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Biosynthesis of 3-amino-4-hydroxybenzoic acid in *Streptomyces murayamaensis*: incorporation of [4-^{13}C]oxalacetate

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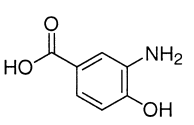
Abstract

The biosynthesis of 3-amino-4-hydroxybenzoic acid (3,4-AHBA), a precursor to several *Streptomyces* secondary metabolites, requires the condensation of a three-carbon compound with a four-carbon unit derived from the tricarboxylic acid cycle. Using the ferrous chelate of 4-hydroxy-3-nitrosobenzamide, a metabolite of *S. murayamaensis*, as a model system, the exact nature of the four-carbon unit was explored. Incorporation experiments with [1-^{13}C]methionine, [1-^{13}C]homoserine, and [4-^{13}C]oxalacetate revealed that only oxalacetate is a precursor to 3,4-AHBA.

Keywords: biosynthesis; labeling; natural products; oxocarbons; oxocarbon acids and derivatives.

The modified benzoic acid, 3-amino-4-hydroxybenzoic acid (3,4-AHBA, **1**), has been shown to be a precursor of several *Streptomyces* metabolites including 4-hydroxy-3-nitrosobenzamide (**2**),[1] manumycin,[2] and asukamycin.[2] The biosynthesis of this aromatic metabolite, which does not arise from the shikimate pathway, has been examined in several organisms. The precursors in *Streptomyces murayamaensis*, the producer of **2** and its ferrous chelate (**3**), were aspartate and pyruvate.[3] These results provided a new explanation for the previously observed incorporation of succinate and glycerol into asukamycin and manumycin,[4] which was borne out by the subsequent incorporation of **1**. This article describes experiments exploring further details of the biosynthesis of **1** in *S. murayamaensis*.



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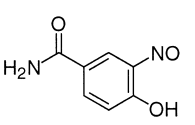

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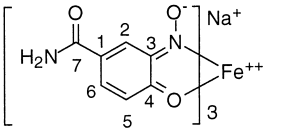

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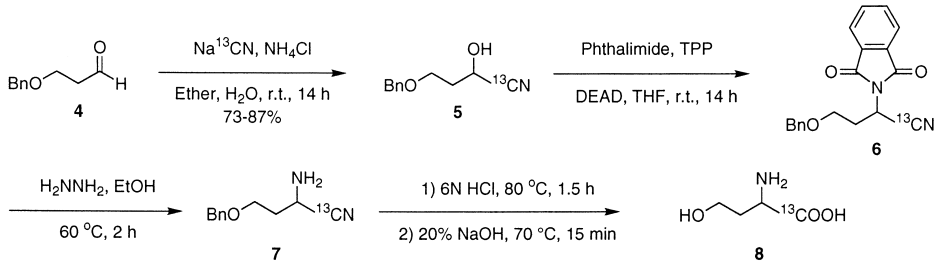
A key unanswered question in the assembly of **1** is the exact nature of the four-carbon precursor. Several possibilities exist for a precursor derived from aspartate, including methionine, homoserine, the α-keto acids derived from these amino acids, and oxalacetate.[3] In order to test these hypotheses, incubation experiments were conducted with labeled versions of these proposed precursors. The ferrous chelate **3**, which is produced in significant quantities by a mutant of *S. murayamaensis* and which was previously shown to be biosynthesized from **1**, was analyzed for the incorporation of labeled precursors.‡ Initial labeling experiments utilized various amounts of [1-^{13}C]methionine. In none of these experiments was definitive labeling observed in the ^{13}C NMR spectra of purified **3**.

