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Biosynthetic Origins of the Ionophore Antibiotic Indanomycin

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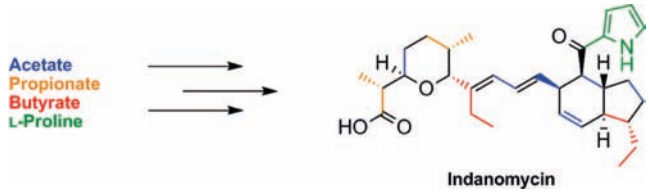
ABSTRACT



The pyrroloketoindane antibiotic indanomycin is produced by *Streptomyces antibioticus* NRRL 8167. These hybrid nonribosomal peptide-polyketide ionophore antibiotics are characterized by the presence of an unusual indane ring system, and there is interest in identifying the biochemical mechanisms guiding its biosynthesis. Following incorporation of [1-^{13}C]-labeled precursors, the primary metabolic origins of indanomycin were determined to be one unit of L-proline, six units of malonyl-CoA and two units each of methylmalonyl-CoA and ethylmalonyl-CoA.

The pyrroloketoindanes produced by soil-dwelling Gram-positive *Streptomyces* species include the ionophorous metabolites indanomycin (antibiotic X-14547A, **1**), 16-deethylindanomycin (**2**), homoindanomycin (**3**), and cafamycin (**4**) (Figure 1). Indanomycin was first isolated from *Streptomyces antibioticus* NRRL 8167 (*S. antibioticus*) in 1974 from a Martinsville, VA soil sample and later from *Streptomyces griseofuscus* (MS/ZD/033).[1] The pyrroloketoindanes thus far isolated display a broad range of biological properties including antibacterial, insecticidal, and antiprotozoal activities.[1,2] A similar pyrroloketoindane, stawamycin (**5**), possesses activity against the Epstein−Barr virus.[3] Several carboxylic acid ionophores, particularly the polyethers monensin A, lasalocid, and nigericin, are of interest for their antimalarial activity.[4] Ionophores facilitate the extraction of monovalent and divalent cations across solvent barriers, disrupting cellular cation gradients.[1a] While some ionophores specialize in transport of a particular cation, typically monovalent ions such as K^+ and Na^+, indanomycin

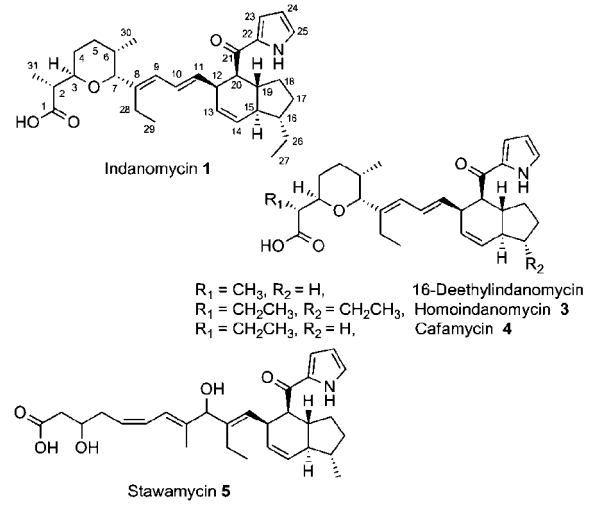


Figure 1. Pyrroloketoindane metabolites.

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has the additional capability of mediating divalent cation transport across biological membranes.[1a]

Indanomycin and the other pyrroloketoindanes harbor unique structural elements that contribute to their varied biological activities. The tetrahydropyran ring and the (*E*),(*E*)-butadiene system are present in the indanomycins **1**−**3** and cafamycin (**4**), whereas all members of this family possess the *trans*-fused tetrahydroindan ring and a pyrroloketo moiety (Figure 1). The indane ring system is a rather unusual feature among polyketide natural products. The indane scaffold is represented in multiple classes of secondary metabolites, including sphingosine derivatives (amaminol), isoprenoids (xestovanins), and polyketides (plakotenins, spiculanes, and pyrroloketoindanes).[5]

The pyrroloketoindane structure is suggestive of a hybrid nonribosomal peptide synthetase-polyketide synthase utilizing pyrrole-2-carboxylate as the initiation unit for the polyketide. To date, no studies have addressed the biosynthesis of this family of indane-containing antibiotics. Incorporation studies with [1-^{13}C]-labeled precursor molecules were performed to establish the metabolic origins of indanomycin. The results obtained from these feeding experiments support a proposed biosynthetic pathway and will facilitate identification of the biosynthetic locus of indanomycin in the producing bacterium.

