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Stereochemical Outcome at Four Stereogenic Centers During Conversion of Prephenate to Tetrahydrotyrosine by BacABGF in the Bacilysin Pathway†

Jared B. Parker and Christopher T. Walsh*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave, Boston, MA 02115

Abstract

The first four enzymes of the bacilysin antibiotic pathway, BacABGF, convert prephenate to a tetrahydrotyrosine (H₄Tyr) diastereomer on the way to the anticapsin warhead of the dipeptide antibiotic. BacB takes the BacA product *endocyclic*- Δ^4,Δ^8 -7*R*-dihydrohydroxyphenylpyruvate (*en*-H₂HPP) and generates a mixture of 3*E*- and 3*Z*-olefins of the *exocyclic*- Δ^3,Δ^5 -dihydrohydroxyphenylpyruvate (*ex*-H₂HPP). The NADH-utilizing BacG then catalyzes a conjugate reduction, adding a pro-*S* hydride equivalent to C₄ to yield tetrahydrohydroxyphenylpyruvate (H₄HPP), a transamination away (via BacF) from 2*S*-H₄Tyr. Incubations of the pathway enzymes in D₂O yield deuterium incorporation at C₈ from BacA and then C₉ from BacB action. By ¹H-NMR analysis on samples of H₄Tyr, the stereochemistry at C₄, C₈ and C₉ can be assigned. BacG (followed by BacF) converts 3*E*-*ex*-H₂HPP to 2*S*, **4*R***, 7*R*-H₄Tyr. The 3*Z* isomer is instead reduced and transaminated to the opposite diastereomer at C₄: 2*S*, **4*S***, 7*R*-H₄Tyr. Given that bacilysin has the 2*S*, 4*S* stereochemistry in its anticapsin moiety, it is likely that the 2*S*, 4*S*-H₄Tyr is the diastereomer “on pathway”. NMR determination of the stereochemistry of the CHD samples at C₈ and C₉ allow assignment of all stereogenic centers (except C₃) in this unusual tetrahydro- aromatic amino acid building block, giving insights and constraints on the BacA, BacB, and BacG mechanisms.

INTRODUCTION

The dipeptide antibiotic bacilysin, produced by strains of *Bacillus subtilis*, consists of L-alanine in amide linkage to the nonproteinogenic amino acid anticapsin ¹. Anticapsin is 3-epoxycyclohexanonyl-alanine and bears the reactive epoxyketone warhead that covalently captures the active site cysteine thiolate in the glutaminase domain of the enzyme glucosamine-6-P synthase ². Blockade of this enzyme shuts down production of GlcNAc units required for both bacterial and fungal cell wall assembly ^{3,4}. The producing *B. subtilis* ligates anticapsin to L-Ala to protect itself, and then exports the resultant bacilysin as a prodrug. Uptake by a dipeptide permease transporter of a neighboring microbial cell followed by dipeptidase action liberates anticapsin in a neighboring victim cell (Figure 1).

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*To whom correspondence should be addressed: Christopher_Walsh@hms.harvard.edu; Phone: 617.432.1715; Fax: 617.432.0483.

SUPPORTING INFORMATION AVAILABLE

HPLC analysis of *ex*-H₂HPP isomer purity; ¹H-NMR spectra of 4*R*- and 4*S*-²H-NADH; ¹H-NMR spectra of H₄HPP isomers synthesized from ²H-NADH; Chiral HPLC analysis of BacF generated tyrosine; additional NMR spectra of deuterated forms of *ex*-H₂HPP, H₄HPP, and H₄Tyr isomers; and tabulated NMR spectral data for H₄HPP and H₄Tyr isomers. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The biosynthetic pathway to anticapsin, and then bacilysin, has been studied genetically to reveal a cluster of five *bacA-E* genes ^{5, 6}, and more recently at the enzyme level to decipher the molecular logic for construction of the epoxycyclohexanone moiety of this unusual amino acid ⁷. The biochemical studies have shown that *ywfGH*, the latter transcribed on the opposite DNA strand from *bacA-E*, encode an NADH-dependent reductase (*ywfH*) and an L-specific transaminase (*ywfG*) that carry out steps three and four of the pathway. Therefore we rename the genes *bacF* (*ywfG*) and *bacG* (*ywfH*) and use that nomenclature throughout this manuscript (Figure 2). In sum, the set of four enzymes BacABGF, in that order, act in tandem to divert some of the cellular flux of prephenate away from Phe and Tyr biosynthesis and into the anticapsin pathway, yielding tetrahydrotyrosine (H₄Tyr) as an intermediate ⁷.

