Biosynthesis of 3-Amino-5-hydroxybenzoic Acid, the Precursor of mC_7N Units in Ansamycin Antibiotics†

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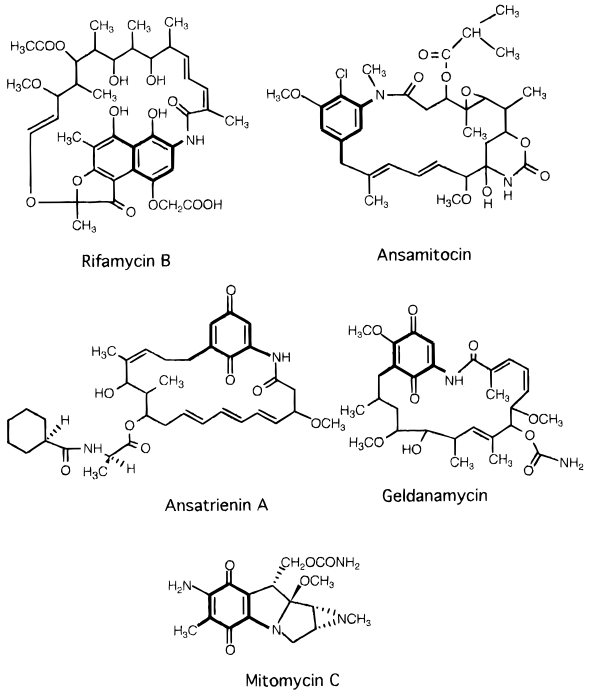
Abstract: The biosynthetic pathway of 3-amino-5-hydroxybenzoic acid (AHBA) formation was studied with cell-free extracts from the rifamycin B producer, *Amycolatopsis mediterranei* S699, and the ansatrienin A producer, *Streptomyces collinus* Tü1892. Phosphoenolpyruvate (PEP) plus erythrose 4-phosphate (E4P) gave AHBA in low but nevertheless significant (6%) yield. 3,4-Dideoxy-4-amino-D-*arabino*-heptulosonic acid 7-phosphate (aminoDAHP) was converted efficiently into AHBA (45%), as were 5-deoxy-5-amino-3-dehydroquinic acid (aminoDHQ, 41%) and 5-deoxy-5-amino-3-dehydroshikimic acid (aminoDHS, 95%). On the other hand, the normal shikimate pathway intermediate, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) did not give rise to AHBA under these conditions. AminoDAHP (9%) was produced by incubation of [^{14}C]PEP and E4P, but not of [^{14}C]DAHP, with the cell-free extracts. The results demonstrate the operation of a new variant of the shikimate pathway in the formation of the mC_7N units of ansamycin, and presumably also mitomycin, antibiotics which leads from PEP, E4P, and a nitrogen source directly to aminoDAHP and then via aminoDHQ and aminoDHS to AHBA.

A variety of antibiotics contain a biosynthetically unique moiety consisting of a six-membered carbocycle, usually aromatic or quinoid, carrying an extra carbon and a nitrogen in a meta arrangement. This so-called mC_7N unit was first recognized as a structural element of the ansamycin antibiotic rifamycin B.[1] It is present in all the ansamycins, both of the benzenic and the naphthalenic type,[2] and in ansamitocins[3] as well as in structurally quite different antibiotics such as the mitomycins[4] (Scheme 1).

Feeding experiments with a variety of labeled precursors[5−7] and genetic experiments[8] have demonstrated the shikimate pathway origin of the mC_7N unit. However, all attempts to obtain incorporation of labeled shikimic acid (SA^+),[5a,6,7b,9] quinic

Scheme 1. Antibiotics Containing mC_7N Units



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† Abbreviations used: SA, shikimic acid; QA, quinic acid; DHQ, 3-dehydroquinic acid; AHBA, 3-amino-5-hydroxybenzoic acid; DHS, 3-dehydroshikimic acid; aminoDAHP, 3,4-dideoxy-4-amino-D-*arabino*-heptulosonic acid 7-phosphate; DAHP, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; E4P, D-erythrose 4-phosphate; PEP, phosphoenolpyruvate; DNP, 2,4-dinitrophenyl; PMSF, phenylmethanesulfonyl fluoride; aminoSA, 5-deoxy-5-aminoshikimic acid; aminoDHS, 5-deoxy-5-amino-3-dehydroshikimic acid; aminoDHQ, 5-deoxy-5-amino-3-dehydroquinic acid; TCA, trichloroacetic acid; PYA, pyruvic acid.

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acid (QA),[10] or 3-dehydroquinic acid (DHQ)[7b] into the mC_7N unit were unsuccessful. The nonincorporation of shikimate pathway intermediates could be due to impermeability of the cell membranes of the producing organisms to these compounds, demonstrated for SA in the rifamycin-producer, *Amycolatopsis mediterranei*,[8c] or could indicate that the biosynthesis of the mC_7N unit branches off from the shikimate pathway at an earlier stage. The former possibility was excluded for the case of ansatrienin A (cf. Scheme 1) by showing that ^{13}C-labeled SA is efficiently incorporated into the cyclohexanecarboxylic acid moiety of this antibiotic, but not into its mC_7N unit.[11] Through genetic studies Gygax *et al*.[8c] established that the branch point for formation of the mC_7N unit must lie before DHQ in the shikimate pathway. Their studies showed that a transketolase inactivated mutant of *A. mediterranei* could not produce aromatic amino acids and rifamycin, but a DHQ synthase inactivated mutant could synthesize rifamycin.