Indanomycin was produced by *S. antibioticus* at titers of 20−100 mg/L in shaking liquid culture flasks.[1a] Purification of the metabolite from a whole cell ethyl acetate extract followed a previously described method with minor modifications.[1b] In separate incorporation experiments, the precursors [1-^{13}C]-acetate, [1-^{13}C]-propionate, and [1-^{13}C]-butyrate were added to a final concentration of 10 mM at 36 h following inoculation of the production medium, while L-[1-^{13}C]-proline at 0.5 mM was added after 48 h. In all cases, indanomycin was harvested from the production cultures after 120 h. Indanomycin isolated from the labeled precursor fermentations was analyzed by ^{13}C NMR spectroscopy. Enrichments were calculated from the integrated resonances detected by inverse gated decoupling ^{13}C NMR (Supporting Information). Significant overlap for the resonances of carbons 3 and 7, and carbons 11 and 22 were observed. There was, however, sufficient separation of these signals to permit an estimation of ^{13}C abundance at these positions (Table 1).

The carbon skeleton of indanomycin is predicted to incorporate six units of malonyl-CoA, and thus six units of acetate. The positions in indanomycin predicted to originate directly from the C-1 of malonyl-CoA are 3, 9, 11, 13, 17, and 19 (Figure 2). Indeed, these sites were enriched by [1-^{13}C]-acetate at levels ranging from 8.8 to 10.3% compared to the average background level of ^{13}C at 1.1% (Table 1). Although the overlapping resonances of C-3/C-7 and C-11/C-22 prevented a precise measurement, ^{13}C enrichment was clearly discernible at C-3, C-7, and C-11. Incorporation of acetate into methylmalonyl-CoA and ethylmalonyl-CoA was also detected by increased ^{13}C abundance at carbons 1 and 5 of 3.1 and 3.0% (for methylmalonyl-CoA) and at carbons 7, 15, 26, and 28 of 8.7−9.2% (for ethylmalonyl-CoA). Acetate is readily assimilated into either butyryl-CoA or crotonyl-CoA, which ultimately provides the ethylmalonyl-CoA elongation unit. Conversion of [1-^{13}C]-acetate into succinyl-CoA (and later to methylmalonyl-CoA) occurs by way of the tricarboxylic acid cycle. Carbons 21 and 25 of the pyrroloketo moiety were also increased in ^{13}C abundance. This latter observation suggests that acetate first entered the tricarboxylic acid cycle, providing α-ketoglutaric acid for amino acid biosynthesis and accounts for enrichment at the corresponding positions of the indanomycin pyrroloketone (see below). These peripheral sites of ^{13}C enrichment observed following the [1-^{13}C]-acetate feeding emphasizes the dynamic role acetate has in *S. antibioticus* primary metabolism under the conditions employed in this study.

It is anticipated that methylmalonyl-CoA is utilized twice during the course of indanomycin biosynthesis, giving rise to the methyl substituents observed at C-2 and C-6 (Figure 2). Consistent with this, feeding [1-^{13}C]-propionate to growing cultures of *S. antibioticus* led to substantial incorporation at C-1 (42.1% abundance) and C-5 (37.0% abundance). A reduced level of enrichment (3.1% abundance) was observed at C-21, corresponding to the carboxyl group of a proline intermediate. The C-21 enrichment is due to the in vivo conversion of methylmalonyl-CoA to succinyl-CoA by methylmalonyl-CoA mutase followed by entry into the tricarboxylic acid cycle and amino acid metabolism to generate the L-proline precursor.