We have previously determined that BacA decarboxylates prephenate without aromatization ⁷ and in the process specifically isomerizes the pro-*R* double bond in the 1,4-diene system to yield the *endocyclic* Δ^4, Δ^8 -7*R*-dihydrohydroxyphenylpyruvate (*en*-H₂HPP) ⁸. The next enzyme, BacB, carries out an allylic isomerization to move the double bond into conjugation at the exocyclic Δ^3 -position (*ex*-H₂HPP). In so doing it generates a 3/1 mixture of the *E*- and *Z*-geometric isomers at the Δ^3 -olefin upon equilibration. The third enzyme, BacG, uses NADH to reduce that conjugated exocyclic double bond and yield an H₄HPP product ⁷. The fourth enzyme, BacF, is a transaminase and uses L-Phe as an amino donor to reductively aminate the 2-keto group of H₄HPP and yields an H₄Tyr diastereomer (Figure 2).

The H₄Tyr is presumably two steps away, epoxidation of the olefin and oxidation of the C₇-OH to the ketone, from anticapsin. H₄Tyr, an unusual noncanonical amino acid, has three stereocenters: C₂, C₄, and C₇. As shown below, BacF action, with L-Phe as cosubstrate, sets the *S*-stereochemistry at C₂ and we have previously established ⁸ that BacA sets *R*-stereochemistry at C₇, which is carried through to H₄Tyr.

At issue then is the stereochemistry at C₄, presumably established by the NADH-utilizing BacG. Also of note is that while the protio version of H₄Tyr has the previously mentioned three stereogenic centers, if the BacA, BacB, and BacG incubations starting from prephenate are conducted in D₂O buffers, then one deuterium (D = ²H) is incorporated at C₈ from action of BacA, one at C₉ from the action of BacB, and then another is incorporated at C₃ by BacG catalysis (the hydride from NADH is added at C₄) ⁷. Thus the C₃, C₈, and C₉ methylene groups (-CH₂-) become chiral by virtue of deuterium substitution (-CHD-). Therefore in ²H₃, ²H₈, ²H₉-containing samples, H₄Tyr produced via the BacABGF pathway possesses six chiral centers: C₂, C₃, C₄, C₇, C₈, and C₉. In this study we report delineation of stereochemistry at the four centers C₂, C₄, C₈ and C₉ that provide mechanistic insights and constraints into the action of BacF, BacG, BacA, and BacB, respectively.

MATERIALS AND METHODS

Materials and Instrumentation

Prephenic acid barium salt, L- and D-tyrosine (Tyr), L- and D-phenylalanine (Phe), 4-hydroxyphenylpyruvic acid (HPP), β -nicotinamide adenine dinucleotide hydrate (NAD⁺), and β -nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH) were purchased from Sigma-Aldrich. NMR solvent (D₂O), D-glucose (1-D, 98%), and ethanol-*d*₆ (D, 99%) were purchased from Cambridge Isotope Laboratories. The enzymes Glucose Dehydrogenase from *Pseudomonas* sp., Alcohol Dehydrogenase from *S. cerevisiae*, and Aldehyde Dehydrogenase from *S. cerevisiae* were also purchased from Sigma Aldrich.

¹H and 2D-NMR spectra were collected at 25 °C on a Varian VNMRs 600 MHz spectrometer equipped with a triple-resonance probe. ¹³C 1D-NMR spectra were recorded

on a Varian MR 400 MHz spectrometer (100.497 MHz for ^{13}C) equipped with a OneNMR probe. NMR data were analyzed and integrated with ACD/Labs software. High-resolution LC/MS data were collected on an Agilent Technologies 6520 Accurate-Mass Q-TOF LC/MS and analyzed using its accompanying software. HPLC was performed on a Beckman Coulter System Gold instrument and peaks were analyzed using the accompanying Karat 32 software. UV-Vis measurements were collected using a Cary 50 BIO UV-Vis spectrometer and analyzed with the accompanying software.

Cloning, expression, and purification of the enzymes BacABGF

The cloning of plasmids for production of BacABGF has been previously described ⁷. The expression and purification of BacA and BacB were also performed as previously described ⁸. The expression and purification of BacG and BacF were identical to the procedure for BacA. Protein concentrations of BacABGF were determined by UV-Vis absorbance using the following calculated extinction coefficients ($\epsilon_{280\text{nm}}$ calculated from the protein primary sequence using the ExPASy Bioinformatics Research Portal): BacA = $17420 \text{ M}^{-1} \text{ cm}^{-1}$; BacB = $24410 \text{ M}^{-1} \text{ cm}^{-1}$; BacG = $9970 \text{ M}^{-1} \text{ cm}^{-1}$; BacF = $41830 \text{ M}^{-1} \text{ cm}^{-1}$.