3-Amino-5-hydroxybenzoic acid (AHBA) was shown to be a specific and proximate precursor of the mC_7N unit both by feeding experiments with labeled AHBA[12] and by complementation experiments with blocked mutants.[13] AHBA was efficiently and specifically incorporated into all the different ansamycins[12a,14−18] and ansamitocins[19] and into mitomycins (cf. Scheme 1).[12b] However, this still leaves unexplained how AHBA is formed via the shikimate pathway. While it was first thought that the nitrogen is attached to C-3 of, e.g., 3-dehydroshikimic acid (DHS),[5a] the analysis of labeling patterns in mitomycin[7b] and $^{13}C-^{13}C$ coupling patterns in geldanamycin,[20] naphthomycin[18] and ansatrienin[21] revealed that the nitrogen is actually linked to the carbon corresponding to C-5 of shikimic acid. This led Hornemann *et al*.[7b] to suggest aminoDAHP, thought to arise from 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP), as the precursor of the mC_7N unit, whereas Rinehart *et al*.[20] proposed transamination of a hypothetical 5-dehydroshikimic acid derived from 3-dehydroshikimic acid.

In both these proposals the nitrogen would originate from the amino nitrogen of glutamic acid via a transamination reaction. However, Jiao *et al*.[22] reported that the amide nitrogen of glutamine was the best source of the nitrogen of rifamycin B. This and mechanistic considerations led us[21a] to propose the pathway shown in Scheme 2 for the formation of AHBA. The key feature is the suggested operation of a modified DAHP synthase containing an additional protein subunit or domain which binds and hydrolyzes glutamine, generating in the active site a molecule of ammonia. The latter forms a Schiff's base with erythrose 4-phosphate (E4P); condensation with phosphoenolpyruvate (PEP) then gives aminoDAHP directly. Cyclization and dehydration, either by the normal shikimate pathway enzymes or by a separate set of enzymes then produces aminoDHS, which is aromatized to AHBA. In the present paper we report results of experiments with cell-free extracts of the rifamycin producer, *A. mediterranei*, and the ansatrienin producer, *Streptomyces collinus*, which provide considerable support for this hypothesis. Some of the data have been communicated in preliminary form.[23]

Materials and Methods

General Chemicals and Procedures. ^1H-NMR spectra were recorded on an IBM-Bruker AF-300 NMR spectrometer, using D_2O as the solvent. Chemical shifts were determined relative to the residual proton absorption of D_2O as internal standard. All ^1H-NMR chemical shifts are reported as δ values in parts per million (ppm). High resolution fast atom bombardment mass spectra (FAB-MS) were obtained in a Fisons Instruments VG 70SEQ spectrometer. GC-MS analyses were carried out on a Hewlett-Packard 5970A gas chromatograph connected to a 5790 mass selective detector.

Unlabeled AHBA and [1-^{13}C]AHBA,[24] DAHP,[25] DHQ,[26] and DHS[27] were prepared by previously described chemical or enzymatic procedures. The synthesis of aminoDAHP has been described.[28] The *N*-(2,4-dinitrophenyl) derivative of aminoDAHP (DNP-aminoDAHP) was prepared following a procedure employed by Basmadjian and Floss.[29] PEP, E4P, QA, SA, phenylmethanesulfonyl fluoride (PMSF), 2,4-dinitrophenyl fluoride, and silylating reagent (SIGMA-SIL-A) were purchased from Sigma (St. Louis, MO). [amide-^{15}N]Glutamine and $^{15}NH_4Cl$ (98% ^{15}N) were obtained from Cambridge Isotope Laboratories. [1-^{14}C]PEP (23 mCi/mmol) and sodium [1-^{14}C]pyruvate (23 mCi/mmol) were obtained from Amersham. [1-^{14}C]DAHP was isolated from the incubations of *A. mediterranei* cell-free extracts with [1-^{14}C]PEP and E4P and purified to radiochemical homogeneity by repeated paper chromatography.

Microbial Cultures and Fermentations. *A. mediterranei* strain S699 was a gift from Professor Giancarlo Lancini (Lepetit Research Laboratory, Geranzano, Italy) and *S. collinus* Tü1892 from Professor Axel Zeeck (Institut für Organische Chemie, Universität Göttingen, Germany). *E. coli* AB2834/pIA321, a genetically engineered overproducer of shikimate dehydrogenase, was kindly provided by Professor John R. Coggins (Department of Biochemistry, University of Glasgow, UK) and *E. coli* RB791/pJB14, a genetically engineered overproducer of DHQ synthase, by Professor Jeremy R. Knowles (Department of Chemistry, Harvard University). *E. coli* AB2834/pIA321,[27] *E. coli* RB791/pJB14,[30] and *S. collinus* Tü1892[15] were grown as previously described.

