4-*O*-L-methyl-L-rhamnose.[12,17] The C27 hydroxyl is appended with an invariant disaccharide composed of L-olivomycose and D-oleandrose. The apoptolidin gene cluster contains a single cluster of putative sugar biosynthetic genes at its flank along with two glycosyl transferases. A third glycosyl transferase is located within the putative PKS encoding cluster. Analysis of the sugar biosynthetic gene cassette reveals five genes encoding putative enzymes appropriate for the biosynthesis of the mono and disaccharide sugars (Fig. 5). The biosynthesis of these sugars diverges from a shared intermediate NDP-L-4-keto-3-deoxyglucose **13**, which may subsequently be converted into NDP-D-oleandrose **16** by Apo1,2,3/4 and TDP-L-olivomycose by Apo1,2 ApoM2, a missing 3,5-epimerase, and Apo3/4. Searching the *Nocardopsis* genome for a 3,5-epimerase reveal a gene designated *Nsf5842*.

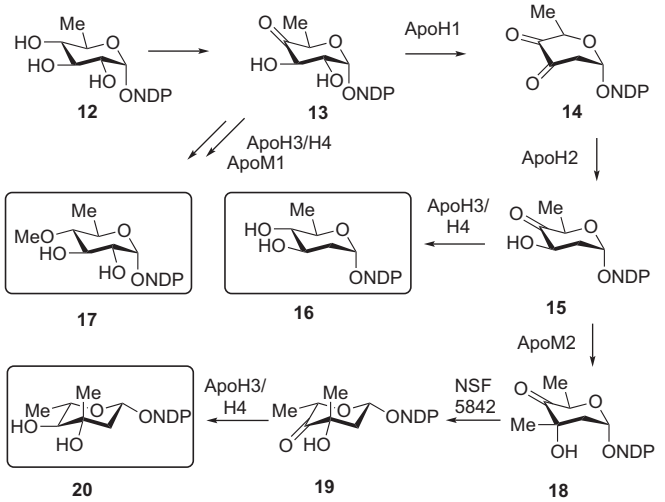

Fig. 5. Suggested sugar precursor biosynthesis: NDP-L-olivomycose **20**, NDP-D-oleandrose **16**, and NDP-D-6-deoxy-4-*O*-methyl-D-glucose **17**. A 3,5-epimerase is missing from the gene cluster, but a candidate NSF5842 is present in the genome.

Finally, NDP-L-4-keto-3-deoxyglucose **13** may be converted into NDP-D-6-deoxy-4-*O*-methyl-D-glucose **17** by apoH3 or apoH4 or a yet to be identified ketoreductase. Moreover a remaining question concerns the biosynthetic origin of the 4-*O*-methyl-L-rhamnose appending C9 of apoptolidins E and F.[17] Intriguingly, two additional unassigned oxidoreductases are apoB and apoC, which are apparent flavin and nicotinamide binding enzymes may provide the necessary functions for the generation of the C9 appending sugars.

O-methyltransferase. Two additional putative *O*-methyltransferases are encoded by *ApoM1* and *ApoM3*, which are directly upstream of the proposed methoxymalonyl-CoA cassette. ApoM1 is most closely related to sugar *O*-methyltransferases, suggesting its role in the methylation of 4-position of the C9 monosaccharide. If this is the case, the remaining methyltransferase ApoM3 is a likely candidate for methylation of the C17 hydroxyl group.

Oxidases. Analysis of the pattern of oxidation in the polyketide backbone of apoptolidin and the isolation of apoptolidins B and C[15] support positions C16 and C20 arise from post-PKS oxygenation reactions. Candidates for these C–H bond oxidative reactions include ApoP, a P_{450} related to the erythromycin macrolide oxidase EyrK (38/56% identity/similarity), and ApoD1-3, an apparent three component Rieske non-heme iron oxygenase similar to oxygenases involved in the oxidation of aryl and biaryl functional groups.