Generation and purification of protonated 3Z-7R- and 3E-7R-ex-H₂HPP isomers for use as substrates for NMR and kinetic analyses of BacG action

Conversion of barium prephenate to potassium prephenate was performed as previously described ⁷. Protonated 3E-ex-H₂HPP was generated in reactions containing 12 mM potassium prephenate, 4 μM BacA, and 1 μM BacB in 2 mL of potassium phosphate buffer pH 8.0. The reactions were incubated at $\sim 20^\circ\text{C}$ for 16 hours. The reactions were quenched via addition of acetonitrile to 30% v/v, frozen, and lyophilized in preparation for purification (discussed after generation of 3Z-ex-H₂HPP).

Production and purification of large amounts of 3Z-ex-H₂HPP from BacAB reactions was found to be very laborious due to a maximum of only 25% of the total product being the 3Z isomer ⁸. However, NMR and kinetic experiments with the enzymes AerD and AerE (BacA and BacB homologs, respectively, from *Planktothrix agardhii* ⁹) showed that these enzymes produce identical products to BacAB action when acting on prephenate substrate, except the 3Z/3E isomer ratio is 9/1 (Parker and Walsh, unpublished data) as opposed to 1/3 for BacAB. Thus, fully protonated 3Z-ex-H₂HPP was generated from AerDE reactions containing 12 mM potassium prephenate, 2 μM AerD, 4 μM AerE in 4 mL of 50 mM potassium phosphate buffer pH 8.0. (Important to note is that 3Z isomer formation from either AerE or BacB action was severely inhibited by high concentrations of salt ($\sim > 250 \text{ mM}$, data not shown).) The reactions were incubated at $\sim 20^\circ\text{C}$ for 16 hours, quenched via addition of acetonitrile to 30% v/v, frozen, and lyophilized to dryness.

For purification of the ex-H₂HPP isomers, the lyophilized reactions of BacAB (for 3E isomer formation) and AerDE (for 3Z isomer formation) were resuspended (separately) in 750 μL of 10 mM potassium phosphate buffer pH 8.0 and loaded onto a $100 \times 21.2 \text{ mm}$ Hypercarb column (Thermo Scientific) equilibrated in the resuspension buffer. Bound compounds were eluted (3Z and 3E isomers separated) by exposure to a shallow linear gradient of acetonitrile. Compound elution was monitored by UV absorbance at 295 nm. Fractions containing the appropriate isomers (normally $\sim 30 \text{ mL}$) were pooled, frozen, and lyophilized to dryness. Pure ex-H₂HPP isomers were then moderately desalted by resuspension in 500 μL of 10 mM potassium phosphate buffer pH 8.0 and loading onto a $100 \times 10 \text{ mm}$ Hypercarb column equilibrated in the same buffer. Bound ex-H₂HPP was eluted with a sharp linear gradient of acetonitrile (0 – 90% over 25 minutes) and 4.5 mL of fractions containing ex-H₂HPP were pooled and lyophilized to dryness. The lyophilized

compound and residual potassium phosphate buffer were resuspended in 300 μL of ddH₂O and concentration determined by UV-Vis absorbance ($\epsilon_{295\text{nm}} = 15\,300\text{ M}^{-1}\text{ cm}^{-1}$ for the 3*E*-isomer⁷ and $\epsilon_{295\text{nm}} = 13\,700\text{ M}^{-1}\text{ cm}^{-1}$ for the 3*Z*-isomer (Figure S10)). Isomer purity was analyzed via analytical HPLC using a 100 $\times$ 2.1 mm Hypercarb column equilibrated in 10 mM potassium phosphate pH 8.0. Bound isomers were eluted using a linear gradient of acetonitrile and elution was monitored by UV-Vis absorbance at 295 nm (Figure S1). Isomers were stored at $-80\text{ }^{\circ}\text{C}$ to prevent nonenzymatic isomer equilibration when not in use.

Kinetics of BacG action on 3*Z*- and 3*E*-*ex*-H₂HPP

Kinetic reactions analyzing BacG action on 3*Z*-*ex*-H₂HPP contained 1 μM BacG, 2 mM NADH, 0 – 1.1 mM 3*Z*-*ex*-H₂HPP, and 50 mM potassium phosphate buffer pH 8.0 in a volume of 250 μL . Kinetic reactions analyzing BacG action on 3*E*-*ex*-H₂HPP contained 5 μM BacG, 2 mM NADH, 0 – 8 mM 3*E*-*ex*-H₂HPP, and 50 mM potassium phosphate buffer pH 8.0 in a volume of 250 μL . 2 mM NADH was found to be > 90% saturating for the reactions of both isomers. Reactions were initiated via addition of BacG and reaction progress was monitored by observing the oxidation of NADH to NAD⁺ (UV absorbance at 370 nm) in a 1 mm cuvette. Reaction velocities (k_{obs}) in units of Abs/min were determined by calculating the slope of the initial, linear region of the 370 nm time course of each reaction. The change in NADH absorbance at 370 nm was converted to concentration using the experimentally determined extinction coefficient $\epsilon_{370\text{nm}} = 2690\text{ M}^{-1}\text{ cm}^{-1}$ (referenced to NADH concentration determined from $\epsilon_{340\text{nm}} = 6220\text{ M}^{-1}\text{ cm}^{-1}$ ¹⁰). Data were fit to the Michaelis-Menten equation (eq 1) using GraphPad Prism software, where k_{cat} is the maximum velocity, $[S]$ is the substrate concentration, and K_m is the substrate concentration that yields $k_{\text{obs}} = \frac{1}{2} k_{\text{cat}}$.