Labeled homoserine was next evaluated as a precursor. The [1-^{13}C]homoserine was prepared utilizing sodium [^{13}C]cyanide to introduce the desired label, as illustrated in Scheme 1. Oxidation of monoprotected 1,3-propanediol by TPAP[5] provided 3-benzyloxypropanal (**4**), which was treated with a mixture of sodium [^{13}C]cyanide and ammonium chloride in a biphasic system to give hydroxynitrile **5** in 73–87% yield together with aminonitrile **7** in 10% yield. The hydroxynitrile **5** was treated with phthalimide under Mitsunobu reaction conditions to give **6** which was heated with hydrazine to generate the aminonitrile **7** (60% for two steps).[6] Hydrolysis of nitrile **7** was accompanied by cleavage of the benzyl protecting group, which led to lactone formation under the acidic conditions. The crude lactone was treated with 20% NaOH aqueous solution to give [1-^{13}C]homoserine (**8**). The labeled homoserine (100 mg) thus prepared was administered to the *S. murayamaensis* culture, but isolated **3** did not show any isotopic labeling. Although the extent of the transamination reactions of homoserine and methionine to their respective α-keto acids is not known for *S. murayamaensis*, the absence of labeling from these amino acids may suggest that the keto acids derived from them are also not precursors.



Scheme 1. Synthesis of [1-^{13}C]homoserine

A third possible intermediate in the biosynthetic pathway to **1** is oxalacetate. Oxalacetate has been proposed as a direct precursor in the biosynthesis of several fungal metabolites,[7] including zaragozic acid,[8] CP-225,917,[9] and piliformic acid,[10] although few attempts have been made to observe direct incorporation. This may be because the use of oxalacetate as a biosynthetic precursor has several drawbacks. It is known to readily decarboxylate in aqueous solution at neutral

‡ The incubation experiments were performed with the *S. murayamaensis* strain MC2. Fermentation protocols and procedures for the isolation of **3** have previously been reported.[3] [3-^{13}C]DL-aspartic acid and [1-^{13}C]methionine were purchased from Isotec, Inc. NMR spectra were recorded in DMSO-d_6.

pH with a $t_{1/2} = 30$–60 min.[11] It can also be transaminated to aspartate or participate in the tricarboxylic acid cycle, greatly reducing its direct availability for use in secondary metabolic pathways. The very low level incorporation of [1,2-^{14}C]oxalacetate into the pyrone ring of bufadienolides has been reported, although possibly via pyruvate.[12,13] Incorporation of [3-^{13}C]oxalacetate into zaragozic acid was interpreted as being indirect.[14] Despite the challenges associated with its use as a labeled precursor, the absence of label incorporation from methionine and homoserine necessitated the attempted labeling with oxalacetate. If oxalacetate is efficiently utilized as a precursor to **1**, some labeled material should be incorporated prior to significant depletion of oxalacetate via decarboxylation and further metabolism. The 4-^{13}C-labeled compound was selected due to its convenient synthetic access and the fact that decarboxylation removes the label, suppressing indirect incorporation of ^{13}C through pyruvate.

Oxalacetate labeled at C4 was synthesized according to a literature procedure using [1-^{13}C]acetyl chloride.[15] Two incubation experiments were conducted in which the labeled precursor was added at 100 mg/L and at 300 mg/L. Neither experiment provided significant labeling of **3**. These initial negative results may have been due to the problems cited above. A possible approach to enhance the chances of observing incorporation would be to slow one of the competing metabolic pathways. Adding sufficient levels of aspartate to the medium was viewed as a way to slow the transamination reaction enough to allow observation of direct incorporation. A third experiment in which the labeled oxalacetate was co-administered with aspartate (400 mg total of each in four 100 mg pulses) was conducted to test this hypothesis. Under these conditions, the isolated **3** showed significant labeling at C5 (2.6% enrichment) and at C7 (1.3% enrichment) (Table 1, Fig. 1). The difference in magnitude of labeling at the two positions suggests that in addition to direct labeling of C5 by oxalacetate, labeling at both C5 and C7 occurs due to incorporation of oxalacetate after passage through the TCA cycle (via the symmetrical succinate). Because [^{13}C]oxalacetate is incorporated only in the presence of added aspartate, oxalacetate is supported as a more direct precursor to **1** than aspartate. If oxalacetate were converted to aspartate prior to incorporation, the dilution by unlabeled aspartate would have suppressed the accumulation of labeled compound.

