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Combined Chemical and Biosynthetic Route to Access a New Apoptolidin Congener

Victor P. Ghidu, Ioanna Ntai, Jingqi Wang, Aaron T. Jacobs, Lawrence J. Marnett, Brian O. Bachmann, and Gary A. Sulikowski*

Departments of Chemistry and Biochemistry, Institute of Chemical Biology, Vanderbilt University, Nashville, Tennessee 37235

gary.a.sulikowski@vanderbilt.edu

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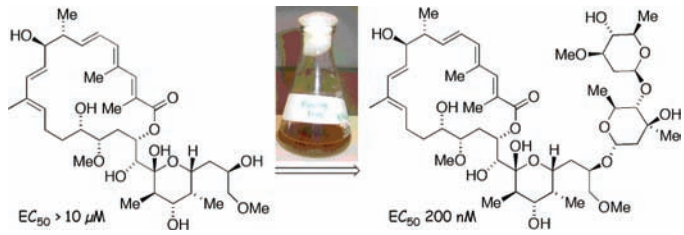
ABSTRACT



Glycosylation of a synthetic aglycone using precursor-directed biosynthesis is facilitated by a chemical ketosynthase "knockdown" of the apoptolidin producer *Nocardiopsis* sp. This synthetic approach facilitated the preparation of an unnatural disaccharide derivative of apoptolidin D that substantially restores cytotoxicity against H292 cells and deconvolutes the role of the decorating sugars in apoptolidin bioactivity.

Actinomycetes have long been a source of important bioactive natural products possessing a high level of molecular complexity.[1] For example, the macrolide apoptolidin A (**1**), produced by an actinomycete of the genus *Nocardiopsis* (FERM BP-5871), selectively induces apoptosis in rat glia transformed cells and exhibits low cytotoxicity against nontransformed cell lines.[2,3] The gross structural features of apoptolidins A−D (**1**−**4**)[4] and related iso-apoptolidins (**5**−**7**)[5] are a central aglycone conjugated to three sugar units (Figure 1). Apoptolidin A is reported to inhibit growth of H292 cancer cells (lung carcinoma) in the nanomolar range (EC_{50} = 30 nM),[6a] and removal of the sugar units results in complete loss of cytotoxicity (apoptolidinones A and D, EC_{50} > 10 μM) emphasizing the significance of the sugar fragments in relation to apoptolidin bioactivity.[6]

From the perspective of total synthesis, the challenge of assembling the complete molecular matrix of apoptolidin, a complex aglycone conjugated to three deoxy sugar units, remains significant.[7] In particular, the introduction of sugar units by chemical methods poses multiple problems in selectivity requiring complex protecting group schemes and methods of stereocontrol in the key glycosylation step. One approach to circumventing these difficulties is to employ in vitro enzymatic glycosylation methods using purified en-

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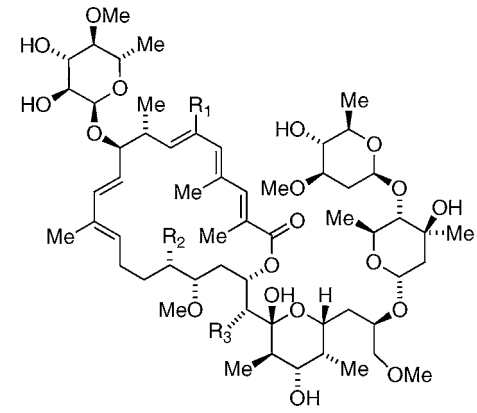
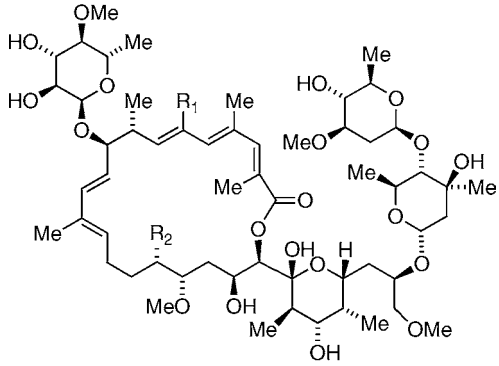