Growth of A. *mediterranei* S699. A plate culture from a solid medium (4 g of yeast extract, 10 g of malt extract, 4 g of glucose, 20 g of agar, and 1 L of distilled water, pH 7.3) was used to inoculate vegetative medium (5 g of meat extract, 5 g of peptone, 5 g of yeast extract, 2.5 g of enzyme hydrolysate of casein, 20 g of glucose, 1.5 g of NaCl, and 1 L of distilled water).[31] Vegetative cultures (50 mL of medium per flask) were grown for 2 days in 500 mL shake flasks with spring coils at 28 °C and 250 rpm (ISF-4-V shaker, Adolf Kuhner AG). After the growth was complete, contamination was checked by light microscopic examination. Four milliliter portions of the vegetative

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culture were introduced into 500 mL flasks with spring coils, each containing 50 mL of production medium [115 g of glucose, 25 g of peanut meal, 9.6 g of $(NH_4)_2SO_4$, 9.5 g of $CaCO_3$, 1 mL of trace element solution (1 g of $MgSO_4 \cdot 7H_2O$, 1 g of $CuSO_4 \cdot 5H_2O$, 1 g of $FeSO_4 \cdot 7H_2O$, 1 g of $MnSO_4 \cdot H_2O$, 1 g of $Co(NO_3)_2 \cdot 6H_2O$, and 1 L of distilled water), and 1 L of distilled water]. After incubation at 28 °C with shaking (280 rpm) for 3 to 4 days, the production of rifamycin B was checked by spectrophotometry.[32] The flasks showing highest production were selected and glycerol was added to 20% (v/v). Aliquots of the glycerol-containing seed culture were stored in a freezer at −20 °C and 1 mL was used to inoculate 50 mL of vegetative medium. Alternatively, 10 mL of inoculum was used for 500 mL vegetative medium in a 2 L flask without a spring coil. After incubation at 28 °C and a shaker speed of 300 rpm for 54 h (New Brunswick G 25 gyrotory shaker), the mycelia were harvested by centrifugation, washed with 50 mM Tris-HCl, pH 7.5 (buffer A) and used to prepare cell-free extracts.

Preparation of Cell-Free Extracts. The washed mycelia of *A. mediterranei* S699 were suspended in 5 mL of buffer A per 1 g of wet cells. PMSF (1 mM) was added to inhibit proteases and the cells were broken by two passages through a French pressure cell followed by centrifugation to remove cell debris. Cell-free extracts of *S. collinus* Tü1892 were prepared in the same way by three passages through a French pressure cell, but using 3 mL of buffer A,1 mM in PMSF, per 1 g of wet cells. Cell-free extracts of *E. coli* AB 2830/pIA321 and *E. coli* RB791/pJB14 were prepared as previously described.[27,30]

Synthesis of Substrates. 5-Deoxy-5-aminoshikimic Acid (aminoSA). AminoSA was synthesized by regiospecific ring opening of methyl *cis*-3-hydroxy-4,5-epoxycyclohex-1-enecarboxylate (4,5-epoxide) which was prepared from SA as previously described.[33] To the 4,5-epoxide (1 g, 5.3 mmol) in a mixture of 49 mL of MeOH and 16 mL of H_2O was added LiOH (224 mg, 5.3 mmol) at 0 °C. The reaction mixture was stirred at 5 °C for 3 days. The solution was neutralized at 0 °C with 1 N HCl and the solvent was evaporated at 0 °C under reduced pressure. The residue was dissolved in 20 mL of concentrated NH_4OH and stirred at room temperature for 7 days. The solvent was evaporated at room temperature under reduced pressure. The residue was dissolved in 2 mL of distilled water and purified by anion exchange column chromatography (15 mL of bed volume, Bio-Rad AG1 × 8, formate form, elution with a gradient of 1 N to 3 N formic acid) to give aminoSA (304 mg, 30%). ^{1}H-NMR (D_2O, 300 (MHz) 6.40 (dd, 1H, J = 6.4, 3.2 Hz), 4.23 (dd, 1H, J = 6.4, 5.0 Hz), 3.70 (dd, 1H, J = 11.2, 5.0 Hz), 3.35 (ddd, 1H, J = 11.2, 10.6, 5.5 Hz), 2.80 (dd, 1H, J = 17.6, 5.5 Hz), 2.22 (ddd, 1H, J = 17.6, 10.6, 3.2 Hz). High resolution FAB-MS $(M + H)^+$ found 174.0780, calcd for $C_7H_{11}NO_4$ + H 174.0684.

