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Identification of a Tetraene-Containing Product of the Indanomycin Biosynthetic Pathway

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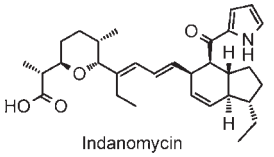
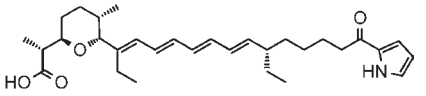
ABSTRACT



Indanomycin



The indanomycin biosynthetic gene (*idm*) cluster was recently identified from *Streptomyces antibioticus* NRRL 8167. The disruption of one of these genes, *idmH*, and the increased production of a previously unreported metabolite in this mutant is reported. The structure of this compound was elucidated and was shown to possess a linear tetraene. This metabolite is not a logical biosynthetic intermediate of indanomycin but instead is likely an alternate product of the pathway.

Metabolites harboring a tetrahydroindane ring system can differ significantly in their overall structures and demonstrate a range of biological activities that include insecticidal and antineoplastic properties.[1] The pyrrole ether antibiotics (Figure 1), for example, are ionophoric agents effective against Gram-positive bacteria while also demonstrating insecticidal and antiprotozoal activities.[1b,c,2,3] A striking aspect of the *trans*-fused bicyclic scaffold observed in indanomycin (**1**) is its appearance throughout evolutionarily distinct families of natural products arising from both terrestrial and marine sources.[1a,d,2,4] The biosynthetic mechanisms guiding the generation of *trans*-fused tetrahydroindane metabolites remain largely unknown, and the indanomycin biosynthetic system provides an ideal model to investigate the formation of this remarkable ring system due to the relative simplicity of the metabolite's polyketide backbone.

Previously, we cloned and sequenced the indanomycin biosynthetic gene (*idm*) cluster from *Streptomyces antibioticus* NRRL 8167 (*S. antibioticus*), revealing that **1** is the product of a hybrid nonribosomal peptide synthetase–polyketide synthase (NRPS-PKS) assembly line.[5] It is

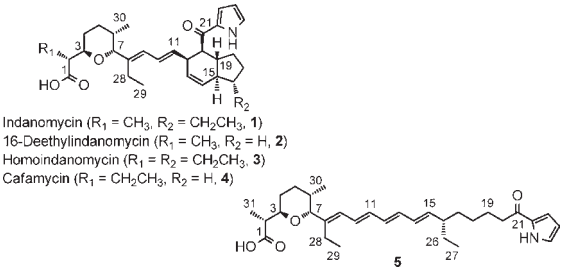


Figure 1. Pyrrole ether antibiotics: the indanomycins (**1**–**3**) and cafamycin (**4**). Structure of **5**.

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expected that a pyrrolyl moiety, while tethered to the carrier protein IdmK, is recognized by the first PKS module of IdmL as the initiating substrate to support 10 extension cycles catalyzed by the PKS subunits IdmLMNOP.[5,6] Following assembly of the polyketide backbone, two intramolecular cyclizations are expected to provide the tetrahydropyran and the *trans*-fused tetrahydroindane found in indanomycin.

Few genes in the *idm* cluster appear to encode enzymes that would tailor the polyketide backbone generated by the indanomycin PKS.[5] One such gene, *idmH*, encodes a 144 amino acid protein similar to SnoaL and SnoaL2, both proteins that adopt an $\alpha + \beta$ barrel fold.[7,8] SnoaL is a cyclase that mediates closure of the final ring of the anthracycline antibiotics, whereas SnoaL2 has been implicated in aromatic ring hydroxylations occurring at a later stage of anthracycline biosynthesis.[8,9] The $\alpha + \beta$ barrel fold is also common to enzymes such as Δ^5-3-ketosteroid isomerase and scylatone dehydratase.[10] Given the functional diversity within this structural protein family, and the resulting difficulty in predicting its biochemical function, we opted to construct an *idmH* mutant of *S. antibioticus* to evaluate its role in indanomycin production. Reported here is the identification and structure determination of a previously unreported metabolite of *S. antibioticus* and a discussion of its biosynthetic implications.

A disruption mutant of *idmH* was generated by replacing the gene with an apramycin resistance cassette using the λ RED PCR-targeting system.[11] The *idmH* mutant, *S. antibioticus* DOH1, was confirmed by PCR using primers flanking the targeted region and by sequence analysis of the amplified product. Both *S. antibioticus* DOH1 and wild-type *S. antibioticus* were cultivated in triplicate under conditions conducive to indanomycin production, and the culture extracts were evaluated by HPLC (Figure 2). **1** was present in the extracts of both wild-type *S. antibioticus* and *S. antibioticus* DOH1, albeit reduced from 89 ± 6 to 37 ± 7 mg/L (Figure 2). The indanomycin produced by *S. antibioticus* DOH1 was isolated, and structural analysis confirmed that it is identical to **1** produced by wild-type *S. antibioticus* (Supporting Information (SI)).