$$k_{\text{obs}} = \frac{k_{\text{cat}} \times [S]}{K_m + [S]} \quad (1)$$

All reactions were performed in triplicate except the reaction containing 8 mM 3*E*-*ex*-H₂HPP, which was performed in duplicate due to lack of material.

Generation and purification of protonated 4*S*- and 4*R*-H₄HPP and 4*S*- and 4*R*-H₄Tyr isomers for NMR analysis

4*S*-H₄HPP was generated in a reaction containing 2 mM 3*Z*-*ex*-H₂HPP, 5 mM NADH, and 20 μM of BacG in 1 mL of 50 mM potassium phosphate buffer pH 8.0 incubated. 4*S*-H₄Tyr was generated in a reaction containing ~ 2 mM 4*S*-H₄HPP (described in the previous sentence), 10 mM L-Phe, 0.1 mM PLP, and 20 μM BacF in 1 mL of 50 mM potassium phosphate buffer pH 8.0. 4*R*-H₄HPP was generated in a reaction containing 2 mM 3*E*-*ex*-H₂HPP, 5 mM NADH, and 20 μM of BacG in 1 mL of 50 mM potassium phosphate buffer pH 8.0. 4*R*-H₄Tyr was generated in a reaction containing ~ 2 mM 4*R*-H₄HPP (described in the previous sentence), 10 mM L-Phe, 0.1 mM PLP, and 20 μM BacF in 1 mL of 50 mM potassium phosphate buffer pH 8.0. All reactions were incubated at ~ 20 $^{\circ}\text{C}$ for 16 hours.

All of the above reactions were quenched via addition of acetonitrile to 30% v/v, frozen, and lyophilized to dryness. Dried reactions were resuspended in 500 μL of 10 mM potassium phosphate buffer pH 8.0 and purified as described above for the *ex*-H₂HPP isomers except using a 100 $\times$ 10 mm Hypercarb column. As H₄HPP and H₄Tyr do not have a characteristic UV-Vis absorbance, their elution was monitored via LC/MS in negative detection mode (0.1% NH₄OH as solvent additive) using a 50 $\times$ 2.1 mm Hypercarb column with ddH₂O as the mobile phase and acetonitrile as the eluent. A mass of 183.0668 was observed for the H₄HPP isomers (calc = 183.0663) and 184.0986 (calc = 184.0979) for the H₄Tyr isomers. 3

mL of fractions containing the desired product were frozen and lyophilized to dryness, then resuspended in 300 μ L of 99.99% D₂O and placed in a 5 mm D₂O matched Shigemi tube for NMR analysis.

NMR spectral assignments were made via analyses of ¹H, ¹³C, ¹H-¹H-gCOSY, ¹H-¹³C-gHSQC, ¹H-¹³C-gHMBC, and ¹H-¹H NOESY (500 ms mixing time) spectra. Proton spectra were referenced to residual H₂O (4.79 ppm) while ¹³C spectra were referenced to the methyl carbon of acetonitrile at (1.47 ppm)¹¹. (Acetonitrile was spiked into the NMR sample at 0.3% v/v after all necessary spectra were acquired and an additional ¹³C experiment was performed to obtain the reference so as not to contaminate the original spectra with the acetonitrile signal.) Unless otherwise stated, water suppression (via presaturation) was utilized in all proton spectra collected.

Generation and purification of deuterated 3Z-ex-H₂HPP and 3E-ex-H₂HPP from BacAB action and deuterated 3E-ex-H₂HPP from action of BacA only

The reactions to produce deuterated *ex*-H₂HPP isomers from BacAB action contained 5 mM prephenate, 10 μ M BacA, and 40 μ M BacB in 1 mL of 95% D₂O containing 50 mM potassium phosphate pD 8.0. The reactions were incubated at 37 °C for 24 hours, then quenched, purified, and desalted as described above for the protonated *ex*-H₂HPP isomers. (BacAB reactions in D₂O were very inefficient at producing 3Z-*ex*-H₂HPP. Therefore, it took many of these reactions to obtain enough deuterated compound for NMR analysis). After desalting and lyophilization, the purified deuterated 3Z- and 3E-*ex*-H₂HPP isomers were resuspended in D₂O, and ¹H, ¹H-¹³C-gHSQC, and ¹H-¹H-NOESY data were collected and referenced as described above for the protonated H₄HPP isomers.