5-Deoxy-5-amino-3-dehydroshikimic Acid (aminoDHS). A solution of aminoSA (6 mM) and $NADP^+$ (6mM) in 30 mL of distilled water was adjusted to pH 10 with 1 N NaOH. The reaction was started by adding 10 μL portions of cell-free extract of *E. coli* AB2834/pIA321 (16 mg protein/mL) at room temperature. Since the pH of the reaction mixture decreased once the reaction started, it was repeatedly adjusted so as not to fall below 9.0. After completion of the reaction [monitored by TLC (silica gel, ethyl acetate/acetic acid/methanol/H_2O 4:1:1:1), R_f of aminoDHS = 0.6; aminoSA = 0.3] the reaction mixture was loaded onto a column (5 mL bed volume) of anion exchange resin (Bio-Rad AG 1 × 8, formate form). AminoDHS was eluted with 1N formic acid. The fractions containing aminoDHS were collected and freeze-dried (60% yield). ^{1}H-NMR (D_2O, 300 (MHz) 6.45 (d, 1H, J = 2.9 Hz), 4.38 (d, 1H, J = 12.3 Hz), 3.66 (ddd, 1H, J = 12.3, 11.3, 5.1 Hz), 3.14 (dd, 1H, J = 18.1, 5.1 Hz), 2.78 (ddd, 1H, J = 18.1, 11.3, 2.9 Hz). High resolution FAB-MS $(M + H)^+$ found 172.0611, calcd for $C_7H_9NO_4$ + H 172.0610.

5-Deoxy-5-amino-3-dehydroquinic Acid (aminoDHQ). To a 6 mM solution of aminoDAHP in 10 mL of distilled water were added 100 μL portions of the cell-free extract of *E. coli* RB791/pJB14 (20 mg protein/mL). The mixture was incubated at room temperature and reaction progress monitored by TLC (same system as employed for aminoDHS synthesis, R_f of aminoDHQ = 0.2, aminoDAHP = 0.1). After completion of the reaction the aminoDHQ was isolated by anion exchange chromatography as described for aminoDHS, except that a linear gradient from 1 to 3 N of formic acid was used for elution. The fractions containing aminoDHQ were collected and freeze-dried (30% yield). ^{1}H-NMR (D_2O, 300 (MHz) 4.37 (d, 1H, J = 10.7 Hz), 3.42 (ddd, 1H, J = 12.5, 10.7, 4.4 Hz), 3.03 (d, 1H, J = 14.3 Hz), 2.48 (dd, 1H, J = 14.3, 2.8 Hz), 2.36 (dd, 1H, J = 13.4, 12.5 Hz), 2.19 (ddd, 1H, J = 13.4, 4.4, 2.8 Hz). High resolution FAB-MS $(M + H)^+$ found 190.0709, calcd for $C_7H_{11}NO_5$ + H 190.0715.

Analysis of Enzymatic AHBA Formation. The AHBA formed in enzyme incubations was quantitated by an inverse isotope dilution analysis. Reaction mixtures containing 10 mL of *A. mediterranei* or *S. collinus* cell-free extract and the components indicated in Table 1 were incubated in 100 mL glass bottles at 28 °C with shaking at 100 rpm for 30 h. At the end of the incubation, 200 μg of [7-^{13}C]AHBA (90% ^{13}C) was added, followed after 1 more hour by 700 μL of 15% trichloroacetic acid (TCA). The acidified solution was centrifuged and the supernatant extracted with 10 mL of ethyl acetate. The separated ethyl acetate layer was evaporated and the residue vacuum-dried for 1 to 2 h. The resulting dark brown, oily material was silylated using 200 μL of SIGMA-SIL-A and after 5 min centrifuged to remove pyridinium hydrochloride. The clear solution was subjected to GC-MS analysis (SE-54 capillary column 0.25 mm × 30 m, flow rate 1.0 mL/min, temperature program 0 min at 60 °C, then 20 °C/min to 285 °C). The retention times for di- and trisilylated AHBA were 12.0 and 12.5 min, respectively.

AminoDAHP Formation from [1-^{14}C]PEP. [1-^{14}C]PEP (0.15 μCi, 23 μCi/μmol) or [1-^{14}C]pyruvic acid (0.15 μCi, 23 μCi/μmol) and the other components indicated in the legend of Figure 1 were incubated at 28 °C with 90 μL of cell-free extract of *A. mediterranei* S699 or *S. collinus* Tü 1892 in a final volume of 100 μL. Aliquots (2 μL) from each incubation mixture were analyzed at different times by silica gel TLC. Separations were carried out in methanol/dioxane/NH_4OH/H_2O 3:6:1:1 (solvent 1) or 1 M LiCl in acetic acid/acetone/H_2O 1:6:4 (solvent 2). The TLC plates were exposed to X-ray film for 2 to 3 days.

To establish the identity of the enzyme reaction product with authentic aminoDAHP, 20 μL of [1-^{14}C]PEP (3.3 μCi, 23 μCi/μmol), 0.5 mL of 12 mM E4P, and 0.5 mL of 10 mM glutamine were incubated in 25 mL of cell-free extract of *S. collinus* Tü1892 for 20 min at 35 °C. AminoDAHP (0.3 mg) was then added, proteins were precipitated by addition of 5 mL of concentrated HCl and removed by centrifugation. AminoDAHP was isolated from the supernatant by two consecutive paper chromatography steps (ethyl acetate/pyridine/H_2O 20:9:20 and butanol/acetic acid/H_2O 2:1:1). The paper sections containing compound comigrating with authentic aminoDAHP were cut out and eluted three times with H_2O. More aminoDAHP (2 mg) was added to the eluate and the mixture derivatized with 2,4-dinitrofluorobenzene. The radioactive DNP-aminoDAHP was subjected to consecutive paper chromatography in four different solvent systems: (A) butanol/propionic acid/H_2O 21:11:14, (B) ethyl acetate/acetic acid/H_2O 6:6:1, (C) ether/acetic acid/H_2O 4:2:1, and (D) ether/isopropanyl alcohol/acetic acid/H_2O 3:2:1:1. After each chromatography the material was eluted and its specific molar radioactivity was determined by measuring the absorbance at 360 nm and the radioactivity of aliquots.