Interestingly, *S. antibioticus* DOH1 produced a second metabolite (**5**) at 165 ± 23 mg/L that is also detected in wild-type *S. antibioticus* at 11 ± 1 mg/L (Figure 2). We previously reported that the disruption of the L-proline adenylyltransferase *idmJ* abolished production of **1**.[5] A detailed examination of the *idmJ* mutant extract indicated that production of **5** was also abrogated, suggesting both **1** and **5** result from the indanomycin PKS machinery. Upon the *in trans* expression of *idmH* in *S. antibioticus* DOH1 (*S. antibioticus* DOH1/pIDMH5), the production of **1** and **5** were each restored to levels comparable to those observed in the wild-type strain (Figure 2, SI).

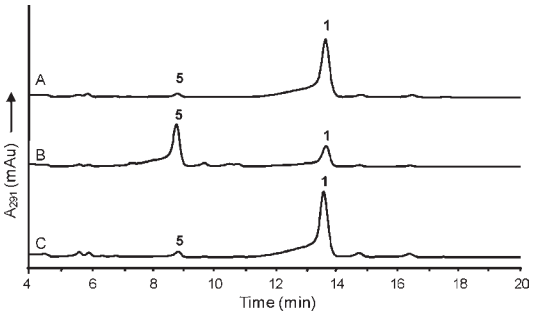

Figure 2. HPLC analysis of culture extracts from (A) *S. antibioticus*, (B) *S. antibioticus* DOH1, and (C) *S. antibioticus* DOH1/pIDMH5. Absorbance was monitored at 291 nm.

Compound **5** has a molecular formula of $C_{31}H_{45}NO_4$ as determined by HR-APCIMS (m/z 496.3426 $[M + H]^+$, calculated m/z 496.3421). An initial analysis of **5** by ^1H and ^{13}C NMR, at 500 and 125 MHz, respectively, revealed resonances characteristic of the indanomycin pyrrolyl and tetryahydropyran moieties (Table 1).[12] The pyrrole and olefinic resonances in the spectra of **5** indicated 10 protons and 12 carbons, suggesting the presence of an additional double bond relative to **1**. Furthermore, the resonances corresponding to the tetrahydroindane ring methine protons of **1** were completely absent in the spectrum of **5**. These data suggest **5** is a pyrrolylketone with a linear tetraene structure. Additionally, the maxima observed in the UV−visible absorption spectrum of **5** (291, 298, 313, and 328 nm) are consistent with the presence of a conjugated tetraene.[13]

Additional analysis of **5** by DEPT-135, COSY, HMBC, and HSQC supported the molecular formula suggested by HR-APCIMS and the structure shown in Figure 1. Comparison of the ^1H NMR spectrum of **5** to that of **1** revealed a new triplet integrating to two protons at δ_H 2.68 due to methylene protons at the pyrrolylketone α-carbon (C-20) of **5** rather than the corresponding methine proton at C-20 in **1**. The triplet coupling pattern of the H-20 protons of **5** suggests that C-19 is also a fully saturated position. Indeed, COSY correlations revealed that the triplet is coupled to a multiplet integrating to two protons at δ_H

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1.55. Saturated carbons at C-19 and C-20 also account for the observed mass of m/z 496.3 by LC-MS, an increase of 2 Da relative to **1**.

To further illuminate the crowded olefinic and overlapping aliphatic resonances, additional ^{1}H NMR, COSY, and HMBC analyses were performed using 800 MHz NMR (Table 1). Following these experiments, the four intervening methylenes between C-16 and C-21 were constructed. The overlapping proton resonances for positions 17 and 26 prevented a definitive assignment for several of the observed HMBC and COSY correlations corresponding to those protons. Although the resonance signals for the protons at positions 11 to 13 could not be distinguished from each other, correlations were clearly detected upon analysis of the COSY and HMBC experiments (Table 1 and SI). The double bonds of the tetraene all appear to be in the *trans* geometry, given that the coupling constants all range from 14.5 to 15.1 Hz. The proposed configurations of **5** at C-2, C-3, C-6, C-7, and C-16 were based on the absolute configuration of **1**. These assignments are supported by similar NMR chemical shifts for H-2 through H-7 in **5** and **1** and by the negative $[\alpha]_D$ values of $-286°$ for **5** and $-329°$ for **1** (SI). Given that the integrity of the PKS was not genetically altered in *S. antibioticus* DOH1, the configurations at the chiral centers in the newly identified metabolite are not expected to differ from the corresponding stereocenters of the parent polyketide.