The ethyl side chains arise from ethylmalonyl-CoA, two of which are likely required for indanomycin biosynthesis (Figure 2). The carbons in indanomycin thought to originate from C-1 of ethylmalonyl-CoA are C-7 and C-15. Following feeding with [1-^{13}C]-butyrate, significant incorporation was observed at both C-7 (22.9% abundance) and C-15 (22.2% abundance), suggesting efficient carboxylation of butyryl-CoA in vivo by an acyl-CoA carboxylase.[6] Catabolism of butyrate to acetate via β-oxidation prior to incorporation into indanomycin was also observed, albeit to a lesser extent than the direct incorporation of butyrate. Noticeable enrichment of ^{13}C from [1-^{13}C]-butyrate was observed at the C-1 positions attributed to methylmalonyl-CoA (8.1% at C-1 and 7.1% at C-5).This can be explained either by the ethylmalonyl-CoA pathway[7] or isobutyryl-CoA mutase, both of which connect metabolism of butyrate to methylmalonyl-CoA production in *Streptomyces*.[8] The generation of methylmalonyl-CoA also provides access to succinyl-CoA, suc-

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Table 1. NMR Data for Indanomycin **1** Produced Following Incubation of *S. antibioticus* with [1-^{13}C]-Labeled Precursors

carbon	δ (ppm)	[1-^{13}C]-acetate absolute abundance[a] (%)	[1-^{13}C]-propionate absolute abundance[a] (%)	[1-^{13}C]-butyrate absolute abundance[a] (%)	L-[1-^{13}C]-proline absolute abundance[a] (%)
1	178.96	3.1	42.1	8.1	0.9
2	41.40	1.0	1.0	1.0	1.0
3	74.72	8.8[b]	1.4[b]	2.2[b]	1.1[b]
4	22.32	1.0	1.0	1.3	1.2
5	26.37	3.0	37.0	7.1	1.0
6	30.54	1.1	1.0	0.9	1.1
7	74.63	9.2[b]	1.2[b]	22.9[b]	1.1[b]
8	140.02	0.8	1.5	0.6	1.1
9	124.54	8.8	1.3	2.8	1.2
10	127.06	0.9	1.4	1.0	1.0
11	132.34	10.3[c]	1.7[c]	1.2[c]	1.2[c]
12	45.23	0.8	1.4	1.0	1.1
13	129.18	8.8	1.1	3.5	1.0
14	129.36	1.1	1.3	0.9	1.0
15	49.79	8.7	1.4	22.2	1.1
16	43.72	0.9	1.4	0.9	1.1
17	29.58	9.3	1.7	3.5	1.3
18	27.04	1.0	1.8	1.1	1.1
19	40.45	9.0	1.3	3.8	1.0
20	52.44	0.9	1.3	1.0	1.0
21	191.45	2.5	3.1	1.3	9.4
22	132.38	1.3[c]	1.1[c]	2.8[c]	1.1[c]
23	115.92	1.1	1.3	0.9	1.0
24	110.12	1.1	1.3	1.0	1.1
25	125.18	4.1	1.2	1.9	1.4
26	27.29	9.1	1.7	1.4	1.1
27	12.49	1.2	1.5	1.0	1.1
28	21.99	8.7	1.3	1.0	1.0
29	13.45	1.1	1.7	1.0	1.0
30	13.59	1.1	1.7	0.9	1.1
31	13.87	1.1	1.6	0.9	1.0

[a] Carbon resonances were integrated and standardized to three different positions (C-2, C-6, and C-14). The ratio of the integrated signal from the labeled sample to that of authentic indanomycin was determined and averaged for the three reference peaks. This was multiplied by 1.1% to give the percent ^{13}C at each position. [b] Due to overlap between the resonances of C-3/C-7, these values reflect an estimation of the integrated signal (Supporting Information). [c] Due to overlap between the resonances of C-11/C-22, these values reflect an estimation of the integrated signal (Supporting Information).