Results

To evaluate the hypothetical pathway of AHBA formation shown in Scheme 2 we prepared the proposed intermediates, aminoDAHP, aminoDHQ and aminoDHS, none of which were known compounds at the time these studies were initiated. AminoDAHP was synthesized from 2-deoxy-D-glucose by a 14-step reaction sequence in 10−12% overall yield.[28] From this material we obtained aminoDHQ by preparative enzymatic cyclization using a cell-free extract of *E. coli* RB791/pJB14,[30] a genetically engineered overproducer of DHQ synthase. AminoDAHP proved to be a good substrate for *E. coli* DHQ synthase, and the product could be readily isolated by anion exchange chromatography. Similarly, we made use of the fact that *E. coli* shikimate dehydrogenase in the presence of $NADP^+$ can oxidize aminoSA at 84% of the rate of its natural substrate,

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Table 1. Enzymatic Synthesis of AHBA in Cell-Free Extracts of *A. mediterranei* Strain S699

substrate(s)	conc (mM)	AHBA formed (μg)	% conversion
PEP + E4P	1.0 + 0.7	66	6 (based on E4P)
DAHP + Gln	0.32 + 0.68	<4	<1
aminoDAHP	0.3	208	45
aminoDHQ	0.26	163	41
aminoDHS	0.58	850	>95
aminoSA	0.58	9	1

shikimic acid, to generate aminoDHS. AminoSA was synthesized by ring opening of *cis*-3-hydroxy-4,5-epoxycyclohex-1-enecarboxylate, obtained from shikimic acid,[33] with ammonia.[34] Incubation of this material with a cell-free extract of *E. coli* AB2834/pIA321,[27] a genetically engineered overproducer of shikimate dehydrogenase,[35] in the presence of $NADP^+$ gave essentially quantitative conversion into aminoDHS, which could be obtained in 60% isolated yield by anion exchange chromatography.

AminoDHS, and to a lesser extent aminoDHQ, like DHS and DHQ[36] are unstable to acid, aromatizing to protocatechuic acid upon standing in concentrated HCl at room temperature. The aromatization of aminoDHS occurred at only 7% of the rate of that of DHS in concentrated HCl and produced no detectible amounts of AHBA (<1% by dilution analysis). Unlike DHS, aminoDHS also aromatized to protocatechuic acid upon standing in 1 M potassium phosphate buffer, pH 7.5; the rate was 30% of that of DHS aromatization in concentrated HCl.

The ability of the synthesized compounds to serve as substrates for the enzymatic formation of AHBA was evaluated in undialyzed cell-free extracts of *A. mediterranei* S699 and occasionally also of *S. collinus* Tü 1892.[37] To avoid having to prepare all the substrates in isotopically labeled form, we developed an inverse isotope dilution assay to quantitate AHBA formation. Incubations were carried out with 10 mL of cell-free extract containing 0.3 mM NAD^+, 1.5 mM $CoCl_2$ and the indicated concentrations of the respective substrates for 30 h at 28 °C. At the end of the incubation, a small, precisely know amount of [7-^{13}C]AHBA (90% ^{13}C, usually 200 μg) was added to the reaction mixture, the AHBA was reisolated, silylated and analyzed by GC-MS for the decrease in ^{13}C enrichment due to dilution with the enzymatically synthesized, unlabeled AHBA. Control reactions were always run without added substrate to determine the amount of endogenous or endogenously formed AHBA, which was typically on the order of 24 μg per incubation. The results are summarized in Table 1.

A small but significant amount of AHBA (66 μg over background) was formed reproducibly from PEP (1 mM) and E4P (0.7 mM) as substrates, corresponding to 6% conversion of the added E4P (4.3% of added PEP). The presence or absence of glutamine (up to 5 mM) had no effect on this conversion. When PEP + E4P were replaced by DAHP (0.32 mM) with or without glutamine (0.68 mM), no formation of AHBA above background was detected. AminoDAHP was efficiently converted into AHBA, as was aminoDHQ (45 and 41%, respectively). Finally, the conversion of aminoDHS into