Production of deuterated 3E-*ex*-H₂HPP from BacA action (without BacB) in tandem with non-enzymatic diene isomerization was accomplished in a reaction containing 10 mM prephenate and 10 μ M BacA in 2 mL of 98% D₂O containing 50 mM potassium phosphate buffer pD 8.0. The reaction was incubated at 37 °C for 144 hours. The progress of the extremely lethargic non-enzymatic isomerization in D₂O was monitored by UV absorbance (λ_{max} of *en*-H₂HPP = 258 nm; λ_{max} of *ex*-H₂HPP = 295 nm⁷). The reaction was then quenched, purified, desalted, and ¹H and ¹H-¹³C-gHSQC NMR data collected as described above for the BacAB generated deuterated *ex*-H₂HPP isomers.

Enzymatic synthesis of deuterated H₄HPP isomers and H₄Tyr isomers in D₂O solvent

Two forms of deuterated 4R-H₄HPP were produced in two distinct reactions. The first reaction contained 1 mM deuterated 3E-*ex*-H₂HPP produced from the deuterated BacAB reaction (described above), 4 mM NADH, and 30 μ M BacG in 1 mL of 97% D₂O containing 50 mM potassium phosphate buffer pD 8.0. The second reaction contained 5 mM potassium prephenate, 20 mM NADH, and simultaneous incubation of 20 μ M BacABG in 95% D₂O containing 50 mM potassium phosphate buffer pD 8.0. Likewise, two forms of deuterated 4R-H₄Tyr were produced in two distinct reactions. The first reaction contained 2 mM deuterated 3E-*ex*-H₂HPP produced from BacAB action (described above), 8 mM NADH, 0.1 mM PLP, 10 mM L-Phe, and 15 μ M BacGF in 1 mL of 97% D₂O containing 50 mM potassium phosphate buffer pD 8.0. The second reaction contained 5 mM potassium prephenate, 20 mM NADH, 20 mM L-Phe, 0.1 mM PLP, and 15 μ M BacABGF in 95% D₂O containing 50 mM potassium phosphate buffer pD 8.0.

Only one form of deuterated 4S-H₄HPP and 4S-H₄Tyr could be produced. The reactions to produce deuterated 4S-H₄HPP and 4S-H₄Tyr were identical to the first set of reactions used to produce deuterated 4R-H₄HPP and 4R-H₄Tyr (described in the previous paragraph)

except that deuterated 3*Z*-*ex*-H₂HPP (produced from the deuterated BacAB reaction described above) was used in place of 3*E*-*ex*-H₂HPP.

All deuterated H₄HPP and H₄Tyr production reactions were incubated at ~20°C for 16 hours, then quenched, purified, and appropriate fractions identified by LC/MS as for the protonated H₄HPP and H₄Tyr reactions described above. H₄HPP masses obtained were 185.0816, 186.0876, 187.0938, and 188.0993 (calc = 185.0788, 186.0851, 187.0914, 188.0977). H₄Tyr masses obtained were 186.1159, 187.1215, 188.1279, and 189.1339, and 190.1392 (calc = 187.1167, 188.1230, 189.1293, 190.1356). These masses correspond to compounds with incorporation of 2, 3, 4, or 5 deuteriums, respectively.

Enzymatic synthesis of 4*R*-²H- and 4*S*-²H-NADH

4*R*-²H-NADH (also known as A-side NADD) was synthesized following a modified protocol of Viola et al ¹². 25 mM ethanol-*d*₆, 10 mM NAD⁺, 300 U of alcohol dehydrogenase, and 32 U of aldehyde dehydrogenase were mixed in 10 mL of 25 mM Taps buffer pH 9.0. The pH of the buffer was readjusted to 9.0 after addition of the NAD⁺ but before addition of enzymes. The reaction was incubated at ~ 20 °C and reaction progress was monitored by following the appearance of NADH (UV absorbance at 340 nm). After 45 minutes of incubation, the reaction was quenched via protein removal using a 3500 MWCO centrifugal filtration device (Amicon).

4*S*-²H-NADH (also known as B-side NADD) was synthesized according to a slightly modified protocol of Ottolina et al ¹³. 30 mM glucose-*d*₁, 15 mM NAD⁺, and 100 U of glucose dehydrogenase were mixed in 6mL of 50 mM potassium phosphate buffer pH 7.5. The pH of the buffer was readjusted to 7.5 before enzyme addition. Reaction progress was monitored and the reaction was quenched (after 80 minute reaction time) exactly as for the A-side NADD synthesis reaction described above.

