Chorismate Aminations: Partial Purification of *Escherichia coli* PABA Synthase and Mechanistic Comparison with Anthranilate Synthase†

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ABSTRACT: Chorismate is converted by regiospecific amination/aromatization sequences to *o*-aminobenzoate and *p*-aminobenzoate (PABA) by anthranilate synthase (AS) and PABA synthase (PABS), respectively. We report here the first partial purification of the large subunit of *Escherichia coli* PABA synthase, previously reported to be quantitatively inactivated in purification attempts. The subunit encoded by the pabB gene was overexpressed from a T7 promoter and purified 9-fold to 25–30% homogeneity. The pabB subunit appears unusually sensitive to inactivation by glycerol so this cosolvent is contraindicated. The K_m for chorismate is 42 μM in the ammonia-dependent conversion to PABA, and we estimate a turnover number of 2.6 min^{-1}. A variety of chorismate analogues have been prepared and examined. Of these compounds, cycloheptadienyl analogue **11** has been found to be the most potent inhibitor of *Serratia marcescens* anthranilate synthase (K_i = 30 μM for an *RS* mixture) and of the *E. coli* pabB subunit of PABA synthase (K_i = 226 μM). Modifications in the substituents at C-3 [enolpyruyl ether, (*R*)- or (*S*)-lactyl ether, glycolyl ether] or C-4 (*O*-methyl) of chorismate lead to alternate substrates. The V_{max} values for (*R*)- and (*S*)-lactyl ethers are down 10–20-fold for each enzyme, and V/K analyses show the (*S*)-lactyl chorismate analogue to be preferred by 12/1 over (*R*)-lactyl for anthranilate synthase while a 3/1 preference was observed for (*R*)-/(*S*)-lactyl analogues by PABA synthase. The glycolyl ether analogue of chorismate shows 15% V_{max} vs. chorismate for anthranilate synthase but is actually a faster substrate (140%) than chorismate with PABA synthase, suggesting the elimination/aromatization step from an aminocyclohexadienyl species may be rate limiting with AS but not with PABS. Indeed, studies with (*R*)-lactyl analogue **14** and anthranilate synthase led to accumulation of an intermediate, isolable by high-performance liquid chromatography and characterized by NMR and UV–visible spectroscopy as 6-amino-5-[(1-carboxyethyl)oxy]-1,3-cyclohexadiene-1-carboxylic acid (**17**). This is the anticipated intermediate predicted by our previous work with conversion of synthetic *trans*-6-amino-5-[(1-carboxyethenyl)oxy]-1,3-cyclohexadiene-1-carboxylic acid (**2**) to anthranilate by the enzyme. Compound **17** is quantitatively converted to anthranilate on reincubation with enzyme, but at a 1.3–10-fold lower V_{max} than starting lactyl substrate **14** under the conditions investigated; the basis for this kinetic variation is not yet determined.

Microorganisms utilize the shikimic acid pathway for the biosynthesis of the aromatic amino acids, the folate coenzymes, and several other essential vitamins (Weiss & Edwards, 1980; Ganem, 1978). The branch point of the pathway is the dihydro aromatic chorismic acid (**1**), which is converted by five separate enzymes to anthranilate, *p*-aminobenzoate, *p*-hydroxybenzoate, isochorismate, and prephenate (Scheme I). In turn, these intermediates are converted respectively to tryptophan, folic acid, uniquinones, substituted benzoic acids (e.g., in enterochelin synthesis), and the aromatic amino acids phenylalanine and tyrosine.

Anthranilate synthase and *p*-aminobenzoic acid (PABA)[1] synthase catalyze the formation of anthranilate and PABA from chorismate in amination/aromatization reactions and appear to be mechanistically related. In fact, the nucleotide sequences of the genes that encode for both enzymes in *Escherichia coli* are homologous and may be derived from a common ancestral gene (Goncharoff & Nichols, 1984). The enzymes are both two-subunit enzymes with each low molecular weight subunit (~20K) possessing glutaminase activity. The large subunit (~50K) is involved with the binding of chorismate and the regiospecific amination/pyruvate elimination sequence.

Anthranilate synthase is readily available from organisms harboring overproducing plasmids with the gene for either or both subunits (Zalkin, 1985) and has consequently been the focus of several mechanistic studies. Policastro et al. (1984) and Teng and Ganem (1984) have established recently that synthetic *trans*-aminoenolpyruvate **2** is a chemically and kinetically competent intermediate in the biosynthesis of anthranilate but to date have not detected **2** as an accumulating intermediate during enzymatic processing of chorismate (Scheme II). The mechanism for the conversion of chorismate to **2** has been proposed to be a formal syn-1,5 displacement of hydroxide by ammonia. This amination step and the subsequent aromatization of **2** by a syn elimination of the elements of pyruvate are not understood. Floss and colleages have found, however, that during aromatization of chorismate the third methyl hydrogen in pyruvate comes from a *re*-face addition of a proton from solvent (Asano et al., 1985), thereby

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[1] Abbreviations: amp, ampicillin; AS, anthranilate synthase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; kan, kanamycin; LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide, reduced form; *n*-Oct, *n*-octyl; PABA, *p*-aminobenzoic acid; PABS, *p*-aminobenzoate synthase; PAGE, polyacrylamide gel electrophoresis; rif, rifampicin; rt, retention time; RT, room temperature; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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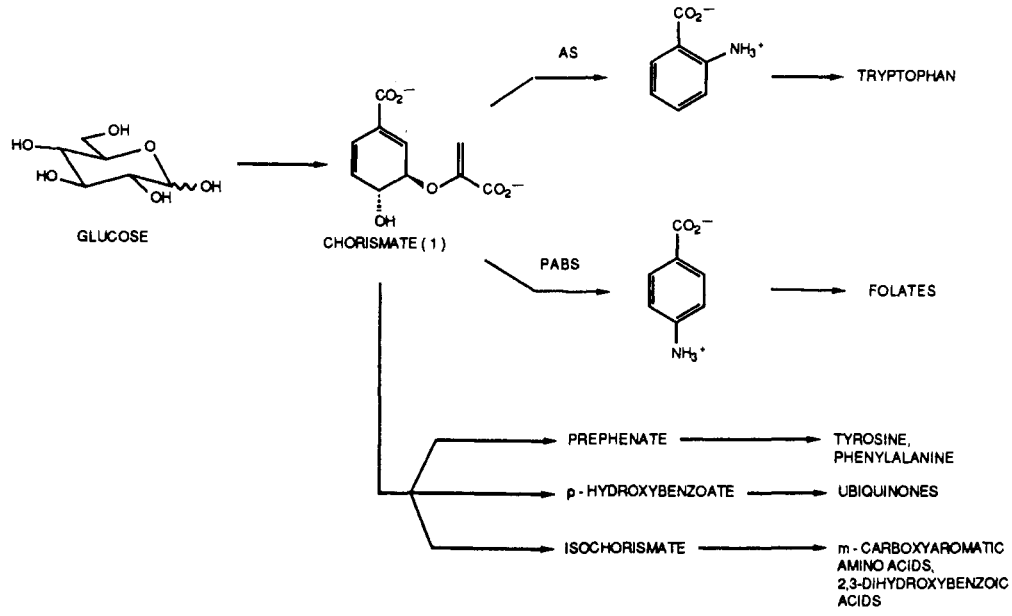
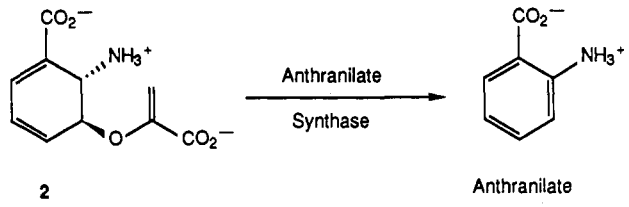
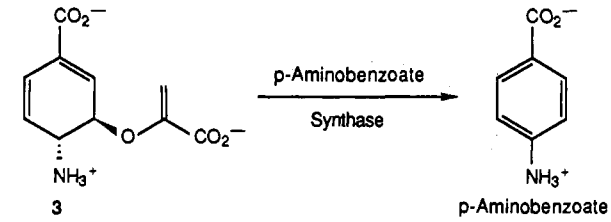
Scheme I



Scheme II



Scheme III



mitigating against a cyclic (six-center) elimination mechanism.

PABA synthase, on the other hand, has received much less attention due to the very low activity (picomoles per hour per milligram) in crude extract and the extreme difficulty of purification of active catalyst. Nichols has sequenced the *E. coli* gene (pabB) that encodes for the large subunit responsible for converting chorismate to PABA (Goncharoff & Nichols, 1984) and has constructed a pabB overproducing plasmid containing a trp promoter that results in a moderate increase in PABA synthase specific activity in crude extracts (B. Nichols, private communication). The PABA synthase enzymatic mechanism has not yet been elucidated but does appear to bear some mechanistic similarities to that of anthranilate synthase since the aminopyruvate 4-amino-4-deoxychorismic acid (**3**) is a competent enzymatic substrate in the biosynthesis of PABA in crude extracts (Teng et al., 1985) (Scheme III).

In this study we have begun to address the two- (or more) step amination and aromatization sequences used by anthranilate and PABA synthase to convert chorismate to the corresponding *o*- or *p*-aminobenzoates. In order to obtain sufficient quantities of PABS of reasonable purity, we have subcloned the pabB gene onto a plasmid containing the T7ϕ10 promoter and report herein overproduction of the large subunit and the first partial purification protocol. The subsequent mechanistic study on both the *E. coli* pabB subunit and the *Serratia marcescens* anthranilate synthase holoenzyme has involved design, synthesis, and testing of alternate substrates and inhibitors to probe for intermediates in the amination steps and also to examine the effect of the leaving group at C-3 of chorismate on the rate of aromatization. Furthermore, we present UV–visible and nuclear magnetic resonance spectroscopic evidence for formation of a dihydro aromatic lactyl intermediate from incubation of the (*R*)-lactyl analogue of chorismate with anthranilate synthase.