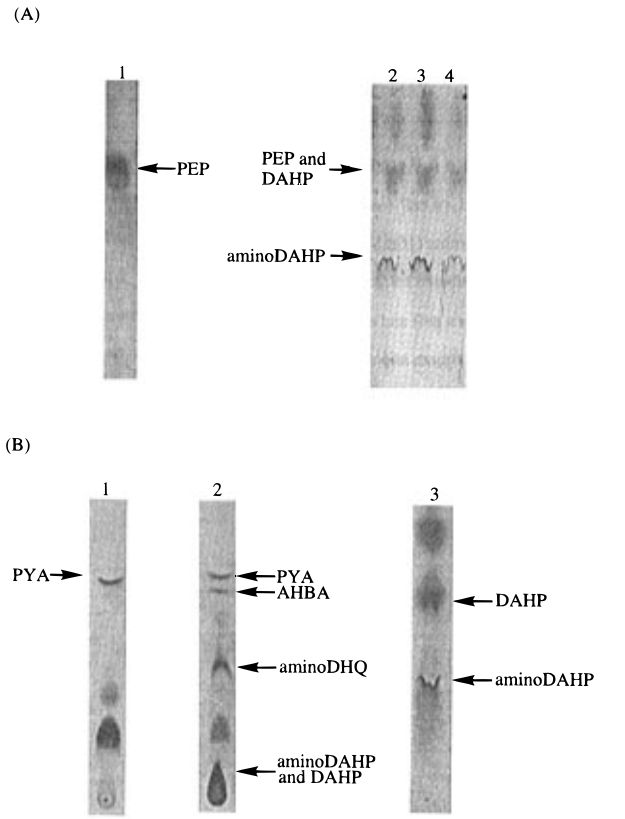


Figure 1. Autoradiographic analysis of incubation mixtures with *A. mediterranei* S699 cell-free extracts for enzymatic formation of aminoDAHP from [1-^{14}C]PEP and E4P. (A) Results after 15 min incubation. Samples were separated in solvent 2; lane 1, [1-^{14}C]PEP standard; lane 2, [1-^{14}C]PEP and E4P (0.1 mM); lane 3, [1-^{14}C]PEP, E4P (0.1 mM), and Gln (5 mM); lane 4, [1-^{14}C]PEP, E4P (1 mM), and Gln (5 mM). (B) Results after 120 min incubation. Lanes 1 and 2 were developed in solvent 1, lane 3 in solvent 2: lane 1, [1-^{14}C]PYA, E4P (0.1 mM), and Gln (0.1 mM); lanes 2 and 3, [1-^{14}C]PEP, E4P (0.1 mM), and Gln (0.1 mM).

AHBA was essentially quantitative under the incubation conditions. On the other hand, none of the other normal shikimate pathway intermediates or shunt metabolites, DHQ, DHS, or quinic acid, gave rise to AHBA formation above background. A very small but significant amount of AHBA was formed from aminoSA, presumably due to unphysiological action of shikimate dehydrogenase in the incubation.

We next focused on the enzymatic formation of aminoDAHP. [1-^{14}C]PEP (0.15 μCi, 0.065 mM) and E4P (0.1 mM) were incubated with cell-free extracts (90 μL) of *A. mediterranei* S699 and 2 μL aliquots of the incubation mixtures (100 μL) were analyzed by TLC and autoradiography. A compound appeared at the same R_f value as aminoDAHP on the TLC plates (Figure 1A), and its amount reached a steady state after 5 min. The production of the compound was not dependent on addition of glutamine and was inhibited by excess E4P (>1 mM). After 2 h of incubation, the reaction mixture also contained compounds with the same R_f values as aminoDHQ and AHBA (Figure 1B). Several compounds seemingly related to metabolism of PEP via pyruvate were also seen, i.e., pyruvate itself, as well as two metabolites also found in an identical incubation with [1-^{14}C]-pyruvate instead of [1-^{14}C]PEP. [1-^{14}C]Pyruvate, however, did not produce the compounds comigrating with aminoDAHP or aminoDHQ. The radioactive compound comigrating with aminoDAHP was isolated by TLC from a 60 min incubation with 0.5 μCi of [1-^{14}C]PEP in 9% radiochemical yield (Table 2). Another experiment with *S. collinus* Tü 1892 cell-free extract gave the same material in comparable yield. However,

(34) For an independent synthesis, see: Pansegrau, P. D.; Anderson, K. S.; Widlanski, T.; Ream, J. E.; Sammons, R. D.; Sikorski, J. A.; Knowles, J. R. *Tetrahedron Lett.* **1991**, *32*, 2589.

(35) Anton, I. A.; Coggins, J. R. *Biochem. J.* **1988**, *249*, 319.

(36) (a) Salomon, I. I.; Davis, B. D. *J. Am. Chem. Soc.* **1953**, *75*, 5567; (b) Scharf, K. H.; Zenk, M. H.; Onderka, D. K.; Carroll, M.; Floss, H. G. *J. Chem. Soc., Chem. Commun.* **1971**, 765.

(37) All the conversions seen in *A. mediterranei* extracts were also detectable in *S. collinus*, but often in lower yields. Since the *A. mediterranei* system is more efficient, we present mostly the data obtained with that system.