The processing of both NADD reactions was identical for the remaining steps. The post-filtration flow-through containing the NADD was frozen and lyophilized to dryness. The dried reaction components were resuspended in 1 mL of H₂O. NADD at this stage was used directly in the BacG enzymatic reactions after pH correction to 8.0. The NADD concentration was determined by UV absorbance ($\epsilon_{340\text{nm}} = 6220 \text{ M}^{-1} \text{ cm}^{-1}$ ¹⁰). To prepare NADD samples for NMR analysis of the deuterium content at C₄, NADD was crudely purified by loading onto a 250 × 10 mm Luna C₁₈ 5 μm HPLC column (Phenomenex) equilibrated in H₂O + 0.01% NH₄OH. Bound NADD was eluted using a linear gradient of acetonitrile spiked with 0.01% NH₄OH. Fractions containing NADD were identified by the characteristic absorbance maxima at 260 nm and 340 nm. The observed 260/340 nm ratio was 2.3 for A-side and 2.5 for B-side NADD (previously reported as ~ 2.2 – 2.3 for pure NADH ¹²). Fractions containing NADD were pooled and lyophilized to dryness. 2 mM samples of both A-side and B-side NADD were prepared in 99.99% D₂O and placed in 5 mm D₂O matched Shigemi tubes for ¹H NMR analysis (Figure S2). Pro-*R* and pro-*S* hydride peaks were identified based on previous assignment ^{13, 14}.

Enzymatic synthesis of H₄HPP isomers from BacG action with 4*R*-²H-NADH and 4*S*-²H-NADH as cosubstrates

Reactions to generate 4*S*- and 4*R*-H₄HPP from protonated 3*Z*- and 3*E*-*ex*-H₂HPP, respectively, contained 1 mM *ex*-H₂HPP, 2 mM ²H-NADH, and 30 μM BacG in 1 mL of H₂O containing 50 mM potassium phosphate buffer pH 8.0. The reactions were incubated, quenched, purified, and fractions identified exactly as described above for protonated H₄HPP isomers generated from protonated NADH. The detected LC/MS mass of H₄HPP produced from 4*R*-²H-NADH was 183.0670 (calc = 183.0663). A major (184.0726) and

minor (183.0666) mass were both detected from H₄HPP produced from 4*S*-²H-NADH (calc = 184.0726 and 183.0663). Collection of ¹H and ¹H-¹³C-gHSQC NMR data were also performed as described above (Figure S3).

Preparation of tyrosine from BacF transaminase action on 4-hydroxyphenylpyruvate for amino acid chirality determination

2 mM HPP, 10 mM Phe (both L and D enantiomers in separate reactions), 0.5 mM PLP, and 10 μM BacF were mixed in 1 mL of 50 mM potassium phosphate buffer pH 8.0. Reactions were incubated at ~ 20 °C for 16 hours, quenched via addition of acetonitrile to 30% v/v, lyophilized to dryness, and resuspended in 500 μL of H₂O. The expected transamination product (tyrosine) was identified from the BacF reaction containing L-Phe by LC/MS in positive detection mode (0.1% formic acid as solvent additive) using a 50 × 2.1 mm Hypercarb column with water as the mobile phase and acetonitrile as the eluent. No tyrosine was observed from the BacF reaction containing D-Phe as the cosubstrate.

Prior to chiral HPLC analysis of the BacF produced tyrosine, it was necessary to purify the nascent tyrosine from the other reaction components to avoid spectral contamination. The resuspended reaction was loaded onto a 100 × 10 mm Hypercarb column equilibrated in 10 mM potassium phosphate buffer pH 8.0. Bound compounds were eluted via a linearly increasing gradient of acetonitrile. Fractions containing tyrosine were initially located by their UV-Vis spectra and later confirmed by LC/MS analysis as described above. The appropriate fractions were pooled and lyophilized to dryness. The amino acid chirality of the BacF generated tyrosine was determined by comparing its elution profile from a 150 × 4.6 mm Chirex 3126 column (Phenomenex) equilibrated in 2 mM copper (II) sulfate in 84/16 (v/v) water/methanol with that of authentic L- and D-tyrosine standards. Tyrosine elution was monitored by UV-Vis absorbance at 280 nm (Figure S4).