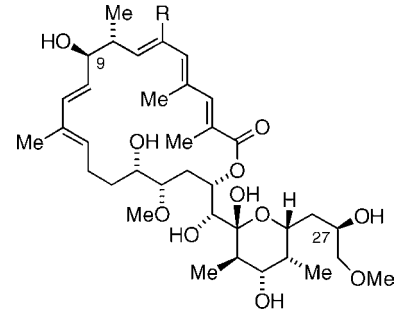
apoptolidine A, R_1 = Me; R_2 = OH; R_3 = OH (**1**)
apoptolidine B, R_1 = Me; R_2 = H; R_3 = OH (**2**)
apoptolidine C, R_1 = Me; R_2 = H; R_3 = H (**3**)
apoptolidine D, R_1 = H; R_2 = OH; R_3 = OH (**4**)



iso-apoptolidine A, R_1 = Me; R_2 = OH (**5**)
iso-apoptolidine B, R_1 = Me; R_2 = H (**6**)
iso-apoptolidine D, R_1 = H; R_2 = OH (**7**)

Figure 1. Structures of apoptolidins and iso-apoptolidins.

zymes.[8] A second option, mutasynthesis, is an extension of precursor-directed biosynthesis whereby a organism, engineered to eliminate the aglycone encoding polyketide synthase, allows glycosylation of a synthetic unnatural aglycone without competitive interference from the endogenous aglycone in the producing organism.[9] However, in vitro expression of glycosyltransferases or mutasynthesis requires appropriately active recombinant enzymes, and mutasynthesis presumes knowledge of the biosynthetic gene cluster and well-developed methods for genetic manipulation of the producing organism.[9] A third alternative, requiring no knowledge of sequence or genetic method, is to employ a selective chemical "knockdown" of the interfering aglycone polyketide synthase. In this way, polyketide synthase (PKS) inhibitors may facilitate the introduction of a foreign or unnatural aglycone and its subsequent enzymatic glycosylation by the PKS cured system. This strategy was successfully demonstrated by Omura in the early 1980s in which the ketosynthase inhibitor cerulenin was used to silence the spiromycin pathway, inhibiting production of the endogenous aglycone and allowing for glycosylation of a exogenously introduced aglycone protylonolide.[10] Herein we report the precursor directed glycosylation of completely synthetic aglycones of apoptolidin using a chemical knockdown methodology.



apoptolidinone A, R = Me (**8**)
apoptolidinone D, R = H (**9**)

Figure 2. Structure of apoptolidinone A and D.

The aglycones of apoptolidins A and D (Figure 2) were prepared by chemical synthesis following our previously published synthetic routes.[6a] To introduce the sugar units, we elected to examine glycosylation of apoptolidinones A and D using precursor-directed biosynthesis employing a whole cell culture of the apoptolidin producer (actinomycete *Nocardiopsis*). Our approach was to employ the promiscuous ketosynthase inhibitor cerulenin[11] allowing the unnatural aglycone to effectively compete as a substrate for the extant glycosyl transferase enzymes. Due to the cross reactivity of cerulenin in essential fatty acid biosynthesis, concentrations were titrated to identify a concentration range that inhibited apoptolidin production, as measured by HPLC/MS, without substantially inhibiting cell growth, as measured by pelleted mycelial mass. Optimized conditions comprising pulsed feeding of 0.2 mM of cerulenin/day under apoptolidin fermentation conditions in *Nocardiopsis* reduced apoptolidin production below 5% relative to control cultures without effecting cell growth (<5%). When the addition of cerulenin was accompanied by pulsed addition of synthetic apoptolidinone A, only trace amounts of apoptolidin A were detected; however, for the first time apoptolidin A disaccharide (**9**) was evident by LC-MS (Figure 3). Notably, indication of disaccharide conjugate **9** by LC-MS is not observed in the