Experimental Procedures

Materials

Chorismic acid was isolated from culture growth of *Klebsiella pneumoniae* 62-1 (formerly *Aerobacter aerogenes* 62-1) according to the procedure of Gibson (1968). The syntheses of compounds **4–12** and **15–16** will be reported separately. Restriction enzymes, T-4 DNA ligase, and *E. coli* JM101 were obtained from New England Biolabs. *E. coli* K-38 (pGP1-2) was obtained from T. Begley, and plasmid pT7-3 was a gift from S. Tabor (Tabor & Richardson, 1985). Cells were grown either in a New Brunswick shaker or in a Chemap fermentor. Buffer A consisted of 100 mM KP_i, 0.2 mM DTT, and 0.1 mM EDTA (pH 7.4). Buffer B consisted of 50 mM KP_i, 0.2 mM DTT, and 0.1 mM EDTA (pH adjusted as indicated). Assay buffer A (used for anthranilate synthase) contained 50 mM triethanolamine, 10 mM $MgCl_2$, and 10 mM glutamine (pH 7.4). Assay buffer B (used for PABA synthase) consisted of 50 mM triethanolamine, 10 mM $MgCl_2$, 33 mM ammonium sulfate, 5 mM DTT, and 0.6% glycerol (pH 8.6).

Methods

Preparation and Purification of Two-Component Anthranilate Synthase (Holoenzyme). Plasmid pJP20 containing the two genes encoding *S. marcescens* anthranilate synthase obtained from Professor H. Zalkin (Purdue University) was transformed into *E. coli* W3110 according to the general procedure described by Maniatis et al. (1982). Competent amp-resistant colonies were selected, and a starter culture (70 mL) was grown in M9CA medium (Maniatis et al., 1982) from one colony (50 mg/L amp). Portions of the starter culture (10 mL each) were used to innoculate six 1-L portions of M9CA medium (50 mg/L amp), and cells were grown in a New Brunswick shaker to 165 Klett and harvested by cen-

trifugation (5000*g*; 5 min). After being washed with buffer A, the cell paste (16.2 g) was stored at −78 °C.

Cells (15.5 g) were resuspended in 30 mL of buffer B (pH 7.6) and disrupted by passage through a French press at 12000 psi. After centrifugation to remove cell debris (37000*g*; 30 min), streptomycin was added to a final 3% concentration. After centrifugation at 37000*g* (30 min), the supernatant was made 70% saturated with ammonium sulfate and centrifuged at 37000*g* for 30 min. The pellet was resuspended in buffer B (pH 7.6) and dialyzed against the same buffer. Purification was then accomplished by ion-exchange chromatography (DEAE-Sepharose CL-6B, 0–0.5 M KCl, buffer B, pH 7.6) followed by gel filtration on Ultrogel AcA-34. The AS obtained after gel filtration (200 mg) was homogeneous (two subunits; 60000 and 21000 daltons) by PAGE. The specific activity was 3500 nmol min^{-1} mg^{-1} as measured by the assay described by Zalkin and Hwang (1971; see also experimental methods, vide infra). Purification of anthranilate synthase I and II has been reported previously by Zalkin using a slightly different procedure (Zalkin, 1985).

PABA Synthase. (*A*) *Construction of Overproducing Strain.* Plasmid pAS4 was isolated from an *E. coli* BN116 strain obtained from Professor B. Nichols (University of Illinois, Chicago) and purified according to the general procedure described by Maniatis et al. (1982). Digestion with *Bam*HI and *Pst*I provided a 4.3-kb fragment (B. Nichols, private communication) containing the functional pabB gene, minus the trp promoter introduced by Nichols. This fragment was purified by preparative agarose gel electrophoresis (0.8% agarose) and isolated by phenol extraction of the agarose and ethanol precipitation. Plasmid pT7-3 bearing the T7 promoter was also digested with *Bam*HI and *Pst*I and purified by phenol extraction and ethanol precipitation. The DNA fragment containing pabB and the pT7-3 fragment were ligated with T-4 ligase to form plasmid pBC1, which was then used to transform kan-resistant *E. coli* K-38(pGP1-2) (Tabor & Richardson, 1985) to kan/amp resistance. Colonies (100) were selected and grown overnight in LB medium at 30 °C (50 mg/L kan and amp) and then used to innoculate fresh LB medium (50 mg/L kan and amp). Transformants were grown to an A_{590} = 1.6 at 30 °C; the cultures were heat shocked (42 °C, 1 h) and then grown at 30 °C for an additional 1.5 h. Cells were isolated by centrifugation (5000*g*; 5 min), resuspended in buffer B (pH 7.6, 1.0 mL), and disrupted by sonication (30 s). PABS activity was then assayed according to the procedure of Teng et al. (1985) with assay buffer B. The culture displaying the highest specific activity was used for all future experiments. Confirmation of the presence of the desired plasmid pBC1 was obtained by restriction digest.

(*B*) *Enzyme Production and Purification. E. coli* K-38(pBC1/pGP1-2) was grown in a Chemap fermentor (10-L culture volume) to 150 Klett in 2% tryptone, 1% yeast extract, 0.5% NaCl, and 0.2% glucose medium at 30 °C. The temperature was raised to and held at 42 °C for 20 min and then lowered back to 30 °C. Rifampicin was added at 30 °C (200 mg/L) and fermentation was continued for 7 h. Cells were harvested by centrifugation (5000*g*; 5 min), yielding 30 g of cell paste.

Cells (30 g) were resuspended in buffer B (pH 7.6) and disrupted by passage through a French press at 12000 psi. Streptomycin was added (3%) after centrifugation to remove cell debris (37000*g*; 30 min), and the subsequent precipitate was removed by centrifugation. Ammonium sulfate was added to 35%, the mixture was centrifuged (37000*g*; 30 min), and the supernatant was made 70% in ammonium sulfate. After centrifugation (37000*g*; 30 min) the resuspended pellet (buffer B, pH 7.6) was dialyzed overnight against the same buffer. Assay of the 35% fraction indicated that ca. 30% of the total cell extract units were present. This material was therefore saved and purified via ion-exchange chromatography as described for the 70% ammonium sulfate precipitate (vide infra).

Half of the protein solution (258 mg) from the 70% ammonium sulfate precipitate was loaded onto a DEAE-Sepharose CL-6B column (25 × 240 mm) and eluted with a 0–0.5 M KCl gradient in buffer B (pH 7.3, *no* glycerol; 6.7-mL fractions; 1.4 mL/min). An identical column was run simultaneously under identical conditions (flow rate, column dimensions, etc.) on the second half of the 70% ammonium sulfate precipitate, except 7.5% glycerol was used in the column buffer. Fractions from the first column containing PABS activity were pooled, concentrated, and passed through a gel filtration column (Sephadex G-150, 2 × 69 cm; buffer B, pH 7.3, 4-mL fractions, 0.25 mL/min). Fractions containing the highest PABS activities (1.6 nmol min^{-1} mg^{-1}) were used in subsequent experiments. PAGE analysis of PABS-containing fractions indicated that a substantial purification had been achieved (ca. 30% pure; PABS, 52K predicted molecular weight; Goncharoff & Nichols, 1984).

Amino-terminal analysis of protein from the band (PAGE) at 52000 daltons (Met-Lys-Thr-Leu-Ser-Pro-Ala-Val-Ile-Thr) confirmed the identity of the protein as PABS (Goncharoff & Nichols, 1984).

Kinetic Analyses

Substrate/Pseudosubstrate Characterization. (*A*) *Anthranilate Synthase.* AS activity was monitored by continuous fluorescence detection of anthranilic acid (325/400 nm) according to the general procedure of Zalkin and Hwang (1971) in assay buffer A at 23 °C. Anthranilate synthase was present at 1.65 μg/mL for experiments with chorismate, 33 μg/mL for experiments with compounds **2–4**, and 330 μg/mL for experiments with 4-*O*-methylchorismate (**16**). When indicated, the lactate dehydrogenase coupled assay proceeded by use of LDH (0.3 mg/mL) and NADH (0.3 mM) in the standard anthranilate assay buffer containing anthranilate synthase and substrate.

(*B*) *PABA Synthase.* PABS activity was monitored discontinuously according to the general procedure described by B. Nichols (private communication) with assay buffer B. After 60–75 min, the assay mixture (1 mL) was acidified with 2 N HCl (0.1 mL) and extracted with ethyl acetate (3 mL), and the concentration of PABA was determined by measuring the fluorescence of the ethyl acetate solution at 290/340 nm. For kinetic determinations on chorismate, 75 μL of a solution containing 0.38 mg/mL protein (ca. 30% PABS, solution A) was used, while for determinations on compounds **2–4** protein from the 35% ammonium sulfate cut/DEAE-Sepharose chromatography was used (100 μL of 6.4 mg/mL; 420 pmol min^{-1} mg^{-1}, solution B).

Inhibition Kinetics Determination. (*A*) *Anthranilate Synthase.* Inhibition constants were measured for various inhibitors by determining K_m and V_{max} for chorismate at various substrate and inhibitor concentrations. Assays were run according to the general procedure given for substrate kinetic analyses (vide supra). In cases where potential turnover could not be detected by the fluorescence assay (e.g., **11**), absence of turnover was confirmed by monitoring pyruvate formation with the LDH coupled assay, thus indicating that inhibition was not due to slow turnover. This procedure gave kinetic parameters for chorismate identical with those obtained in the fluorescence assay.

(*B*) *PABA Synthase.* Single-point K_i values were determined by measuring PABS activity with chorismate (84 μM; $2K_m$) in the presence of 1 mM of each inhibitor. Approximate K_i values were calculated from $V_{(I)} = V_{max}[S]/\{K_m(1 + [I]/K_i) + [S]\}$. For each experiment 60 μL of PABS solution A was used, and rates were measured over 75 min.

Inactivation Kinetics Determination. (*A*) *Anthranilate Synthase.* Time-dependent inactivation was measured by mixing 16.5 μg of anthranilate synthase and 2 mM inactivator (final concentration) in 100 μL of standard assay mixture at 23 °C. Aliquots (10 μL) were withdrawn at various times and added to 1.0 mL of assay buffer (1:100 dilution) containing chorismate (30 μM), and anthranilate synthase activity was measured.