RESULTS

BacG acts on both 3*Z*, 7*R*- and 3*E*, 7*R*-*ex*-H₂HPP geometric isomers to give H₄HPP products diastereomeric at C₄

In initial studies of the Bac pathway enzymes to convert prephenate to H₄Tyr, we conducted incubations of either BacAGF or BacABGF in parallel, based on the observation that the BacA-derived *en*-H₂HPP will isomerize nonenzymatically and quantitatively into conjugation to give the exocyclic *ex*-H₂HPP product ⁷. BacB speeds up the allylic isomerization by up to 10³ and so was of use in shortening the kinetics in the four enzyme incubations ¹⁵. These incubations could be conducted in buffered H₂O or buffered D₂O, to yield H₄Tyr with one deuterium at each of C₈ and C₉ (as well as at C₂ and C₃, *vide infra*). A deeper analysis of the BacB-mediated isomerization recently led us to the finding that while the *E/Z* ratio of *ex*-H₂HPP from BacA followed by nonenzymatic allylic isomerization was 50/1, in BacB-containing equilibrated incubations the *E/Z* ratio was 3/1. In turn that led us to separation and structural assignment of the 3*E* and 3*Z* isomers on Hypercarb HPLC columns and a proposed mechanism for geometric isomer equilibration via dienolate intermediates ⁸.

The presumption from the above results was that each of the 3*E*- and 3*Z*-7*R*-*ex*-H₂HPP geometric isomers could be a substrate for the NADH-utilizing BacG. This enzyme is assayed by loss of the 340 nm chromophore of the reduced nicotinamide ring of NADH as *ex*-H₂HPP is reduced to H₄HPP (and NADH is oxidized to NAD⁺). Because of the partially overlapping chromophores of the *ex*-H₂HPP substrate (λ_{max} = 295 nm) and cosubstrate NADH, measurements of the reaction were made off the absorbance maximum of NADH, at 370 nm, where sufficient signal to noise persisted. Indeed, the separated geometric 3*E* (96%

purity) and 3*Z* (97% purity, Figure S1) isomers were each saturable substrates (Figure 3). The k_{cat} for the 3*Z* isomer at 71.3 min^{-1} was about twice that for the 3*E* isomer (33.2 min^{-1}). The 3*Z* isomer also had a 30-fold lower K_m value (0.09 mM vs. 2.7 mM). By k_{cat}/K_m ratios, which compare the relative catalytic efficiencies of two substrates for the same enzyme, the 3*Z*-7*R*-*ex*-H₂HPP isomer is a 64-fold better substrate than the corresponding *E* isomer. Thus, although the 3*Z* geometric isomer is kinetically disfavored during BacB action, and essentially absent from the *nonenzymatic* isomerization of *en*-H₂HPP to the thermodynamically favored *ex*-H₂HPP⁸, it is very much the preferred substrate for the conjugate reduction reaction of BacG.

Figures 4A and 4C show the proton NMR spectra of the H₄HPP samples generated from BacG action on 3*Z*-*ex*-H₂HPP and 3*E*-*ex*-H₂HPP substrates, respectively. It is clear from examination of the H₈ and H₉ resonances that the products are distinct, most likely due to distinct diastereomers at C₄, the anticipated site of hydride addition by BacG. On subsequent BacF-mediated transamination of each of the H₄HPP keto acid samples to the corresponding 2*S*-H₄Tyr samples, the proton NMR spectra in Figures 4B (derived from Figure 4A sample) and 4D (derived from Figure 4C sample), also gave distinct diastereomers as evidenced from the distinct splitting patterns from the methylene hydrogens at C₃, C₈, and C₉. The assignment of the indicated stereochemistries as 4*S* (from the 3*Z* substrate) and 4*R* (from the 3*E* substrate) are detailed in a subsequent section.

Proof that BacG acted by conjugate addition of an NADH-derived hydride ion to C₄ of the *ex*-H₂HPP substrates was explicitly tested with 4*R*-D₁-NADH and 4*S*-D₁-NADH samples as reductants. ¹H NMR analysis of the two chirally deuterated NADH samples established that the 4*R*-D₁-NADH was 40% D₁, while the 4*S*-D₁-NADH was 62% D₁ (Figure S2).

NMR analysis of the H₄HPP products from 4*R*-D₁-NADH incubations on both the 3*E*- and 3*Z*-*ex*-H₂HPP substrates show transfer of the diastereotopic 4-pro-*S* hydride to both the 3*E* and 3*Z* substrate isomers. Figure S3 shows the 3*Z*-substrate had 0.94 ¹H equivalents at C₄ while the 3*E* substrate possessed 1.0 ¹H equivalents, referenced to the single hydrogen at C₇ in the product H₄HPPs. One could worry that the lack of deuterium reflected a kinetic isotope effect between NADH and 4*R*-D₁-NADH molecules in the NADH reductant pool, but the complementary results with deuteride transfer from the 4*S*-D₁-NADH sample ruled out that possible ambiguity. The 4*S*-D₁-NADH donor sample (62% deuterium content) gave H₄HPP with 68% D at C₄ when the 3*Z* substrate was reduced and 58% D at C₄ when the 3*E* substrate was reduced. These results clearly indicate that BacG transfers the pro-*S* hydride of NADH during *ex*-H₂HPP reduction.