2.3. Cosmid library construction and screening

A cosmid library was constructed from the high molecular weight genomic DNA of *Nocardiopsis* sp. FU40 by using SuperCos1 cosmid vector. To identify the cosmid clones containing the *Apo* cluster, three genes in this cluster, ApoM2, ApoGT1, and ApoM1, were used as hybridization probes resulting in seven positive colonies. End-sequencing of cosmids revealed that these cosmids cover the whole of the proposed *apo* gene cluster. Two cosmids, designated apo14C7 and apo4C5, were used for gene disruption.

2.4. Transformation of *Nocardiopsis*

The genetic manipulation of *Nocardiopsis* FU40 was initially intransigent as the most commonly practiced methods of transformation of actinomycetes were unsuccessful in our hands. Firstly, polyethylene glycol induced protoplast transformation[44] was not effective, possibly because spherical protoplasts were not observable under any standard lysozyme digestion conditions. Secondly, interspecies conjugative methods were not successful using conjugation with *Escherichia coli* ET12567/pUZ8002, which resulted in no transformants using a series of integrative and non-integrative vectors and markers. Finally, adapting a method for electroporation from Hopwood et al. for *Streptomyces*,[44] we developed a method for the transformation of *Nocardiopsis* sp. FU40, which was subsequently able to introduce modified cosmids into *Nocardiopsis* sp. FU40.

2.5. Targeted gene disruption

To confirm the proposed roles of the *apo* gene cluster in the biosynthesis of apoptolidin and functionally analyze genes in this cluster, ApoS8 and ApoP, were deleted, respectively, from the genome of *Nocardiopsis* sp. FU40 using the well-established λ-Red (Red/ET) system with appropriate cosmids.[45] Correspondingly, targeted genes in cosmids were replaced by apramycin resistance gene markers by double crossover homologous recombination in an engineered hyper recombinant *E. coli* strain. The resulting cosmids were transformed into *Nocardiopsis*, selecting for apramycin resistant colonies, and the disruption of genes was confirmed by PCR analysis of transformants.

2.6. Analysis and complementation of disruption mutants

Terminated by the *seco* acid releasing TE domain, ApoS8 is predicted to contain the last extension module of the apoptolidin PKS. Correspondingly a site directed knockout mutant, FU40ΔApoS8 was generated as described above and in the Experimental section. This strain was cultured in production medium, extracted with ethyl acetate and analyzed via HPLC/MS for the production of known apoptolidins (Fig. 6A). The FU40ΔApoS8 strain was unable to produce apoptolidin A or any known apoptolidin analog. Additionally, when FU40ΔApoS8 was grown and pulse fed 3.75 mg apoptolidin monosaccharide **11** (Fig. 3), we observed the largely restored production of the apoptolidin A (0.85 mg) and isoapoptolidin A (0.39 mg). This chemical complementation validates that the targeted gene disruption phenotype and indicates that transcriptionally downstream genes involved in biosynthesis and glycosylation of apoptolidin monosaccharide remain competent. The unoptimized 24.7% yield for the conversion of apoptolidin monosaccharide to apoptolidin A demonstrates the potential for the employment of FU40ΔApoS8 as a glycosylation biotransformation reagent.[3]