(*B*) *PABA Synthase.* Time-dependent inactivation was measured by mixing 60 μL of PABS solution A, 20 μL of 10 mM inactivator (2 mM final concentration), and 20 μL of 5× assay buffer B. After being heated at 37 °C (90 min), the solution was diluted to 1.0 mL (1:10 dilution) with assay buffer B containing chorismate (200 μM) and incubated at 37 °C. The remaining activity was then compared with that of a control sample that had been heated at 37 °C (90 min) in the absence of any inactivator.

Synthesis of (S)- and (R)-Lactyl Compound ***13*** *and* ***14***

Methyl α-Diazopropionate (**19**). To a solution of methyl-DL-alanine hydrochloride (11.12 g, 80.0 mmol) (Sigma) in 40 mL of H_2O was added Na_2CO_3 (9.36 g, 1.1 equiv). The aqueous solution was extracted with four 50-mL portions of chloroform. The combined chloroform extracts were dried ($MgSO_4$), filtered, and diluted to 400 mL with chloroform. Isoamyl nitrite (12.5 mL, 1.2 equiv) and acetic acid (0.45 mL, 0.10 equiv) were added. The solution was heated at reflux for 1 h, during which time the solution turned bright yellow. The reaction mixture was cooled to room temperature and washed with 100-mL portions each of cold H_2O and cold saturated $NaHCO_3$. The $CHCl_3$ layer was dried ($MgSO_4$), filtered, and evaporated under reduced pressure. The yellow oil was filtered through an alumina plug (elution with 1:1 benzene/hexanes) and concentrated to give 6.32 g (69%) of pure **19**: IR (neat) 2082 cm^{-1}; ^1H NMR (250 MHz) δ 3.70 (3 H, s), 1.94 (3 H, s).

Methyl (1R,2R,6R)-2-[[1-(Methoxycarbonyl)ethyl]oxy]-7-oxabicyclo[4.1.0]hept-3-ene-4-carboxylate (**21**). A mixture of (−)-**20** (Pawlak & Berchtold, 1987) (1.00 g, 5.88 mmol), **19** (0.67 g, 1.0 equiv), and $Rh_2(n\text{-}Oct)_4$ (50 mg) in 150 mL of anhydrous benzene was stirred at room temperature under N_2. Every hour, an additional 134 mg (0.2 equiv) of **19** was added. Stirring was continued until starting material was consumed, as indicated by TLC. The solution was washed with saturated $NaHCO_3$ (100 mL) and H_2O (100 mL). The solution was dried ($MgSO_4$), filtered, and evaporated under reduced pressure. Flash chromatography on silica gel (1:1 ether/petroleum ether) gave 786 mg (52%) of **21**: IR (neat) 1750, 1720 cm^{-1}; ^1H NMR (250 MHz) δ 6.83 (1 H, m), 4.41 (1 H, br s), 4.30 (1 H, m), 3.78 (3 H, s), 3.75 (3 H, d, J = 0.6 Hz), 3.44 (1 H, br s), 3.28 (1 H, br s), 2.96 (1 H, dm, J = 19.5 Hz), 2.68 (1 H, dm, J = 19.5 Hz), 1.47 (3 H, t); mass spectrum, m/z (relative intensity) 241 (0.3), 225 (4), 197 (7.5), 169 (24), 153 (83). Anal. Calcd for $C_{12}H_{16}O_6$: C, 56.25; H, 6.29. Found: C, 55.97; H, 6.34.

Methyl (3R,4S,5S)-3-[[[(1R)- and (1S)-1-Methoxycarbonyl]ethyl]oxy]-4-hydroxy-5-(phenylseleno)-1-cyclohexene-1-carboxylate (**22a,b**). To an ice-cold solution of PhSeSePh (412 mg, 0.75 equiv) in 50 mL of dry methanol was added $NaBH_4$ (slight excess, added slowly). The resulting colorless solution was added dropwise under inert atmosphere to a solution of **21** (0.45 g, 1.76 mmol) in 30 mL of dry methanol. The reaction mixture was stirred at room temperature. Every 12 h, $NaBH_4$ was added to decolorize the yellow solution. After 48 h, the solution was concentrated. The residue was dissolved in CH_2Cl_2 and washed with saturated NH_4Cl solution (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated. The isomers were separated by flash chromatography (1:1 ether/petroleum ether) providing, in 78% overall yield, 273 mg of **22a** and 268 mg of **22b**. **22a**: $[\alpha]_D$ +70.4° (*c* 1, MeOH); IR (neat) 3490, 1719 cm^{-1}; ^1H NMR (250 MHz) δ 7.73 (2 H, m), 7.34 (3 H, m), 6.83 (1 H, t, J = 2.8 Hz), 4.59 (1 H, q, J = 7.5 Hz), 4.18 (1 H, m), 3.77 (3 H, s), 3.72 (3 H, s), 3.58 (1 H, dd, J = 7.5 and 11.5 Hz), 3.22 (1 H, dt, J = 5.2 and 11.5 Hz), 2.94 (1 H, dd, J = 5.2 and 16.5 Hz), 2.37 (1 H, m), 1.48 (3 H, d, J = 7.5 Hz); ^{13}C NMR δ 174.0, 166.0, 137.6, 136.1, 130.8, 129.0, 128.4, 125.3, 79.3, 74.6, 73.2, 51.8, 51.7, 45.3, 31.7, 18.7; mass spectrum, m/z (relative intensity) 414 (4.5), 412 (3.0), 382 (16), 380 (7.8), 225 (46), 153 (51). Anal. calcd for $C_{18}H_{22}O_6Se$: C, 52.31; H, 5.36. Found: C, 52.60; H, 5.51. **22b**: $[\alpha]_D$ +14.5° (*c* 1, MeOH); IR (neat) 3465, 1725 cm^{-1}; ^1H NMR (250 MHz) δ 7.68 (2 H, m), 7.33 (3 H, m), 6.64 (1 H, br s), 4.28 (1 H, q, J = 7 Hz), 3.98 (1 H, m), 3.80 (3 H, s), 3.72 (3 H, s), 3.65 (1 H, dd, J = 7.5 and 11 Hz), 3.25 (1 H, dt, J = 5.6 and 11 Hz), 2.82 (1 H, dd, J = 5 and 18 Hz), 2.41 (1 H, m), 1.48 (3 H, d, J = 7 Hz); ^{13}C NMR δ 174.6, 166.0, 136.6, 135.4, 131.5, 129.0, 128.3, 125.8, 81.1, 74.1, 72.5, 52.3, 51.9, 42.7, 32.3, 19.1; mass spectrum, m/z (relative intensity) 414 (7.7), 412 (3.8), 382 (19), 380 (9.5), 225 (51), 153 (53). Anal. Calcd for $C_{18}H_{22}O_6Se$: C, 52.31; H, 5.36. Found: C, 52.05; H, 5.60.

(3R,4S,5S)-3-[[(1R)-1-Carboxyethyl]oxy]-4-hydroxy-5-(phenylseleno)-1-cyclohexene-1-carboxylic Acid (**23a**). Diester **22a** (200 mg, 0.485 mmol) was dissolved in 12 mL of THF and cooled in an ice bath; 1 N NaOH (3 equiv, 1.45 mL of a 1 N solution) and 10 mL of H_2O were added. The solution was warmed to room temperature and stirred for 2 h. HCl (1.45 mL of 1 N solution) was added, and the aqueous solution was extracted with ethyl acetate (4 × 40 mL). The ethyl acetate extracts were dried ($MgSO_4$), filtered, and concentrated to give 187 mg (100%) of **23a** as a colorless solid: $[\alpha]_D$ +68.5° (*c* 1, MeOH); ^1H NMR (250 MHz, [$^2H_6$]-acetone) δ 7.65 (2 H, m), 7.33 (3 H, m), 6.92 (1 H, t, J = 2.2 Hz), 4.60 (1 H, q. J = 7 Hz), 4.15 (1 H, m), 3.70 (1 H, dd, J = 6.8 and 11 Hz), 3.55 (1 H, dt, J = 5.6 and 11 Hz), 2.76 (1 H, dd, J = 5.6 and 17 Hz), 2.38 (1 H, m), 1.41 (3 H, t, J = 7 Hz); ^{13}C NMR δ 174.6, 166.6, 138.5, 135.5, 130.6, 129.1, 127.8, 79.8, 75.0, 74.3, 43.9, 32.3, 18.4; mass spectrum, m/z (relative intensity) 386 (1.5), 384 (0.7), 368 (71), 366 (35), 211 (27); HRMS, calcd for $C_{16}H_{16}O_5{}^{78}Se$ 366.0170, found 366.0170.

(3R,4S,5S)-3-[[(1S)-1-Carboxyethyl]oxy]-4-hydroxy-5-(phenylseleno)-1-cyclohexene-1-carboxylic Acid (**23b**). Hydrolysis of **22b** was achieved by the same procedure used for the preparation of **23a**: $[\alpha]_D$ +33.0°; ^1H NMR (250 MHz, [$^2H_6$]acetone) δ 7.69 (2 H, m), 7.33 (3 H, m), 6.76 (1 H, br s), 4.46 (1 H, q, J = 6.5 Hz), 4.15 (1 H, m), 3.68 (1 H, dd, J = 7 and 11 Hz), 3.48 (1 H, dt. J = 5.8 and 11 Hz), 2.74 (1 H, dd, J = 7 and 18 Hz), 2.35 (1 H, m), 1.40 (3 H, d, J = 6.5 Hz); ^{13}C NMR δ 175.6, 166.6, 136.5, 136.1, 134.1, 131.6, 129.4, 128.2, 81.5, 74.2, 73.8, 42.9, 31.5, 19.2; mass spectrum, m/z (relative intensity) 368 (100), 366 (51); HRMS, calcd for $C_{16}H_{16}O_5{}^{78}Se$ 366.0170, found 366.0170.