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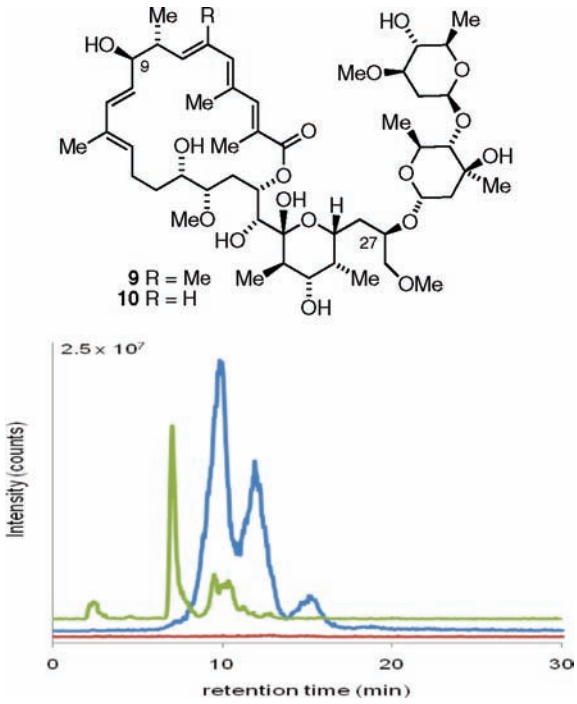

Figure 3. HPLC/MS detection of apoptolidin disaccharides in fermentation extracts of the apoptolidin producer inoculated with 0.2 mM cerulenin/day: no aglycone supplementation (red); supplemented with synthetic apoptolidinone A (blue ES^+ M + NH_4/z = 986.6); supplemented with apoptolidinone D (green ES^+ M + NH_4/z = 972.6).

control fermentation[12] suggesting the C9 glucose sugar *may* be introduced at the seco acid stage and the C27 disacharride following macrolactonization.[13,14]

The addition of apoptolidinone D to the compromised culture led to the isolation of apoptolidin D disaccharide (**10**), once again unique to the aglycone supplemented culture (Figure 3). Analysis of the crude extract of this culture showed the primary product to be apoptolidin D disaccharide (**10**) accompanied by minor amounts of apoptolidin and iso-apoptolidin (see Supporting Information for HPLC analysis). Apoptolidin D disaccharide (**9**) was isolated by preparative HPLC and fully characterized by NMR analysis.[15]

Upon evaluation against H292 lung carcinoma cells, apoptolidin D disaccharide inhibited cell growth in the submicromolar range (EC_{50} = 100−300 nM), showing less than one order loss of activity relative to apoptolidin A. Notably, the observed significant recovery of cytotoxicity on incorporation of the C27 disaccharide is in accord with earlier indications of the biological significance of this sugar residue.[6b,16]

In conclusion, we have demonstrated the glycosylation of apoptolidinone D (6-normethylapoptolidinone A) by employing whole cells of the natural apoptolidin producer actinomycete *Nocardiopsis* in combination with cerulenin as a ketosynthase inhibitor. Such chemical knockdowns of polyketide biosynthesis provide rapid and organism-nonspecific methods to access the glycosylation apparatus of a wide range of biosynthetic systems.

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Supporting Information Available: Experimental procedures and characterization data and NMR spectra of apoptolidin D disaccharide (**9**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) We typically isolate a combined mass of 150−200 mg per liter of apoptolidin and iso-apoptolidin. Under our fermentation conditions, we have not observed by LC-MS apoptolidins B−D. To date, no detection of partially glycosylated apoptolidin metabolites by LC-MS has been observed in control fermentations.

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(14) Although not proven, we assume the second major peak corresponding in mass to apoptolidin A disaccharide is the 21-membered macrolactone or iso-apoptolidin A disaccharide (cf. Figure 1).

(15) Addition of 15 mg of apoptolidinone D to a cured culture afforded 3.7 mg (ca. 18% isolated yield) of apoptolidin D disaccharide (**9**) following HPLC purification. Our attention was focused on apoptolidinone D as a substrate for precursor-directed biosynthesis as at the time we assumed this to be an unnatural aglycone. It was only during the course of our investigations that Wender's group discovered apoptolidin D (ref 4a).

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