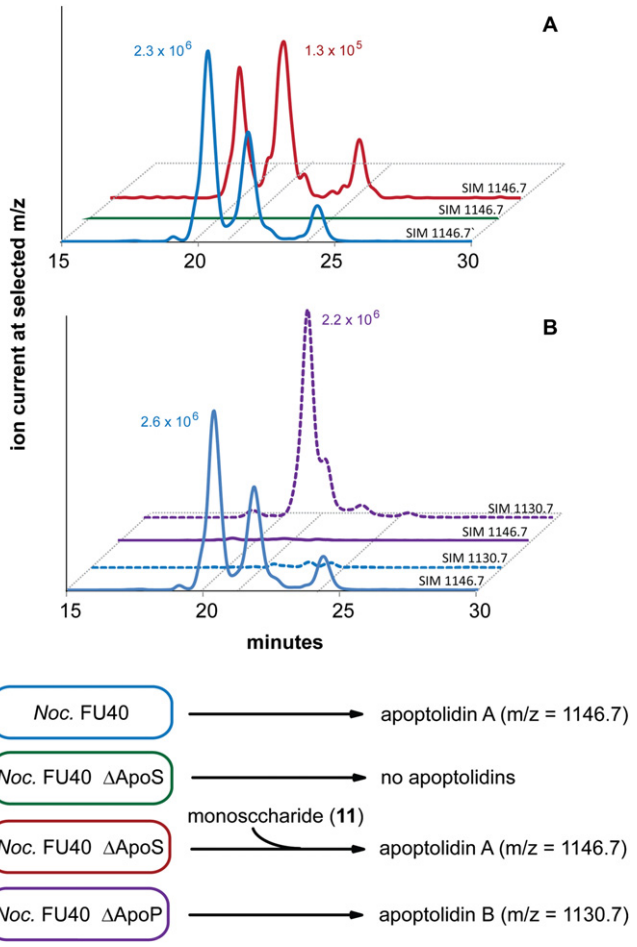

Fig. 6. Analysis of disruption mutants. Panel A, knockout of PKS: Wild-type (blue) ΔApoS8 (green) and ΔApoS8+apoptolidin C9 monosaccharide (red) scanning for apoptolidin A ion (ES^+ M+NH_4/*z*=1146.7). Panel B, knockout of P450: Wild-type (blue) and ΔApoP (purple) monitoring for apoptolidin A (solid line, ES^+ M+NH_4=1146.7) and B (dashed line, ES^+ M+NH_4/*z*=1130.7).

ApoP was identified as a cytochrome P450 monooxygenase gene with 38/56% identity/similarity to EryK from erythromycin biosynthetic gene cluster of *Saccharopolyspora erythraea*[46] and 43/61% similarity to TylI of tylosin biosynthesis in *Streptomyces*

fradiae (PMID 7984112). As both of these proteins are involved with post-PKS oxygenation of polyketide C–H bonds, ApoP comprised a likely candidate for hydroxylation of either C16 or C20. Correspondingly, we generated a selective *ApoP* gene replacement mutant, FU40ΔApoP and the fermentation products of this mutant did not produce detectable levels apoptolidin A, as analyzed by HPLC/MS. However, FU40ΔApoP strains instead produced apoptolidin analogs 18 Da less than parent apoptolidins, indicating that the ApoP is a hydroxylating P450 and that targeted replacement of the corresponding gene did not generate polar effects downstream of *ApoP*.

Subsequent isolation of the deoxy compound followed by preliminary structural characterization by ^{1}H, HSQC, COSY, and HMBC NMR determined that the deoxy product accumulated in FU40ΔApoP is consistent with (1) an isoapoptolidin scaffold and (2) an additional methylene at C16. Full assignment of this analog, complicated by separation problems is ongoing.

3. Conclusion

In summary, we have identified the apoptolidin gene cluster in *Nocardiopsis* FU40. A notable feature is the observation of a methoxymalonyl-ACP loading module initiating polyketide synthase. Functions are proposed for the 38 gene cluster and gene replacement experiments confirm the function of two genes in the *apo* cluster, *ApoP* and *ApoS8*. The transformation and genetic methodology described herein sets the stage for the further production of new apoptolidin analogs by genetic engineering for the purpose of continuing targeted biological investigations for this selective cytotoxic macrolide.

4. Experimental section

4.1. General

All reagents were of the maximum purity available from the manufacturer. All strains, plasmids, and primers are described in Supplementary datas, Table S1.