(3R,4R)-3-[[(1R)-1-Carboxyethyl]oxy]-4-hydroxy-1,5-cyclohexadiene-1-carboxylic Acid (**14**). A solution of **23a** (66 mg, 17 mmol) in 3 mL of acetone was cooled to −35 °C. Hydrogen peroxide (2.5 equiv, 46 μL) was added, and the solution was stirred for 1 h. 3,5-Dimethoxyaniline (3.0 equiv, 79 mg) was added, and the solution was allowed to warm to room temperature. The solution was stirred at room temperature for 1.5 h. The solution was concentrated, taken up in 1 mL of methanol, and diluted with water (10 mL). The aqueous solution was washed with CH_2Cl_2 (6 × 5 mL) and extracted with ethyl acetate (6 × 10 mL). The ethyl acetate extracts were dried ($MgSO_4$), filtered, and concentrated to give 19.5 mg (50%) of pure **14**: $[\alpha]_D$ −95.6° (*c* 1, MeOH); IR (neat) 3450, 1709 cm^{-1}; ^1H NMR (250 MHz, [$^2H_6$]-acetone) δ 7.04 (1 H, br s), 6.08 (2 H, AB), 4.53 (1 H, q, *J* = 8 Hz), 4.50 (2 H, AB), 1.42 (3 H, d, *J* = 8 Hz); ^{13}C NMR δ 175.6, 166.6, 140.2, 134.1, 129.1, 122.3, 83.6, 75.8, 83.5, 19.6; mass spectrum, *m/z* (relative intensity) 228 (1), 210 (3.5), 182 (7), 167 (5.4), 138 (70); HRMS, calcd for $C_{10}H_{10}O_5$ 210.0528, found 210.0528; UV (H_2O, pH 7.5) 231 nm (ε 1570), 273 (ε 2040).

(3R,4R)-3-[[(1S)-1-Carboxyethyl]oxy]-4-hydroxy-1,5-cyclohexadiene-1-carboxylic Acid (**13**). Preparation of **13** was achieved from **23b** by the same procedure used for the preparation of **14**: $[\alpha]_D$ −136.6° (*c* 1, MeOH); IR (neat) 3420, 1709 cm^{-1}; ^1H NMR (250 MHz, [$^2H_6$]acetone) δ 6.91 (1 H, br s), 6.09 (2 H, AB), 4.3–4.7 (3 H, m), 1.48 (3 H, d, *J* = 8 Hz); ^{13}C NMR δ 175.6, 165.6, 138.2, 133.1, 129.0, 121.3, 83.6, 73.5, 72.3, 19.4; mass spectrum, *m/z* (relative intensity) 228 (2.9), 210 (9.5), 182 (19.2), 138 (100); HRMS, calcd for $C_{10}H_{10}O_5$ 210.0528, found 210.0528; UV (H_2O, pH 7.5) 232 nm (ε 1530), 273 (ε 1690).

3-[[(1R)-1-Carboxyethyl]oxy]benzoic Acid (**26**). A solution of **14** (35.5 mg, 0.16 mmol) in 1 mL of [$^2H_6$]Me_2SO was stirred at 50 °C until 1 H NMR analysis showed disappearance of starting material. The solution was diluted with H_2O (10 mL) and extracted with ethyl acetate (6 × 10 mL). The combined ethyl acetate extracts were dried ($MgSO_4$), filtered, and concentrated. Flash chromatography on silica gel (2:1 ethyl acetate/petroleum ether + 3% HOAc) gave 10.0 mg (30%) of pure **26**: $[\alpha]_D$ +24.3° (*c* 1, MeOH).

3-[[(1S)-1-Carboxyethyl]oxy]benzoic Acid (**27**). The aromatization of **13** was performed by the same procedure as used for the preparation of **26**. The spectral data of **26** and **27** were identical: $[\alpha]_D$ −22.9° (*c* 1, MeOH).

Confirmation of Absolute Stereochemistry of **26** *by Synthesis.* To a stirred solution of ethyl (*S*)-lactate (1.0 g, 8.5 mmol) and distilled triethylamine (2.8 equiv, 3.30 mL) in 50 mL of CH_2Cl_2 was added dropwise methanesulfonyl chloride (2.7 equiv, 1.77 mL) over 10 min. The solution was stirred at room temperature for 2 h. The reaction mixture was washed with H_2O (2 × 50 mL), dried ($MgSO_4$), filtered, and concentrated. The crude oil was filtered through a 1-in. silica plug (1:2 ethyl acetate/petroleum ether) to give 1.51 g (91%) of pure mesylate.

A slurry of methyl 3-hydroxybenzoate (152 mg, 1.0 mmol), mesylate (1.2 equiv, 196 μL), and potassium carbonate (2.0 equiv, 176 mg) in 2 mL of acetone was refluxed for 48 h. The reaction mixture was diluted with CH_2Cl_2 (50 mL), washed with H_2O (2 × 20 mL), dried ($MgSO_4$), filtered, and concentrated. Flash chromatography (1:5 ethyl acetate/petroleum ether) gave 155 mg (62%) of **24**: $[\alpha]_D$ +38.0° (*c* 1.5, MeOH). Flash chromatography (1:5 ethyl acetate/petroleum ether) gave 155 mg (62%) of **24**: $[\alpha]_D$ +38.0° (*c* 1.5, MeOH); IR (neat) 2988, 2955, 1753, 1724 cm^{-1}; ^1H NMR (300 MHz) δ 7.57 (1 H, dd, *J* = 1.3 and 6.3 Hz), 7.52 (1 H, m), 7.34 (1 H, t, *J* = 8 Hz), 7.10 (1 H, td, *J* = 1.4 and 8 Hz), 4.81 (1 H, q, *J* = 6.7 Hz), 4.23 (2 H, dq, *J* = 1.1 and 7 Hz), 3.90 (3 H, s), 1.63 (3 H, d, *J* = 6.7 Hz), 1.59 (3 H, t, *J* = 7 Hz); ^{13}C NMR δ 171.8, 166.7, 157.6, 131.5, 129.5, 122.8, 120.4, 115.6, 72.8, 61.4, 52.1, 18.4, 14.1; mass spectrum, *m/z* (relative intensity) 253 (2.48), 252 (27.4), 221 (9), 179 (100), 135 (18); HRMS, calcd for $C_{13}H_{16}O_5$ 252.0997, found 252.0994.

To a stirred solution of **24** (152 mg, 0.60 mmol) in 6 mL of THF/H_2O was added 1 N NaOH (3.0 equiv, 1.81 mL). The solution was stirred at room temperature for 2 h. The reaction mixture was diluted with H_2O (20 mL), and 1 N HCl (3.0 equiv, 1.81 mL) was added. The water layer was extracted with ethyl acetate (4 × 10 mL) and the combined organic extracts were dried ($MgSO_4$), filtered, and evaporated to give 127 mg (100%) of **26** as a colorless solid: mp 165–166.5 °C; $[\alpha]_D$ +23.8 °C (*c* 1.0, MeOH); IR (neat) 3300, 2291, 1690 cm^{-1}; ^1H NMR ([$^2H_6$]acetone) δ 7.66 (1 H, dd, *J* = 1.5 and 5.8 Hz), 7.56 (1 H, q, *J* = 1.8 Hz), 7.45 (1 H, t, *J* = 5.8 Hz), 7.20 (1 H, ddd, *J* = 6, 2.4, and 1.8 Hz), 4.89 (1 H, q, *J* = 5.4), 1.64 (3 H, 5, *J* = 5.4); ^{13}C NMR δ 172.9, 167.2, 158.8, 132.7, 130.3, 123.2, 120.7, 116.3, 72.8, 18.6; mass spectrum, *m/z* (relative intensity) 211 (6.2), 210 (54.6), 165 (100), 147 (25), 138 (61), 121 (92); HRMS, calcd for $C_{10}H_{10}O_5$ 210.0528, found 210.0528.

Confirmation of Absolute Stereochemistry of **27** *by Synthesis.* To a stirred solution of ethyl (*S*)-lactate (1.0 g, 8.5 mmol) in 60 mL of anhydrous THF were added triphenylphosphine (1.5 equiv, 3.56 g) and acetic acid (1.5 equiv, 779 μL). Diisopropyl azodicarboxylate (1.5 equiv, 2.67 mL) was added over 15 min. The solution was stirred at room temperature for 72 h. The solution was concentrated (water aspirator) to remove THF. Kugelrohr distillation (90 °C, 5 mmHg) yielded 1.05 g of a yellow oil. This oil was added to a solution of sodium ethoxide (1.0 equiv, 8.5 mmol, from 193 mg of sodium metal) in absolute ethanol (100 mL). The ethanolic solution was stirred at room temperature for 2.5 h. Saturated aqueous NH_4Cl solution was added carefully to pH 7. After enough water was added to dissolve the precipitate, the solution was extracted with diethyl ether (5 × 50 mL). The ether extracts were dried ($MgSO_4$), filtered, and evaporated to give 290 mg (29%) of ethyl (*R*)-lactate. From ethyl (*R*)-lactate, the synthesis of **27** was identical with that of **26**. The following were determined: mp 165–167 °C; $[\alpha]_D$ −22.6° (*c* 1, MeOH).

^1H NMR spectra were measured at 250 MHz (Brucker WM-250) or 300 MHz (Varian XL-300), and chemical shift values are reported in parts per million downfield from tetramethylsilane. ^{13}C NMR spectra were measured at 67.9 MHz (Brucker WM-270), and chemical shift values (δ) are reported in parts per million downfield from tetramethylsilane. Unless otherwise indicated, ^1H and ^{13}C NMR spectra were obtained in $CDCl_3$. ^1H NMR spectroscopic monitoring of intermediate accumulation was measured at 300 MHz (Varian XL-300). Mass spectral data were recorded on a Finnegan MAT 8200 mass spectrophotometer at 70 eV.

Intermediate Accumulation: Anthranilate Synthase

UV–Visible Spectroscopic Detection. (*S*)- or (*R*)-lactyl substrates **13** or **14** in the appropriate buffers containing glutamine (assay buffer A) or ammonia (100 mM ammonium sulfate, 50 mM triethanolamine, and 10 mM $MgCl_2$) at various temperatures (4, 22, and 37 °C) was treated with anthranilate synthase (33–200 μg) and monitored by repetitive scanning (1.5- or 3-min repeat time) of the 270–360-nm region.