Table 2. Enzymatic Synthesis of AminoDAHP in Cell-Free Extracts of *A. mediterranei* S699 and *S. collinus* Tü1892

substrates		E4P	Gln	organism	vol. of cell-free	temp	incubation		radiochemical
[1-^{14C}]PEP	[1-^{14}C]DAHP	(mM)	(mM)	used	extract (mL)	(°C)	time (min)	isolation method	yield (%)
0.5 μCi		0.22	0.22	*A. mediterranei*	0.1	28	60	TLC	9
2.0 μCi		1	10	*S. collinus*	2.5	35	20	paper chromatography	9
	0.02 μCi		10	*S. collinus*	0.5	37	15	paper chromatography	<0.1

Table 3. Purification of Enzymatically Synthesized AminoDAHP, as Its DNP-Derivative, to Constant Specific Radioactivity

TLC	first	second	third	fourth[a]
solvent systems	A	B	C	D
Total cpm	1.6×10^5	8.7×10^4	5.7×10^4	2.3×10^4
total A_{360nm}	8.32	4.59	2.51	0.73
specific activity (cpm/A_{360nm})	1.9×10^4	1.9×10^4	2.3×10^4	3.1×10^4
recovery of radioactivity	79%	43%	28%	12%

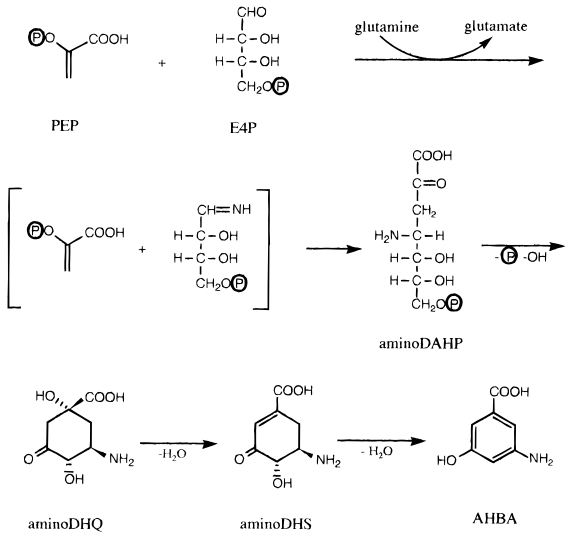
[a] The apparent specific radioactivity decreased by 75% during a fifth rechromatography (solvent: isoamyl alcohol/acetone/acetic acid/water 10:15:2:5). At this stage the concentration of the recovered product was so small that yellow color from impurities artificially increased the A_{360nm} of the sample.

when [1-^{14}C]PEP and E4P were replaced by [1-^{14}C]DAHP, no significant conversion into aminoDAHP was observed (Table 2). The identity of the enzyme reaction product with authentic aminoDAHP was established by cochromatography of the compounds themselves in several solvent systems, and by derivatization of the radioactive product, mixed with authentic carrier material, with 2,4-dinitrofluorobenzene and chromatographic purification of the DNP derivative to constant specific radioactivity (Table 3). The material retained its specific radioactivity through four successive paper chromatography steps in different solvent systems.

Since none of the above experiments had demonstrated any dependence of AHBA or aminoDAHP synthesis in the crude cell-free extracts on glutamine, the role of glutamine as a nitrogen source for AHBA was tested further. When PEP (1 mM) and E4P (0.7 mM) were incubated with cell-free extract of *A. mediterranei* containing [amide-^{15}N]glutamine (1 mM) or $^{15}NH_4Cl$ (1 mM), no ^{15}N was detectible in AHBA after addition of 200 μg of unlabeled carrier, reisolation and GC-MS analysis. Since the level of enrichment with ^{15}N might be below detection, in a second set of experiments the produced ^{15}N-AHBA was analyzed without addition of unlabeled carrier at the end of the incubation. The enrichment of AHBA thus obtained from [amide-^{15}N]glutamine was 2.7% and from $^{15}NH_4Cl$ 2.4%. These values when compared to the amount of AHBA formed in analogous incubations (66 μg in a background of 24 μg) indicate that the vast majority of the AHBA must have been formed from a nitrogen source endogenous to the cell-free extract.

To determine if AHBA is a limiting substrate for the synthesis of antibiotics derived from it, we fed unlabeled AHBA to fermentations of the rifamycin B producer, *A. mediterranei* S699, the ansatrienin producer, *S. collinus* Tü 1892 and the mitomycin producer, *S. verticillatus*. Earlier workers had already reported no effect of AHBA on rifamycin B production in *A. mediterranei* N813[13] and on rifamycin SV production in *A. mediterranei* NG112-4,[22] and we observed the same in *A. mediterranei* S699 over a contration range of 0.3−1 g AHBA/L. Ansatrienin production in *S. collinus* Tü 1892, on the other hand, was increased 60% by 0.3 g/L AHBA, whereas mitomycin production was inhibited by 19% at the same concentration of AHBA.