4.2. Bacterial strains and culture conditions

Nocardiopsis sp. FU40[9] and its mutants were maintained on either Bennett's agar (0.1% yeast extract, 0.1% beef extract, 0.2% NZ amine A, 1% glucose, and 2% agar, pH 7.2) or Seed Agar (1% soluble starch, 1% molasses, 1% peptone, 1% beef extract, and 2% agar, pH 7.2) with appropriate antibiotics at 30 °C. For apoptolidin or analog production, *Nocardiopsis* sp. FU40 or its mutants were cultured in Production Medium (2% glycerol, 1% molasses, 0.5% casamino acid, 0.1% peptone, and 0.1% $CaCO_3$, pH 7.2) at 30 °C.

E. coli XL1-Blue MR (Stratagene Catalog #251301) was used as the host strain for the cosmid library construction. *E. coli* BW25113 containing plasmid pIJ790[45] was used for targeted gene disruption in *Nocardiopsis* sp. FU40. *E. coli* TOP10 (Invitrogen, Catalog # 553001A) was used for gene cloning and *E. coli* ET12567 was used as a non-methylating *E. coli* host to avoid a potential DNA methylation-sensing restriction system in *Nocardiopsis* sp. FU40. All *E. coli* strains were maintained in LB medium supplemented with appropriate antibiotics for selection of plasmids.

4.3. Plasmids and general DNA procedures

SuperCos-1 derivatives were used to construct genomic libraries of *Nocardiopsis* sp. FU40. DNA isolation and gel electrophoresis were conducted according to standard methods.[44] Cosmid DNA was isolated from *E. coli* strains using a Qiagen Miniprep Kit (Catalog #27106). Isolation of DNA fragments from agarose was carried out with a Qiagen gel extraction kit (Catalog #28704). Genomic DNA from *Nocardiopsis* sp. FU40 and its derivatives were isolated using the Wizard genomic DNA purification kit (Promega, Catalog #297885).

4.4. Genome sequencing of *Nocardiopsis* sp. FU40 and apoptolidin gene cluster identification

High molecular weight genomic DNA was purified from *Nocardiopsis* sp. FU40 using the CTAB method described by Hopwood et al.[44] Whole genome sequencing was performed with a 454/Roche FLX DNA sequencer (Roche Diagnostics Corp.) using a protocol that included paired-end sequencing (3 kb insert size library) and optimization for GC rich bacterial genomes developed by the University of Maryland School of Medicine Genomic Resource Core. A total of 1,167,141 reads with an average clear range of 277 bp were obtained. The reads were assembled using the Celera Assembler (version 5.2) (http://wgs-assembler.sourceforge.net/). The Glimmer Gene Finder[47] was utilized to identify potential coding regions in the resulting scaffolds and manual annotation was performed using the MANATEE system (http://manatee.sourceforge.net/).

Identification of the apoptolidin biosynthetic gene cluster was performed using the method previously described by Bachman et al.[28] A total of eight sequencing gaps were present in the cluster and were filled by PCR and sequencing. The nucleotide sequences for the putative apoptolidin gene cluster (*Apo*), upstream and downstream regions *ApoU1–10* and *ApoD1–10* have been deposited into the NCBI database with locus accession numbers of JF819834, JF894099, JF894100, and JF894101. The unclustered 3,5-epimerase (*Nsf5842*) was deposited as JF909300. A table of proposed gene functions is included in Supplementary datas, Table S2.