^1H NMR Spectroscopic Monitoring. Anthranilate synthase

(1 mL of 3.3 mg/mL) in D_2O buffer (100 mM ammonium sulfate, 50 mM Tris, and 10 mM $MgCl_2$, pD 8.2) was obtained by four successive dilutions and ultrafiltration steps with the deuteriated buffer. A solution of (*R*)-lactyl substrate **14** (20 mM) in the deuteriated buffer (0.6 mL) containing anthranilate synthase (0.82 mg) was monitored by ^1H NMR at 300 MHz (37 °C).

HPLC Isolation of Accumulated Intermediate. After 10 h the deuteriated assay mixture used during the NMR experiment was passed through a PM-10 membrane, and the filtrate was analyzed and purified by HPLC [5% aqueous acetic acid; C-18 μBondapak, 3.9 × 150 mm; 1.5 mL/min; UV detection (254 nm)].

Characterization of Accumulated Intermediate. The species isolated by HPLC was characterized by UV–visible analysis (5% acetic acid, 250–400 nm) and ^1H NMR (CD_3OD and D_2O) at 300 MHz: UV–vis λ_{max} 280 nm, ϵ 5670 (5% aqueous acetic acid); ^1H NMR (D_2O) δ 6.96 (1 H), 6.34 (2 H), 4.18 (br s, 1 H), 1.42 (br s, 3 H) (other signals obscured by HOD).

Analytical Methods. Protein concentrations were determined by the Bio-Rad Bradford assay procedure (Bradford, 1976). Denaturing polyacrylamide gels were run according to the Laemmli procedure (Laemmli, 1970) with either 10 or 12% separating gels with a 3% stacking gel. Amino-terminal analyses were carried out by Harvard Microchem (Cambridge, MA). UV–visible spectra were measured on Perkin-Elmer Model 554 or Lambda 5 instruments. Fluorescence measurements were done on a Perkin-Elmer LS-3 fluorescence spectrometer.

Results and Discussion

Production and Purification of Enzymes

In order to initiate our study on the biosynthesis of anthranilate and PABA from chorismate, a source of reasonably pure anthranilate and PABA synthases was required. Anthranilate synthase from *S. marcescens* is readily available from plasmid pJP20 and is easily purified to homogeneity (Zalkin, 1985; see Experimental Procedures). However, no purification of PABS has yet been reported due to enzyme instability (B. Nichols, private communication). We began work on the purification of the large subunit of PABS pabB gene product from *E. coli* encoded by plasmid pAS4 in *E. coli* BN116 (provided by B. Nichols), which has been the best available source of this enzyme (Teng et al., 1985) by crude extract assay.

Initial purification attempts proceeded on 15-g quantities of *E. coli* BN116 and involved disruption by French press and *immediate* ion-exchange chromatography (DEAE-Sepharose CL-6B) followed by gel filtration on Sephadex G-150. Because of the essentially quantitative losses of activity, an alternative procedure for obtaining stable active enzyme was required. One possible solution at this point appeared to be to construct a plasmid that would produce PABS at substantially higher percentages of the total cell protein and thus perhaps facilitate the isolation and purification process.

Construction of pabB Overproducing Plasmid. A 4.3-kb fragment containing the pabB gene was obtained from *Bam*HI/*Pst*I digestion of plasmid pAS4 isolated from *E. coli* BN116. This fragment did not contain the trp promoter region introduced by Nichols in pAS4, although the putative regulatory region upstream from pabB could not be removed by this procedure. This fragment was then subcloned into plasmid pT7-3 bearing the T7 promoter (Tabor & Richardson, 1985). Replacement of the trp promoter with the T7 promoter was carried out since T7 RNA polymerase (produced from pGP1-2 also present in these cells) does not recognize *E. coli* termination sequences and thus might not be affected by the putative pabB regulatory region. The resultant plasmid pBC1 was then transformed into *E. coli* K38(pGP1-2), and colonies were selected on kan/amp plates (Figure 1).

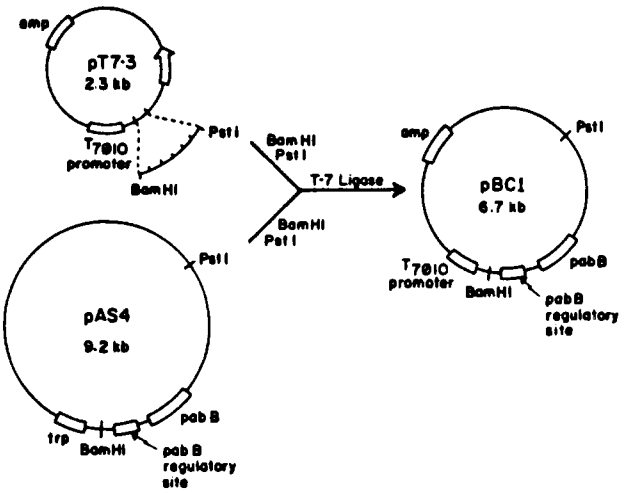


FIGURE 1: Construction of plasmid pBC-1. The section of pAS4 containing pabB (with its regulatory region) was subcloned into pT7-3 downstream from the T7ϕ10 promoter. The procedures used are described under Methods.

Optimization of PABS production was next carried out by investigating the effects of several experimental variables during cell growth on specific activity. The resultant optimal conditions involved growth at 30 °C to midlog phase, heat shock (42 °C) for 20 min, subsequent addition of rifampicin (200 mg/L) at 30 °C, and final incubation at 30 °C for 7 h. Crude cell extracts (200 mg of cells) from sonication displayed specific activities (nmol min^{-1} mg^{-1}) of 32, 56, and 270 for uninduced, heat-shocked, and heat shock/rifampicin-treated cells. Although the specific activity obtained under optimal conditions is only marginally better than that obtained from pabB under trp promoter control in pAS4, we chose to use this strain for further work since the PAGE protein profile of crude extracts appeared substantially less complex, particularly in the 50-kDa region of the PABS large subunit.

Purification of PABA Synthase. Before beginning further attempts at purifying the pabB-encoded subunit of PABS from pBC1, enzymatic activity was monitored under a variety of conditions in crude cell extract obtained from sonication. It immediately became clear that the large subunit of this enzyme was much more stable than previously thought. For example, no activity was lost on standing at 4 °C for 20 h in phosphate buffer B at a variety of pHs (6.8–8.0) nor at 4 h at room temperature (pH 7.6). Only in Tris buffer (pH 8.0) was activity lost after 20 h at 4 °C (ca. 50%). Addition of protease inhibitors either before or after sonication did not result in any changes in specific activity, indicating that proteolytic degradation is not a problem. Thus, the PABS large subunit is apparently quite stable on standing, even in crude cell extract.

Purification proceeded as described under Experimental Procedures (see Table I). Most notable was that inclusion of 7.5% glycerol during DEAE-Sepharose chromatography lowered enzyme yield from <40% to less than 1%. This suggests that rather than stabilizing enzyme activity as observed for anthranilate synthase component I (Nagano & Zalkin, 1970) the glycerol actually results in *essentially quantitative loss* of pabB enzyme activity. Complete recovery of the enzyme units in the subsequent Sephadex G-150 column was observed, concomitant with a ca. 5-fold increase in specific activity. PAGE of the individual fractions indicated that the

Table I: Purification Data for PABA Synthase (pabB Subunit)

purification step	protein (mg)[a]	total activity (units)[b]	specific activity (units/mg)	yield (%)	x-fold purification
(1) crude extract and streptomycin precipitation	392	52	0.13		
(2) ammonium sulfate precipitation (35–70%)	258	34[c]	0.13	65	0
(3) DEAE-Sepharose column	18	7.6	0.42	22	3.2
(4) Sephadex G-150 column	5	5.9	1.2	11	9.2

[a] Protein determination was performed by using the Bio-Rad protein assay with BSA and lysozyme as standards. [b] One unit is the amount of enzyme required to produce 1 nmol of PABA in 30 min under the conditions described under Methods. [c] Thirty percent of the total cell extract units were present in the 0–35% ammonium sulfate cut.

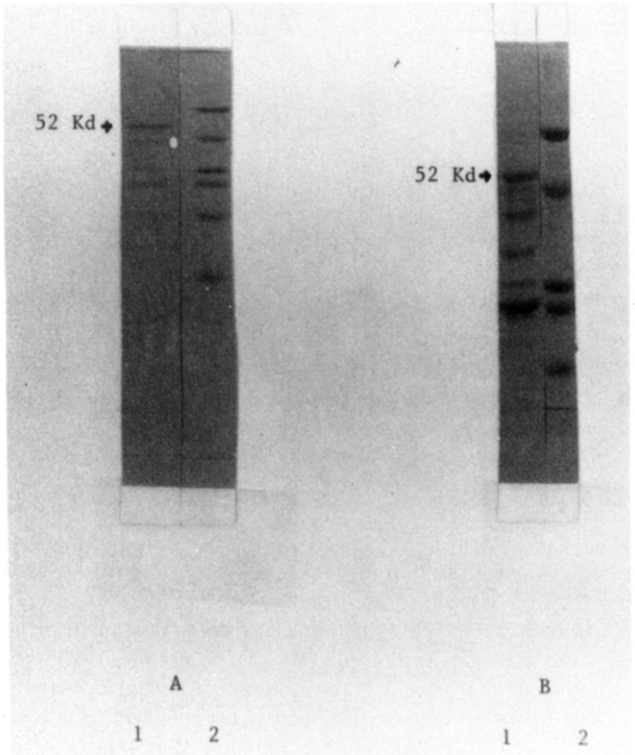


FIGURE 2: Polyacrylamide gel electrophoresis analysis of PABA synthase. Gels were prepared as described under Methods [(A) 12% running gel; (B) 10% running gel]. (A) (Lane 1) Protein after FPLC purification (see Results and Discussion). The band at 52 kDa corresponds to PABA synthase. (Lane I) Molecular mass standards (66, 45, 36, 29, 24, and 20.1 kDa). (B) (Lane 1) Protein after Sephadex G-150 column. The band at 52 kDa corresponds to PABA synthase, as ascertained by amino-terminal sequence analysis (see Methods). (Lane 2) Molecular mass standards (66, 45, 29, 24, and 20.1 kDa).

band corresponding to PABS (52000 daltons) was present at ca. 30% of the total protein (Figure 2).