To evaluate the ability of **5** to serve as a substrate of IdmH, the protein was heterologously produced as an N-terminal hexahistidine fusion construct (N-His$_6$-IdmH) in *Escherichia coli* BL21(DE3) and purified by nickel chelate chromatography. When presented to N-His$_6$-IdmH, **5** did not appear to be subjected to any changes observable by HPLC or LC-MS, even when incubated for 24 h (data not shown). This result suggests that **5** is not a substrate of IdmH, although it is noted that the conditions explored for IdmH activity were not exhaustive. Additionally, cultures of the *S. antibioticus* Δ*idmJ* mutant that were supplemented with **5** failed to restore indanomycin production or accumulate any other metabolites (data not shown). There are multiple reasons a chemical complementation may fail. One possibility, however, is that **5** is not an intermediate in the biosynthesis of **1**.

The metabolite **5** was assayed alongside **1** for antibacterial activity against *Escherichia coli* (*E. coli*), a *Bacillus* sp., methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Enterococcus faecium* (VREF) using a microbroth dilution technique. As expected, **1** demonstrated a minimum inhibitory concentration (MIC) against the Gram-positive bacteria chosen for this analysis of 0.1 μg/mL.[2] The antibacterial efficacy of **5** was diminished relative to **1**, and the MIC values revealed only modest activities against *Bacillus* sp., MRSA, and VREF at 3.1, 31.3, and 12.5 μg/mL, respectively. Neither metabolite demonstrated appreciable activity against *E. coli*.

A central question regarding indanomycin biosynthesis concerns the mechanism that is in place guiding tetrahydroindane ring formation. It has been proposed that

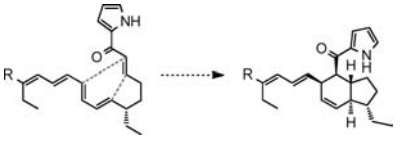
Table 1. NMR Spectroscopic Data for **5** in DMSO-d_6

position	δ_H (mult, J in Hz)	δ_C	HMBC[a]
1	11.99 (br s)	175.9	
2	2.98 (dq, 10.7, 6.7)	39.2[b]	1, 3, 31
3	3.82 (ddd, 10.4, 5.5, 2.0)	75.4	
4_A	1.79 (m)	19.6	
4_B	1.37 (m)		
5_A	1.89 (m)	25.4	6
5_B	1.47 (m)		6
6	1.89 (m)	29.4	
7	4.28 (s)	72.9	8, 9, 30
8		143.6	
9	5.93 (d, 11.6)	123.2	7, 8, 10, 11, 28
10	6.43 (dd, 14.5, 11.8)	128.1	8, 9, 13
11	6.19 or 6.18[c]	132.0	13[d]
12	6.23 (dd, 14.7, 10.4)	132.2	10, 14
13	6.19 or 6.18[c]	131.4	11, 15[d]
14	6.02 (dd, 15.1, 10.3)	130.5	11, 13, 16
15	5.44 (dd, 15.1, 9.0)	138.9	12, 16, 17, 26
16	1.89 (m)	44.1	
17_A	1.37 (m)	34.3	[d]
17_B	1.24 (m)		[d]
18	1.24 (m)	26.6	
19	1.57 (m)	24.9	17, 18, 20
20	2.68 (t, 7.4)	37.3	18, 19, 21, 22
21		189.6	
22		131.7	
23	6.94 (m)	116.2	22
24	6.15 (m)	109.6	
25	7.03 (m)	125.1	22, 23, 24
26_A	1.37 (m)	27.5	17[d]
26_B	1.24 (m)		17[d]
27	0.78 (t, 7.4)	11.6	16, 26
28_A	2.22 (m)	21.4	7, 8, 9, 29
28_B	1.89 (m)		7, 8, 9, 29
29	0.93 (t, 7.5)	13.9	8, 28
30	0.74 (d, 6.9)	12.5	5, 6, 7
31	0.96 (d, 6.7)	14.4	1, 2, 3
NH	11.71 (br s)		