4.5. Cosmid library construction

A cosmid library in *E. coli* XL1-Blue MR was constructed from the high molecular weight genomic DNA of *Nocardiopsis* sp. FU40 by using SuperCos1 Cosmid Vector Kit from Stratagene (Catalog #251301). Cosmid arms were prepared as specified by the manufacturer and the genomic DNA was partially digested by Sau3AI (New England Biolabs, Catalog #R0169S) and analyzed by field inversion gel electrophoresis. The resulting 30–40 kb fragments were ligated with the cosmid arms and packed with Gigapack® III Gold Packaging Extract Kit (Stratagene, Catalog #200201). The packed DNA was introduced into *E. coli* XL1-Blue MR as specified by the manufacturer. A total of 2880 kanamycin resistant colonies were picked and inoculated into thirty 96-well microtiter plates containing LB medium, which were grown overnight and then adjusted to contain a final concentration of 25% glycerol. These microtiter plates were stored at −80 °C and served as cryogenic stocks for the cosmid library.

4.6. Cosmid library screening

To identify the cosmid library members containing the predicted apoptolidin gene cluster, colony hybridization was performed using oligonucleotides generated for three putative genes, *ApoM2*, *ApoGT1* and *ApoM1* as hybridization probes. Three DNA fragments from these genes, with sizes of 500, 518, and 500 bp, respectively, were amplified from the genomic DNA of *Nocardiopsis* sp. FU40 using PCR using primers ApoM2F, ApoM2R, ApoGT1F, ApoGT1R, ApoM1F, and ApoM1R, as described in Supplementary datas Table S1. These DNA fragments were labeled with digoxigenin-dUTP using DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Catalog # 11585614910) as specified by the manufacturer.

Cosmid library plates were arrayed onto nylon membranes and crosslinked by UV irradiation by using a UV Stratalinker® 2400

(Stratagene). Probe hybridizations were performed at 55 °C for 2 h and positive colonies were detected by immunodetection using an anti-digoxigenin-AP, Fab fragments and then visualized with the chemiluminescence substrate CSPD. Enzymatic dephosphorylation of CSPD by alkaline phosphatase results in light emission at a maximum wavelength of 477 nm, which is recorded on X-ray films. All the experiments were performed as described by the manufacturer (Roche Applied Science). Cosmid DNA was purified from these positive colonies and the coverage regions were determined by end-sequencing and mapped to the genome by BLAST alignment.

4.7. *Nocardiopsis* sp. FU40 electrocompetent cell preparation and electroporation

To prepare electrocompetent cells, 10 μL of a glycerol stock of *Nocardiopsis* sp. FU40 spores were inoculated into 10 mL of FU40 Seed Media (1% soluble starch, 1% molasses, 1% peptone, and 1% beef extract, pH 7.2) and cultured at 30 °C with shaking for 24 h. Aliquots of 1 mL of the seed culture was inoculated into 30 mL of CRM media (1% glucose, 10.3% sucrose, 1.012% $MgCl_2 \cdot 6H_2O$, 1.5% Oxoid TSB, and 0.5% yeast extract) and cultured with shaking for 18 h. Three such cultures were pooled together and the cells were harvest from this 90 mL culture by centrifugation and washing with 100 mL ice-cold 10% sucrose then 50 mL of 15% ice-cold glycerol. The cells were resuspended in 10 mL 15% glycerol containing 100 μg/mL lysozyme and incubated at 37 °C for 30 min and washed twice with 10 mL ice-cold 15% glycerol. The pellet was resuspended in 2.5 mL of 30% PEG1000, 10% glycerol, 6.5% sucrose, aliquoted (50μL/tube), and stored until use at −80 °C.

For electroporation, 5 μL plasmid or cosmid DNA was mixed gently with 50 μL of *Nocardiopsis* sp. FU40 electrocompetent cells and the mixture was transferred into an ice-cold electroporation cuvette (BioRad Gene Pulser, Xcell, 2 mm electrode gap) and exposed to a single electrical pulse (2.0 kV, 25 μF, 400 Ω, ~5 μs). Immediately following the discharge, the suspension was mixed with 0.75 mL ice-cold CRM media and incubated with shaking for 3 h at 30 °C. A 300 μL portion was spread on *Nocardiopsis* sp. FU40 Seed agar (1% soluble starch, 1% molasses, 1% peptone, 1% beef extract, and 2% agar, pH 7.2) containing 80 μg/mL apramycin and incubated at 30 °C. Apramycin resistant colonies generally appeared after 4–5 days.