Further attempts to purify the enzyme on dye-affinity and chromatofocusing columns were unsuccessful due to nearly complete loss of PABS activity. Initial experiments using FPLC (mono-Q column) did result in some further purification (Figure 2); however, this procedure did not lead to homogeneous protein, and approximately 50% of the units were lost during this step. At this point the material from gel filtration was of sufficient purity to proceed with the mechanistic studies, and further purification has not yet been pursued.

Table I summarizes a 9-fold increase in specific activity from crude extract through G-150 gel filtration with an 11% overall recovery. From the data in Table I and the V_{max} of 4.6 nmol min^{-1} mg^{-1} derived from a Lineweaver–Burk plot (data not shown), the V_{max} and turnover number for pure active pabB subunit of the PABS enzyme can now be estimated. From the gel shown in Figure 2, we estimate a purity of 25% for the large subunit at 52K (correct N-terminal sequence as noted under Methods) and would thus calculate a V_{max} of 19 nmol

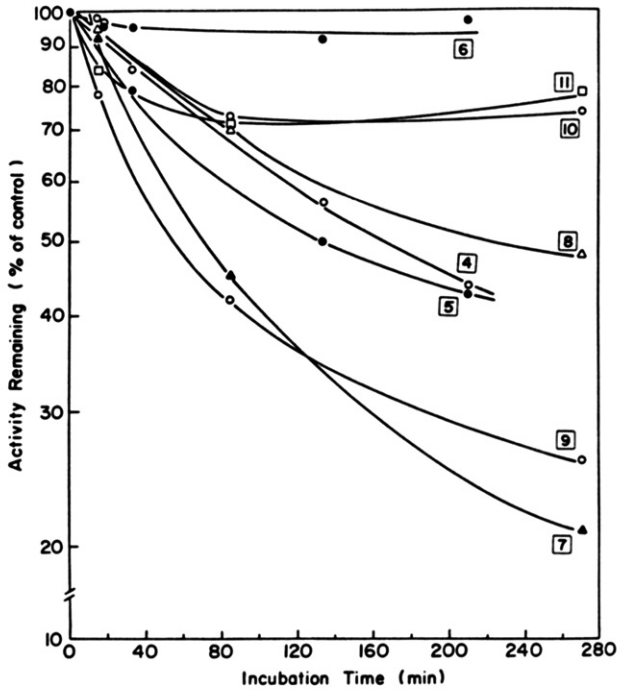


FIGURE 3: Time-dependent inactivation studies of anthranilate synthase. Percent of control activity remaining is plotted vs. time of incubation with compounds **4–11**. Incubations were carried out as described under Methods, and activity was measured according to the standard fluorescence assay (Methods).

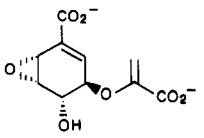
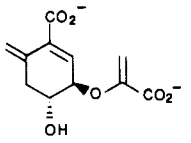
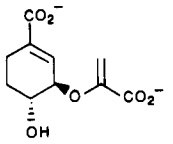
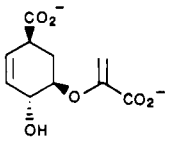
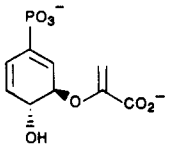
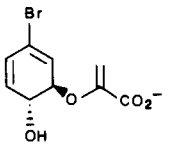
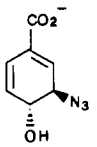
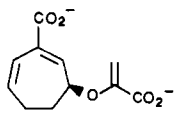
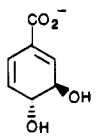
min^{-1} mg^{-1} not correcting for activity loss. Pure, fully active 52K subunit would have an estimated V_{max} = 49 nmol min^{-1} mg^{-1} (turnover number = 2.6 min^{-1}), correcting for the 38% overall recovery of units during the ion-exchange and gel filtration steps. Tandem expression of genes for both the small and large subunits may produce both a more stable and higher activity holoenzyme. Nonetheless, this represents the first significant purification yet reported for PABS and has allowed initial mechanistic studies.

Mechanistic Studies

Previous workers (Levin & Sprinson, 1964) have suggested that a first step in the anthranilate synthase reaction would be generation of intermediate **2** from chorismate and glutamine in an animation sequence (Scheme II). Similarly, for PABS the corresponding regioisomer **3** had been suggested as a possible intermediate by Haslam (1974) and by Dardenne et al. (1975) (Scheme III). In the second (aromatization) step syn elimination of enolpyruvate from either intermediate **2** or intermediate **3** results in formation of the respective aminobenzoate isomer.

To probe the proposed mechanism outlined above and to delineate pertinent active site structural features, a variety of chorismate analogues were designed and tested as potential mechanism-based inactivators (to capture reactive active site nucleophiles) or inhibitors of both synthases. Specific differences in inactivation or inhibition behavior between the two

Table II

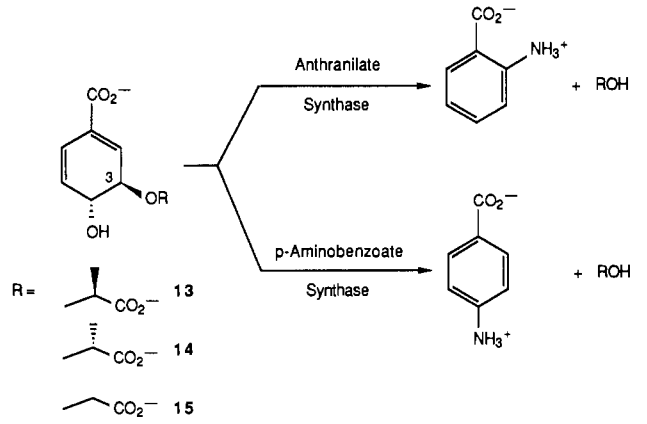
compound	no.	inactivation of PABS (% of control)[a,b]	inhibition of PABS, K_i (μM)[c]	inhibition of AS, K_i (μM), type[d]
	4	70	700	350, comp
	5	79	1100	4000, non
	6	97	860	570, comp
	7	96	2140	1500, comp
	8	69	1100	120, comp
	9	67	450	320, non
	10	75	430	960
	11	84	230	30, comp
	12			>5000

[a] PABA synthase was preincubated in the presence of the chorismate analogue (2 mM) for 90 min and then diluted 10-fold into assay buffer B containing chorismate ($5K_m$). The residual activity was measured after another 90-min incubation. [b] Percents were not corrected for any reversible inhibition that occurs at 200 μM. [c] Values were obtained from measuring PABA synthase activity after a 75-min incubation in the presence of chorismate (84 μM) and inhibitor (1 mM). The K_i was then calculated as described under Methods. [d] Data were obtained from a Lineweaver–Burk plot except for compounds **10** and **12**, which were analyzed from only one point with the reported K_i's in these cases requiring the assumption that inhibition was competitive. comp, competitive; non, noncompetitive.

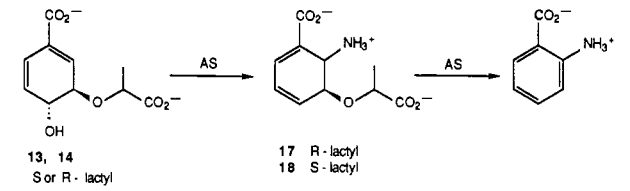
synthases might also yield information regarding the basis of regiospecificity in the amination reactions.

Inactivation and Inhibition Data. Data from studies to probe for inactivation of both AS and PABS are presented in Figure 3 and Table II, respectively. None of the compounds tested gave the first-order loss of activity expected for formation of an irreversibly inactivated enzyme species. These data are particularly surprising for compounds **4**–**7** since they present potential targets for trapping an enzyme active site nucleophile.

Scheme IV



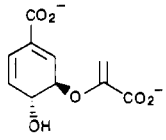
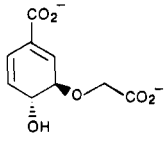
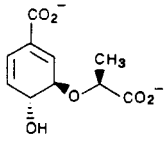
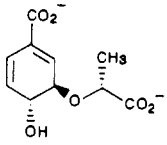
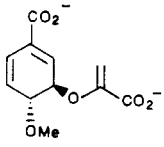
Scheme V



Additional data regarding the active site requirements for binding of substrates were obtained from the inhibition data for a variety of chorismate analogues (Table II). Several conclusions may be drawn. First, it appears that a negatively charged moiety at C-1 and C-8 is required for binding since the phosphonate **8** is a fair inhibitor while no inhibition was observed with diol **12**. Second, there is an apparent requirement for planarity around C-5–C-6–C-1–C-2 as evidenced by the lack of significant inhibition by the dihydrochorismate analogues **5** and **6**. The most potent reversible inhibition was observed with the cycloheptadienyl compound **11** (K_i = 30 μM for a racemic mixture vs. K_m = 6 μM for chorismate), which lacks the C-4 hydroxyl group present in chorismate. Apparently the planarity of the diene moiety, coupled with the presence of the two carboxylates, is sufficient for strong binding. Finally, compounds without the carboxyl at C-1 (e.g., **9**) or with steric bulk at C-6 (e.g., **5**) are not bound at the active site but rather function as noncompetitive inhibitors (data not shown) with anthranilate synthase, presumably via binding to the tryptophan regulatory site (Zalkin & Hwang, 1971). While the data for PABS are not as refined as that for AS, it is apparent that similar trends in inhibitory capacity are followed; again, the seven-membered ring has the best K_i (230 μM vs. a chorismate K_m = 42 μM), but some 27 times less affinity than seen against AS holoenzyme.