Table 1
^{13}C NMR enrichments observed in **3** isolated from [4-^{13}C]oxalacetate incubation experiments (DMSO-d_6)

Carbon	δ (ppm)	% Enrichment [4-^{13}C]oxalacetate	% Enrichment [4-^{13}C]oxalacetate + [3-^{13}C]aspartate
4	179.8	-	2.4
5	120.8	2.6	1.7
6	135.7	-	1.2
7	167.5	1.3	0.9

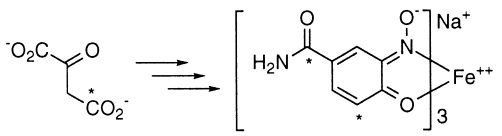
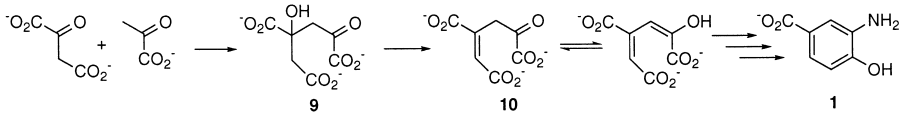


Figure 1. Incorporation of [4-^{13}C]oxalacetate into **3**

An attempt was made to see if the use of differentially labeled aspartate and oxalacetate would provide further insight into the incorporation of these precursors. Simultaneous incubation with [4-^{13}C]oxalacetate and [3-^{13}C]aspartate (100 mg/L of each) provided labeled benzamide chelate **3** which was analyzed by ^{13}C NMR. The results of the experiment were: 2.4% enrichment at C6, 1.7% at C5, 1.2% at C1, and 0.9% at C7 (Table 1). The labeling at C6 and C1 results from incorporation of [3-^{13}C]oxalacetate derived from [3-^{13}C]aspartate, while C5 and C7 labeling is from [4-^{13}C]oxalacetate. Although a higher level of incorporation from [4-^{13}C]oxalacetate was the desired result, the lability of oxalacetate provides an explanation. The labeled oxalacetate is incorporated shortly after being introduced to the culture, prior to extensive decarboxylation. It is prevented from extensive transamination due to the presence of sufficient levels of aspartate. The labeled aspartate, however, has a longer half-life and once the level of labeled oxalacetate is depleted, transamination of the labeled aspartate to oxalacetate is possible. The newly formed [3-^{13}C]oxalacetate is then incorporated into **3**.

These results provide supporting evidence that the four carbon intermediate in the biosynthesis of 3,4-AHBA is oxalacetate, rather than homoserine, methionine, or a keto acid derived from these two amino acids. The results also demonstrate that ^{13}C labeled oxalacetate can effectively be used in biosynthetic studies if precautions are taken to slow its participation in competing metabolic pathways.

The incubation experiments suggest that oxalacetate directly condenses with either pyruvate or phosphoenolpyruvate to form 4-carboxy-4-hydroxy-2-oxoadipate (**9**; Scheme 2). The reverse reaction, cleavage of **9** to oxalacetate and pyruvate, is known in the catabolism of hydroxylated aromatic compounds in several bacterial species.[16,17] The aldolase that mediates this cleavage has been purified and shown to function in both directions, providing biochemical precedence for the condensation of oxalacetate and pyruvate.[18] The next step could be a dehydration to yield *E*-3-carboxy-5-oxo-2-hexenedioic acid (**10**). The reverse of this step also occurs in catabolic pathways and the enzyme that mediates this reversible step has been characterized.[19] An unknown number of conversions would then be required to form 3,4-AHBA (**1**).



Scheme 2. Proposed steps in the biogenesis of 3-amino-4-hydroxybenzoate

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