4.8. Targeted replacement of ApoS8 and ApoP by λ-Red recombination system

The apramycin resistance gene *aac(3)IV* was amplified from plasmid pIJ773 using primers (ApoS8-red-F, ApoS8-red-R, ApoP-red-F, and ApoP-red-R) with flanking regions appropriate for gene replacement (see Table S1, Supplementary datas). The resulting 1.4 kb PCR products were transformed into *E. coli* BW25113/pIJ790 containing cosmid 14C7 or 4C5. Apramycin resistant colonies were picked and cosmid DNA was purified from these colonies. Gene replacements were confirmed by sequencing with the primers Del-up and Del-down. The modified cosmids were passaged through a methylation deficient *E. coli* host ET12567 and introduced into *Nocardiopsis* sp. FU40 by electroporation as described above.

Apramycin resistant colonies were picked from the plates and inoculated onto fresh Bennett's agar plates containing 80 μg/mL apramycin. Two strains, *Nocardiopsis* sp. FU40ΔApoS8 and *Nocardiopsis* sp. FU40ΔApoP, were used for further studies. Spores from these strains were collected in 20% glycerol and stored at −80 °C.

To confirm the targeted gene replacements, spores of the above strains were inoculated into 3 mL of Seed Media with 50 μg/mL apramycin added and cultured at 30 °C with shaking for 2 days. Genomic DNA was purified from each culture by using Wizard genomic DNA purification kit (Promega, Catalog #297885). PCR was performed to confirm the disruption using these genomic DNA samples as templates with primers AprF and AprR.

4.9. Fermentation, analysis, and purification of apoptolidin analogs

Spores of *Nocardiopsis* sp. FU40 or mutants were inoculated onto Bennett's agar and incubated at 30 °C for 5–6 days. The fresh spores were then inoculated into 5 mL of Seed Medium (50 μg/mL Apramycin was added for gene replacement mutants) and cultured at 30 °C with shaking for 4 days. Then the 5 mL seed culture was inoculated into 50 mL of Production Medium in a 250 mL flask and cultured at 30 °C with shaking for 6 days.

After 6 days' incubation, the broth was centrifuged at 3750 rpm, aqueous layer was extracted with 50 mL of ethyl acetate by shaking for 1 h.

To detect the products of these strains, the ethyl acetate extracts were dried with a Genevac® at 30 °C and resuspended in 0.5 mL of methanol. The methanol solution was analyzed by HPLC/MS. Mass spectrometry was performed by using TSQ Triple Quantum mass spectrometer equipped with an electrospray ionization source and Surveyor PDA Plus detector. For positive ion mode, capillary temperature 270 °C; spray voltage 4.2 kV; spray current 30 μA; capillary voltage 35 V; tube lens 119 V; skimmer offset −15 V. For negative ion mode, capillary temperature 270 °C; spray voltage 30 kV; spray current 20 μA; capillary voltage −35 V; tube lens −119 V; skimmer offset −15 V. Samples were introduced by an Accela pump. The injection volume was 20 μL. Extracts were separated by using a Jupiter minibore 5 mm C18 column (2.0 mm 3 15 cm) with a linear water/acetonitrile gradient (ranging from 95:5 to 5:95 water/acetonitrile) containing 10 mM ammonium acetate over 30 min, followed by 30 min isocratic acetonitrile run (5:95 water/acetonitrile), followed by 10 min linear acetonitrile/water gradient (from 5:95 to 95:5 water/acetonitrile), followed by 8 min isocratic water run (95:5 water/acetonitrile). The total run time was 78 min with the flow rate of 1 mL/min. Data analysis was conducted using the Thermo Fisher Xcalibur software, version 2.1.0.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tet.2011.05.106.

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