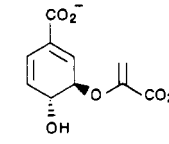
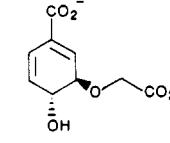
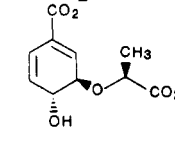
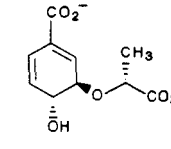
Alternative Substrates. The two- (or more) step conversion of chorismate to anthranilate suggests the intermediacy of amino enolpyruvate **2**, which was synthesized recently and found to be kinetically viable as an intermediate (Policastro et al., 1984; Teng & Ganem, 1984). Our approach involves detection and isolation of such an intermediate during the enzymic reaction. Since we saw no evidence for such an intermediate using the natural chorismate substrate, we have designed, prepared, and tested a series of alternate substrates for anthranilate synthase (Scheme IV). The diastereomeric lactyl chorismate analogues (*S*)-**13** and (*R*)-**14** and the glycolyl compound **15** were chosen with the expectation that the poorer leaving group at C-3 might slow the aromatization step suf-

Table III: Steady-State Parameters for Turnover of Chorismate Analogues by AS

entry	substrate	no.	K_m (μM)	K_m relative to chorismate	V_{max} (nmol min^{-1} mg^{-1})	turnover (min^{-1})	V_{max} (% of chorismate)	V/K	$(V/K)_{chorismate}/(V/K)$
1		**1**	5.6[a]		3272[a]	266		584	
2		**15**	66.5	12×	482	39	14.7	7.2	81
3	 (S)	**13**	17.2	3.1×	145	12	4.4	8.4	69
4	 (R)	**14**	150	27×	100	8.1	3.0	0.67	872
5		**16**	420	75×	1.2	0.1	0.4	0.003	195 000

[a] The K_m and V_{max} reported by Zalkin and Hwang (1971) were 3.6 μM and 3400 nmol min^{-1} mg^{-1}, respectively.

Table IV: Steady-State Parameters for Turnover of Chorismate Analogues by PABA Synthase

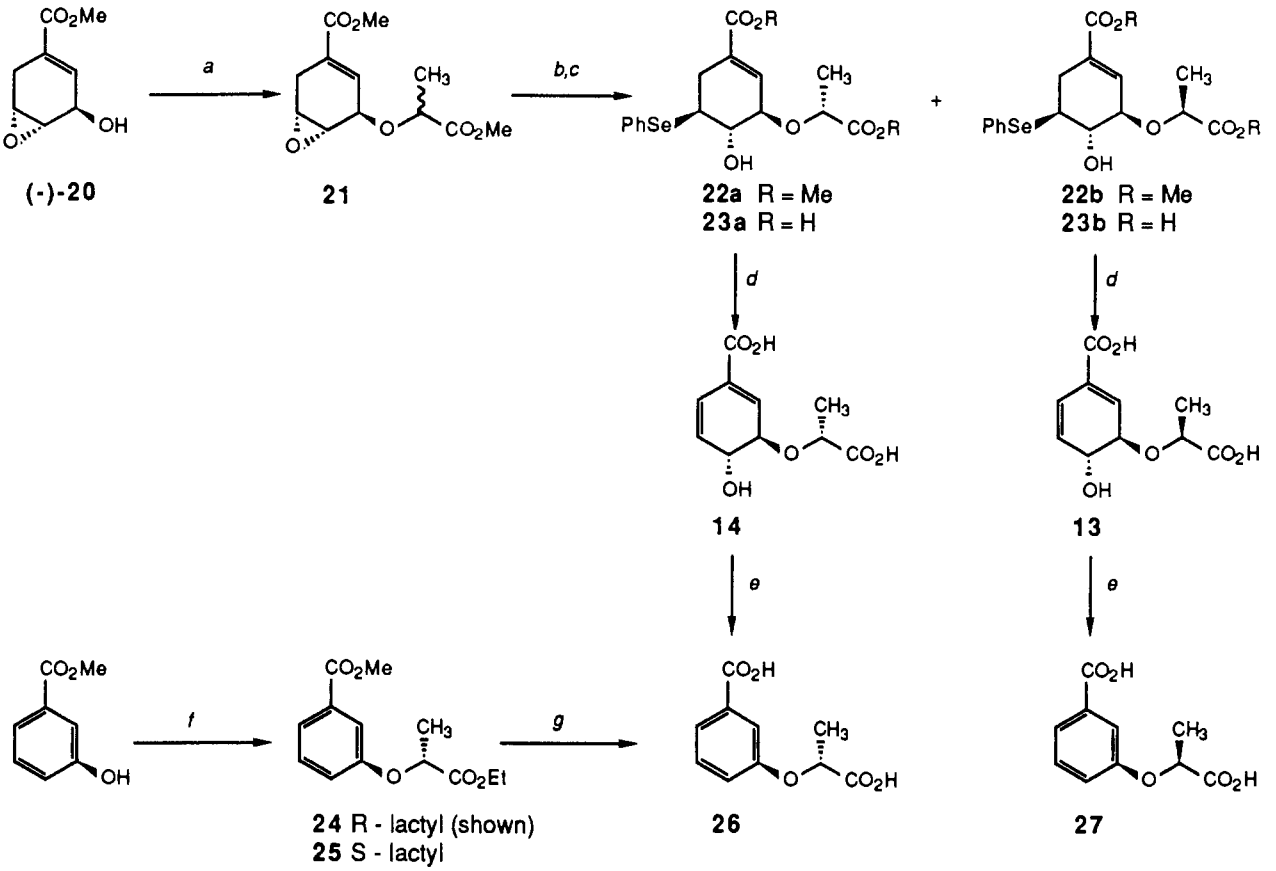
entry	substrate	no.	K_m (μM)	K_m relative to chorismate	V_{max} (pmol min^{-1} unit^{-1})[a]	V_{max} (% of chorismate)	V/K	$(V/K)_{chorismate}/(V/K)$
1		**1**	42		121[b]		2.9	
2		**15**	1250	30×	165	140	0.13	22
3	 (S)	**13**	645	15×	6.5	5.4	0.01	290
4	 (R)	**14**	555	13×	16	13	0.03	97

[a] One unit is the amount of enzyme required to produce 1 nmol of PABA in 30 min at 37 °C. Using this definition, Teng et al. (1985) found the K_m and V_{max} for chorismate to be 12 μM and 52 pmol min^{-1} unit^{-1}, respectively. [b] This value corresponds approximately to a V_{max} of 49 nmol min^{-1} mg^{-1} (2.6 min^{-1}; see text for discussion).

ficiently to allow accumulation of the expected intermediate (i.e., **17** or **18**) corresponding to **2** (Scheme V).

The synthesis of the lactyl chorismate analogues **13** and **14** began with optically active epoxy alcohol (−)-**20** (Pawlak & Berchtold, 1987) (Scheme VI). Rhodium-mediated reaction of **20** with methyl α-diazopropionate (**19**) gave a diastereomeric mixture of ethers **21**. Epoxide opening with phenylselenide anion afforded alcohols **22a** and **22b**, which were

Scheme VI[a]



[a] Reagents: (a) $CH_3C(N_2)CO_2Me$, $Rh_2(Oct)_4$, benzene, RT; (b) PhSeSePh, $NaBH_4$, MeOH; (c) NaOH, THF:H_2O; (d) H_2O_2, dimethoxyaniline, acetone, −35 °C–RT; (e) Me_2SO, 50 °C; (f) $CH_3CH(OMs)CO_2Et$, K_2CO_3, acetone, reflux; (g) NaOH, THF:H_2O.

separated by flash chromatography. Ester hydrolysis with aqueous sodium hydroxide followed by oxidative selenide elimination gave **13** and **14**, as summarized in Scheme VI. The *R* and *S* stereochemical assignment at the lactyl carbinol was validated by aromatization and comparison with independently synthesized samples (**26** and **27**) as described under Methods.

Summarized in Tables III and IV are data for the turnover of alternative substrates with AS and PABS. The compounds were turned over to the corresponding aminobenzoate with saturation kinetics displayed by each enzyme. Clearly, however, the turnover numbers for (*S*)- and (*R*)-lactyl analogues **13** and **14** by both AS and PABS are more than an order of magnitude lower than that of chorismate, suggesting that the aromatization step, with elimination of the poorer leaving group lactate rather than enolpyruvate, may now be the rate-limiting step during turnover. Also of interest are the V/K data for the two lactyl substrates, which suggest that the AS and PABS active sites may have opposite selectivities for the stereochemistry of the lactyl side chain. Thus, AS prefers the (*S*)-lactyl isomer $[(V/K_{chorismate})/(V/K_{lactyl}) = 70]$ over (*R*)-lactyl $[(V/K_{chorismate})/(V/K_{lactyl}) = 870]$ by some 12-fold, but PABS shows opposite selectivity by V/K criteria (290 vs. 100, respectively).

The glycolate analogue **15** is also turned over by both enzymes with surprisingly good V/K values. Perhaps most interesting is the observation that the V_{max} value of **15** with PABS is *actually higher* (140%) than that of chorismate while V_{max} of **15** with AS is substantially lower (15%) than that of chorismate. Since glycolate is a poorer leaving group than pyruvate, these data suggest that aromatization is rate limiting for AS but not for PABS. Further support in this hypothesis is the fact that **4** is processed to PABA at 20 times the rate of chorismate (Teng et al., 1985). Similarly, **2** is processed to anthranilate at only 1.2 times the rate of chorismate (Policastro et al., 1984). Thus, for PABS, formation of **4** appears to be the rate-limiting step while for AS; processing of **2** to anthranilate is rate limiting.

Finally, compound **16** with an *O*-methoxyl instead of hydroxyl leaving group at C-4 was examined as a substrate of AS. Since the leaving group at C-3 is the same as in chorismate, the much lower V_{max} (0.4% of chorismate V_{max}; Table III) in this case may reflect a decrease in the rate of the initial amination sequence to **2**.

The possibility that substitution of the C-3 pyruvyl side chain with a lactyl moiety in **13** and **14** may have slowed the aromatization step in turnover, thus making it rate limiting, prompted a search for accumulation of the corresponding amino intermediate **17** (Scheme V) with the more readily available anthranilate synthase.