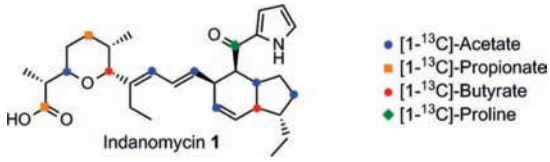

Figure 2. Sites of direct incorporation by [1-^{13}C]-labeled precursors.

cinate, and amino acid biosynthesis. A small amount of the ^{13}C label did appear at C-22 of indanomycin (2.8%), correlating to the α-carbon of proline, as discussed below. Actinomycetes are known to harbor 2-oxoglutarate synthase, which catalyzes the carboxylation of succinate to form α-ketoglutarate,[9] and could explain the enrichment observed at C-22. The pyrrole moiety observed in indanomycin and other pyrroloketoindanes most likely originates from L-proline (Figure 2). One common strategy utilized for pyrrole biosynthesis in polyketide and nonribosomal peptide-polyketide metabolites requires the initial sequestration of L-proline upon a carrier protein by an amino acid activation protein similar to those observed in nonribosomal peptide synthetases.[10] Following formation of a prolyl-*S*-carrier protein, a four-electron oxidation generates a pyrrole.[10a] In polyketide synthases utilizing pyrrolyl-2-carboxylate as a starting unit, the activated pyrrolyl-*S*-carrier protein then serves as an acyl donor for the first extension module of a polyketide synthase. In agreement with the labeling of the

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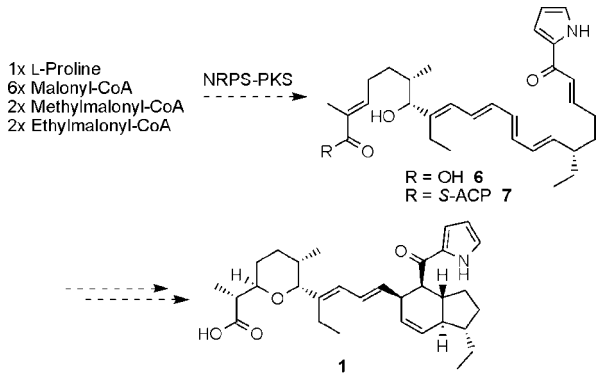
pyrroloketo substituent observed from the other precursors, supplementation of *S. antibioticus* cultures with L-[1-^{13}C]-proline led to 9.4% enrichment at C-21, the carbonyl of indanomycin derived from C-1 of L-proline.

We predict that the assembly of the indanomycin (**1**) carbon backbone follows the action of a hybrid nonribosomal peptide synthetase-polyketide synthase (NRPS-PKS). As supported by selective incorporation of L-[1-^{13}C]-proline, this will initiate with a carrier protein-mediated strategy to provide the pyrrolyl moiety. Six units of malonyl-CoA, two units of methylmalonyl-CoA, and two units of ethylmalonyl-CoA are subsequently incorporated into the polyketide (Scheme 1). The tetrahydroindene characterizing the pyrroloketoindanes is of particular biochemical interest, and is the result of an intramolecular cyclization of the polyketide backbone. This biosynthetic transformation could proceed via an *endo*-selective, intramolecular [4 + 2] cycloaddition (Scheme 1), but alternative mechanisms to produce the indane cannot be ruled out at this time. Syntheses of indanomycin employing a Diels−Alder cycloaddition to construct the bicyclic system provided the naturally occurring stereochemistry as the major product,[11] consistent with a biomimetic strategy. There are at least two possible points at which cyclization of indanomycin can occur. In one scenario, cyclization is directed upon a free intermediate (**6**), either before or after formation of the tetrahydropyran. Another possibility bearing consideration is the cyclization of a nascent polyketide, such as **7**, prior to liberation from the polyketide synthase. Confirmation of the biosynthetic building blocks for indanomycin now provides a solid foundation for the identification and characterization of its associated biosynthetic system and engineering of designer indane antibiotics.

Scheme 1. Proposed Biosynthesis of Indanomycin



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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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