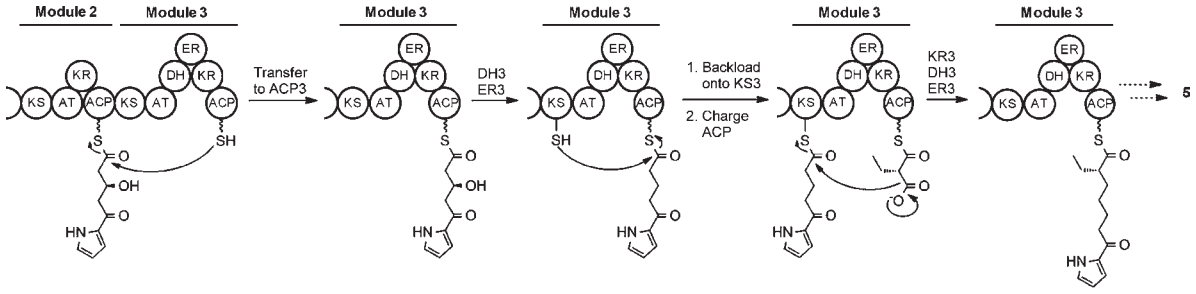
[a] HMBC correlations are from the proton to the indicated carbon. [b] The δ of this resonance was determined by HSQC due to overlap with the solvent peak. [c] Overlap of the proton resonances prevented an accurate assignment of positions. [d] Overlap of the proton resonances for H-11/13 to carbons 9, 12, and 14 and H-17/26 to carbons 15, 16, and 17 prevented assignment of crosspeaks.

the bicyclic ring system might form via an intramolecular [4 + 2] cycloaddition (Scheme 1).[14] In multiple syntheses of indanomycin, a Diels–Alder reaction was employed to construct the *trans*-fused tetrahydroindane ring system, supporting the feasibility of this scenario.[15] The type I modular

Scheme 1. Proposed Diels–Alder Cyclization during Indanomycin Biosynthesis



Scheme 2. Proposed Non-Colinear Use of Indanomycin PKS Module 3 in the Formation of **5**[a]



[a] KS: ketosynthase, AT: acyltransferase, KR: ketoreductase, ACP: acyl carrier protein, DH: dehydratase, and ER: enoylreductase.

indanomycin PKS (IdmLMNOP) harbors the domain organization needed to generate a tetraene intermediate and is thus predicted to be capable of supplying an appropriate "diene" for the proposed cycloaddition (Figure S1).

Generation of the proposed pyrrolylenone intermediate, or the "dienophile", however, is less straightforward. The domain organization of the second module of the indanomycin PKS suggests that a hydroxyl group should be retained at C-19 in the linear product of the indanomycin PKS assembly line (Figure S1).[5] The proposed biosynthetic [4 + 2] cycloaddition for the *trans*-fused tetrahydroindane ring system of **1** would necessitate dehydration of the C-19 alcohol at some point following its introduction by module 2 to produce the pyrrolylenone, either while a nascent form is tethered to the indanomycin PKS or following release of the full-length intermediate. Since it was anticipated that the accumulation of a linear product of the indanomycin PKS would have retained either the C-19 alcohol or a pyrrolylenone, the production of **5** alongside **1** is surprising.

Although alternate mechanisms cannot be ruled out at this time, the presence of **5** in the culture extracts of *S. antibioticus* wild-type and DOH1 can be explained by the noncolinear use of the β-carbon processing domains of module 3 (Scheme 2). One possible mechanism to initiate this event is the transfer of the acyl chain intermediate from the acyl carrier protein of module 2 (ACP2) to ACP3, a phenomenon referred to as module "skipping" (Scheme 2). Module skipping has been implicated in the biosynthesis of the macrolide antibiotic pikromycin and various hybrid polyketide synthases.[16] In these examples, the entire module is used either once or not at all, producing multiple products that differ in lactone ring size. After the reductive domains of module 3 in the indanomycin PKS are used a first time, the nascent polyketide would be backloaded onto KS3 and the third module used again as part of a normal extension step (Scheme 2). Iterative use of only part of a module, as proposed here for the production of **5**, is observed less frequently in polyketide biosynthesis. It has been suggested that the *cis* double bond of the epothilone macrolide is installed by the combination of programmed module skipping and iterative use of a downstream DH domain.[17]

We have demonstrated that the indanomycin biosynthetic system is indeed capable of producing a linear tetraene, as predicted by the bioinformatic analysis of the indanomycin PKS. Compound **5** is not a logical intermediate in the biosynthesis of **1**; thus it is more likely an alternate product of the indanomycin megasynthase. It appears that **5** arises from a rare combination of module skipping and iterative use of domains within module 3 of the indanomycin PKS (Scheme 2). This same process could conceivably produce a pyrrolylenone intermediate which would be an appropriate dienophile for a Diels–Alder cyclization. The function of IdmH as it relates to indanomycin biosynthesis remains enigmatic. Although not essential, IdmH clearly plays a key role in the efficient production of **1**. A more sophisticated understanding of the biochemical roles undertaken by IdmH and the indanomycin PKS in providing a suitable biosynthetic precursor to the *trans*-fused tetrahydroindane ring of indanomycin awaits a thorough biochemical analysis of both proteins.

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Supporting Information Available. Experimental procedures and full spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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