Detection of Aminocyclohexadienyl Intermediate **17**. Evidence in support of the intermediacy of **17** and **18** during the turnover of **13** and **14** with AS was obtained by monitoring the course of the enzymatic reaction by UV–vis spectroscopy. The overall characteristics of the 360–265-nm region during the reaction were consistent with formation of anthranilate ($\lambda_{max} = 312$) at the rate predicted from the V_{max} values (Table III). During the course of the reaction a loss of the 273-nm peak arising from **13** was seen along with formation of a transient UV absorption at 280 nm (Figure 4). This shift in λ_{max} is consistent with formation of the amino intermediate **18** on the basis of the $\lambda_{max} = 280$ nm observed for the aminopyruvate **2** (Policastro et al., 1984). A similar absorption shift was observed when the turnover of the (*R*)-lactyl isomer **14** was monitored and when ammonia was used in place of

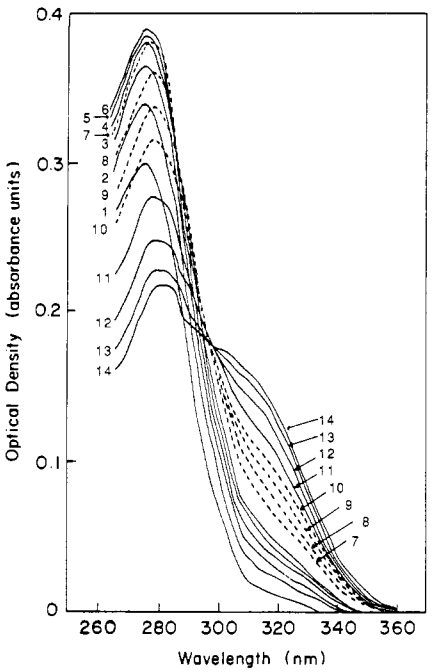


FIGURE 4: UV–vis monitoring of intermediate accumulation with anthranilate synthase. Assay buffer A containing 100 μM (*S*)-lactyl substrate **13** was cooled to 4 °C and treated with anthranilate synthase (198 μg). The optical spectrum was monitored (360–265 nm) at the various intervals indicated below. The scan speed was 120 nm/min; the temperature was held constant at 4 °C for the duration of the experiment. Traces on the graph refer to the following (curve number, time in minutes): 1, no enzyme; 2, T_0 (add enzyme); 3, T = 1.5; 4, T = 3; 5, T = 4.5; 6, T = 6.0; 7, T = 9.0; 8, T = 12.0; 9, T = 15.0; 10, T = 18.0; 11, T = 24.0; 12, T = 30.0; 13, T = 36.0; 14, T = 40.5.

glutamine. However, no such absorption was detected during turnover of chorismate. Finally, we examined the temperature dependence (4, 23, and 37 °C) of formation of the 280-nm peak. At 4 °C the intermediate 280-nm absorption persists for several minutes, coinciding with a decrease in the 273-nm peak. From the trace in Figure 4, it is seen that the 273-nm absorption slowly shifts to 280 nm during which an initial increase in absorption is observed, consistent with the larger ϵ value predicted for the 280-nm absorption of aminocyclohexadienyl lactate **18**.

Further evidence in support of **17** (*R* isomer) as the structure of the accumulating intermediate is the ^{1}H NMR spectrum of the enzymatic reaction of **14** (*R* isomer), in ^2H_2O, which clearly shows the accumulation of a compound that corresponds to structure **17**. The compound accumulates to approximately 15 mol % of the total assay mixture during the experiment. The signals at δ 6.95 and 6.35 are very similar to those found in **2** (Policastro et al., 1984). ^{1}H–^{1}H decoupling clearly shows these signals to be coupled. Furthermore, one can observe three separate lactyl carbinol protons, corresponding to a mixture of **14**, **17**, and free lactate (coproduct with anthranilate). HPLC analysis of this mixture yielded three major components with retention times of 3.4, 4.6, and 6.5 min. The peaks at 4.6 and 6.5 min corresponded to authentic samples of **14** and anthranilate, respectively. The third peak (rt = 3.4 min) was collected and the UV spectrum of the eluent had a λ_{max} = 280 nm. Lyophilization yielded one compound, with the expected NMR signals.

The compound was taken up in water and used for kinetic experiments with anthranilate synthase. As anticipated for a reaction intermediate, the compound was found, by UV assay, to be converted completely to anthranilate by the enzyme, with or without an ammonia source; this last fact ensures the presence of an amino group in the compound. The quantitative enzymic processing allowed the determination of the total concentration of compound in the stock solution and yielded the E_{280} of 5670 M^{-1} cm, in analogy to the UV spectrum of **2**. The compound showed normal saturation kinetics with anthranilate synthase; but because of the limited quantity of compound available, the molar concentration of the compound (4–25 μM) was only 5–30 times the molar concentration of AS (0.8 μM) in the kinetics experiments. The precision of the velocity measurements is subject to question. In one set of experiments, the V_{max} for conversion to anthranilate was only 10% that for the starting lactyl substrate **14** (100 nm min^{-1} mg^{-1}), but in a second set of studies the V_{max} was 80% that of **14**. The reasons for the wide variation are as yet unclear, but the latter result suggests the intermediate may be kinetically competent.

The issue of kinetic incompetence/marginal kinetic competence raises the questions that the compound isolated was not **17** or that **17** may be a spurious byproduct, but these are considered unlikely. It is possible that the enzyme may require some type of activation when it starts with species **17**, but on the other hand, intermediate **2** was kinetically competent with AS I in the presence of NH_4^+ (Policastro et al., 1984).

The characterization of the intermediate is consistent with the expected structure **17**. The UV and NMR data support the presence of a 1,3-diene system. The ^{1}H NMR spectrum also establishes that the lactyl side chain is intact. The fact that the compound is metabolized quantitatively to anthranilate in the absence of an ammonia source indicates that the intermediate does contain an $-NH_2$ group. The only ambiguity left in the proposed structure is the stereochemistry at C-2. The possibility of epimerization at C-2 upon isolation, yielding a kinetically incompetent cis-2,3 isomer was considered as an explanation for the observed results. Dihydroaromatic systems such as **17** are, however, very prone to acid- or base-catalyzed aromatization. Intermediate **17** would be expected to epimerize at a rate comparable to or slower than the rate of aromatization (since both processes would occur via similar intermediates). Since *no* aromatization was observed after isolation from the enzymic incubation, epimerization seems an unlikely explanation. There is still the possibility that addition of NH_3 to **14** is not stereospecific, and the *cis*-2-amino-3-lactylcyclohexadiene accumulates and is then only slowly converted to anthranilate. Because of obscuring HOD peaks, C-2 stereochemistry could not be determined by NMR to date.

A second possible explanation is that we have indeed isolated **17**, but it is not along the major anthranilate synthase reaction pathway. This seems less likely given the kinetic competence of synthetic **2** (Policastro et al., 1984) with AS and synthetic **4** with PABS. Additional studies are neede to resolve these issues.

SUMMARY

The partial purification of PABS reported herein was achieved via two steps, with protein derived from an overproducing plasmid containing the pabB gene under T7 promoter control. The similar but distinct reactions catalyzed by PABS and AS were then probed by a variety of methods. Investigation of the initial amination step proceeded by testing for inactivation of the enzymes by chorismate analogues that could potentially accept an active site nucleophile at C-6 prior to the addition of ammonia.

The aromatization step was also examined by replacing the C-3 pyruvyl side chain with lactyl and glycolyl substituents. The decreased turnover numbers observed for the lactyl substrates relative to chorismate suggested that the aromatization

step was now rate limiting. This observation prompted the search for accumulation of the putative amino intermediate **17** by UV–vis and NMR spectroscopy and HPLC isolation of a species that has the anticipated structure. **17** is quantitatively chemically competent but under present conditions kinetically at best marginally competent in subsequent enzyme conversion to anthranilate by anthranilate synthase.

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Registry No. **1**, 617-12-9; **4**, 108646-26-0; **5**, 108562-56-7; **6**, 108646-24-8; **7**, 108562-51-2; **8**, 108562-52-3; **9**, 108562-53-4; **10**, 108562-54-5; **11**, 108562-55-6; **12**, 108646-25-9; **13**, 108646-22-6; **14**, 108562-47-6; **15**, 108646-23-7; **16**, 108562-50-1; **18**, 108562-57-8; **19**, 34757-14-7; (−)-**20**, 34757-14-7; **21** (isomer 1), 108562-45-4; **21** (isomer 2), 108646-20-4; **22a**, 108562-46-5; **22b**, 108646-21-5; **23a**, 108594-49-6; **23b**, 108647-59-2; **26**, 108562-48-7; **27**, 108562-49-8; PABS, 9059-52-3; AS, 9031-59-8; DL-AlaOMe·HCl, 13515-97-4; *i*-Pr$(CH_2)_2NO_2$, 110-46-3.

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Stereochemical Analysis of Prenyltransferase Reactions Leading to (*Z*)- and (*E*)-Polyprenyl Chains†

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ABSTRACT: A feasible method was developed to determine the stereochemical direction of the C–C bond formation with respect to the face of the double bond of isopentenyl diphosphate in the prenyltransferase reactions. This method was applied to the reactions of undecaprenyl diphosphate synthase and heptaprenyl diphosphate synthase, which catalyze (*Z*)-prenyl chain elongation and (*E*)-prenyl chain elongation, respectively. In both cases, the C–C bond formation was found to take place at the *si* face of the double bond with elimination of one of the hydrogens of C-2 in a syn fashion.

Among a variety of biochemical reactions, cryptic stereochemistry involved in the conversion between prochiral molecules is interesting from a viewpoint of comparative biochemistry as well as enzymatic reaction mechanism. The chain elongation reactions of isoprenoid biosynthesis have to some extent been studied from such a viewpoint.

Prenyltransferase is the enzyme that catalyzes the chain elongation by sequential condensation of IPP[1] with allylic diphosphates to give a product with a certain chain length and configuration. More than 10 enzymes with different specificities have so far been separated from various organisms.

Since the stereochemistry of farnesyl diphosphate synthase reaction was first studied by Cornforth et al. (1966a), the stereochemistry of the hydrogen elimination from C-2 of IPP has been well elucidated with various prenyltransferases

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[1] Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, (2*E*,6*E*)-farnesyl diphosphate; Tris, tris(hydroxymethyl)aminomethane; MS, mass spectrometry; NMR, nuclear magnetic resonance; ORD, optical rotatory dispersion; CD, circular dichroism; HPLC, high-performance liquid chromatography.

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