Scheme 2. Proposed Biosynthetic Pathway to AHBA



Discussion

The results presented here add considerable support to the proposed mode of AHBA formation shown in Scheme 2. The three postulated intermediates, aminoDAHP, aminoDHQ, and aminoDHS, are all converted efficiently into AHBA in cell-free extracts of the rifamycin producer, *A. mediterranei*, the last compound essentially quantitatively. In addition, formation of AHBA from PEP and E4P as substrates was also demonstrated, albeit in much lower yield. The enzymatic formation of aminoDAHP has also been demonstrated, although this proved to be more difficult. For one, the amounts of aminoDAHP formed in the cell-free system reach a steady state very early in the incubation, followed by apparent conversion into other compounds. While some of these are intermediates further downstream on the pathway to AHBA, e.g., aminoDHQ, as well as AHBA itself, other products are also formed in the incubation. The detection of both pyruvic acid and a lower R_f compound (see Figure 1B) which is also formed from [1-^{14}C]pyruvic acid, in the incubation mixture containing [1-^{14}C]PEP suggests dephosphorylation of PEP, although the possibility cannot be excluded that the radioactive aminoDAHP is decomposed to [1-^{14}C]pyruvic acid, E4P and ammonia by a retro-aldol reaction. DAHP synthase, however, is not known to catalyze such a retro-aldol reaction of DAHP.[38] Another difficulty was the unequivocal identification of the reaction product as aminoDAHP. Since we have so far been unable to crystallize our synthetic aminoDAHP or a derivative thereof, and since the amount of enzymatically formed material was too small for direct spectroscopic characterization, we had to resort to chromatographic comparisons. The radioactive material cochromatographed with an authentic sample in every one of several solvent systems tested, and the DNP derivative of a mixture of chromatographically purified radioactive enzyme reaction product and authentic material retained a constant specific radioactivity through several successive chromatographic purifications. These observations leave little doubt that the enzymatically generated compound is indeed aminoDAHP.

(38) Srinivasan, P. R.; Sprinson, D. B. *J. Biol. Chem.* **1959**, *234*, 716.

The results reported here show that the formation of AHBA must diverge from the normal shikimate pathway already in the very first reaction. Several research groups have suggested DAHP as the branch compound in the shikimate pathway from which AHBA is derived.[7b,8c] However, our results show unequivocally that DAHP cannot replace PEP plus E4P or aminoDAHP as a precursor of AHBA in the cell-free system. Likewise, none of the other early shikimate pathway intermediates, e.g., DHQ, DHS, or quinic acid, are substrates for AHBA formation. Thus, AHBA is formed by a new biosynthetic route which parallels the first three steps of the shikimate pathway but with the added feature of the introduction of an amino nitrogen into the substrate in the first step.

The source of the nitrogen in the formation of aminoDAHP is not clear yet. Based on mechanistic considerations and on the report by a Chinese group[22] that the amide nitrogen of glutamine is the best source of the rifamycin nitrogen, we had proposed that aminoDAHP synthase is composed of a DAHP synthase domain and a second domain or subunit which binds and hydrolyzes glutamine to generate ammonia in the active site of the enzyme. This has precedence in a number of enzymes, e.g., anthranilate synthase or *p*-aminobenzoate synthase. However, no dependence of aminoDAHP or AHBA formation from PEP and E4P on added glutamine could be demonstrated in our cell-free experiments. When [amide-^{15}N]glutamine or $^{15}NH_4Cl$ were used as nitrogen sources in the incubations, surprisingly low ^{15}N enrichments, 2.7% and 2.4%, were observed in the resulting AHBA samples. Since the extracts were undialyzed, they might have contained some unlabeled glutamine. However, in comparable incubations about 66 μg of AHBA were formed from added substrate vs 24 μg background which were either already present or formed from endogenous substrate. Thus, the endogenous concentration of glutamine in the extract would have to have been between 27 and 37 mM to account for the observed dilution of ^{15}N. This observation thus casts some doubt on the role of glutamine as the immediate nitrogen donor for aminoDAHP synthesis. A close examination of the data of Jiao *et al.*[22] reveals that their interpretation is not unequivocal. Because of differences in dilution factors in different experiments their data could also be interpreted to indicate that [amide-^{15}N]glutamine and [^{15}N]-glutamate are comparable nitrogen sources in rifamycin biosynthesis. Furthermore, in studies comparing [amide-^{15}N]-glutamine, [^{15}N]glutamate, and $^{15}NH_4Cl$ as nitrogen sources in ansatrienin biosynthesis we were unable to identify any one of these substrates as the preferred source of the mC_7N unit nitrogen.[21b] Thus, the question of the proximate source of the nitrogen in AHBA must await the purification of aminoDAHP synthase and more detailed studies on this enzyme.

While the first step of the pathway to AHBA, the synthesis of aminoDAHP, would seem to require at least a modified enzyme, compared to normal DAHP synthase, the next two reactions could easily be carried out by the normal shikimate pathway enzymes. The fact that *E. coli* DHQ synthase and shikimate dehydrogenase can catalyze their respective transformations with virtually equal ease on the 5-amino analogs of their normal substrates would support that notion. However, we have found that highly purified DHQ dehydratase from *A. mediterranei*, a type II enzyme, discriminates between DHQ and aminoDHQ and does not catalyze the dehydration of the latter to aminoDHS.[39] Thus, a second enzyme must function specifically in AHBA biosynthesis. Genetic evidence also points to the presence of another set of early shikimate pathway genes clustered with the gene encoding AHBA synthase.[40] The last reaction, the aromatization of aminoDHS to AHBA, has no parallel in the normal shikimate pathway. Remarkably, the acid- or general base-catalyzed chemical aromatization of aminoDHS, as that of DHS, produces exclusively protocatechuic acid, whereas the enzymatic process leads quantitatively to AHBA. The enzyme catalyzing this reaction therefore must completely redirect the aromatization chemistry of its substrate. The purification of AHBA synthase, the cloning and expression of the encoding gene, and the mechanism of the reaction will be the subject of a forthcoming publication.

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