These results validate several points. First, BacG is acting as a regiospecific hydride transfer catalyst to C₄ for each of the 3*E*- and 3*Z*-*ex*-H₂HPP substrate isomers. Second, the pro-*S* hydride of NADH is transferred to each of the substrate geometric isomers. Third, from the NMR spectra in Figures 4A and 4C noted above, the H₄HPP products are likely to be diastereomers at C₄.

The BacF Transaminase is 2*S*-specific

Given that the BacF transaminase processes L-Phe (2*S*) as amino donor to cosubstrate H₄HPP, we anticipated it would show the same specificity towards partner keto acids that become reductively aminated. To that end we have now demonstrated that while L-Phe is an effective amino donor, D-Phe is not (data not shown). To validate that the keto acid partner undergoing amination gives a 2*S* amino acid product, we used *p*-hydroxyphenylpyruvate as acceptor with L-Phe as donor. As anticipated, tyrosine was generated (LC/MS detected mass of 182.0805; calc = 182.0812) as L-Phe was oxidatively deaminated in the first half of the BacF reaction. The chirality of that Tyr was determined as “L” (2*S*) by comparison with

authentic D- and L-Tyr samples by chiral HPLC (Figure S4). By analogy, we assign the H₄Tyr from reductive amination of H₄HPP by BacF as the 2*S*-diastereomer. At this point with the 2*S* stereochemistry assigned, we turned to analysis of the absolute stereochemistry at C₄ of the H₄Tyr samples generated from tandem action of BacABGF to obtain the absolute stereochemistry of the H₄Tyr emerging from the four enzymes in the bacilysin pathway.

Interpretation of NOE Correlations to Determine the Stereochemistry of 4*S*-H₄Tyr and 4*R*-H₄Tyr at C₄

To determine the stereochemistry at C₄ of H₄Tyr (and by inference H₄HPP) derived from BacG reduction of 3*Z*-*ex*-H₂HPP, ¹H-¹H NOESY data reporting on the relative distances between protons were collected on a fully protonated sample of H₄Tyr (Figure 5A). Because the proton resonances needed to make the appropriate correlations were not overlapped in this sample, stereochemical assignment was straightforward. We began linking correlations using H₇ as the reference, since the stereochemistry at C₇ had been previously assigned in our earlier study as the *R*-stereochemistry⁸. From inspection of the NOE cross-peaks it is seen that H₇ shows a strong correlation with H_{8a} and H_{9a}, but not H_{8b} and H_{9b}. This indicates that H₇, H_{8a}, and H_{9a} are on the same face of the cyclohexenol ring, while H_{8b} and H_{9b} are on the opposite face (Figure 5A). We then moved our attention to the H₄ proton and noticed that H₄ correlates strongly with H_{8b} and H_{9b}, but not H_{8a} and H_{9a}, indicating that H₄, H_{8b}, and H_{9b} are on the same face of the cyclohexenol ring and thus on the opposite face from H₇. These correlations establish that H₄Tyr (and thus H₄HPP, as shown in Figure 4) originating from BacG reduction of 3*Z*-*ex*-H₂HPP possesses 4*S* stereochemistry (Figure 5A).

Determination of the C₄ stereochemistry of H₄Tyr (and H₄HPP) derived from BacG reduction of 3*E*-*ex*-H₂HPP was not so straightforward due to the overlapping cross-peaks of H_{3a}, H_{8a}, and H_{9b} in the ¹H-¹H NOESY data of protonated H₄Tyr (Figure 5B). However, two important NOE correlations could be clearly seen in these data: a strong cross-peak between H_{8b} and H_{9a} and a weak cross-peak between H₄ and H₇. While the strong cross-peak between H_{8b} and H_{9a} indicates they are most likely on the same face of the cyclohexenol ring, the NOE correlation between H₄ and H₇ confirms that H₄ and H₇ are on the same face of the ring. This shows that 4*R*-H₄Tyr (and thus 4*R*-H₄HPP) is derived from BacG action on 3*E*-*ex*-H₂HPP (Figure 5B).

Although the stereochemistry at C₄ of 4*R*-H₄Tyr could be interpreted despite the overlapped resonances in the ¹H-¹H NOESY spectrum (Figure 5B), NOE analysis of 4*R*-H₄Tyr-*d*₄ (Figure 5C) was necessary to determine the relative positions of the nonequivalent protons of C₈ and C₉. As observed in Figure 5C and in greater detail in Figure S5B, simultaneous incubation of BacABGF with prephenate in 95% D₂O solvent incorporates a deuterium at each of C₂, C₃, C₈, and C₉ of 4*R*-H₄Tyr-*d*₄ and fortuitously yields a ¹H NMR spectra without overlapped resonances. Inspection of the ¹H-¹H NOESY data of 4*R*-H₄Tyr-*d*₄ shows an especially strong cross-peak between H₄ and H_{9b}, indicating they are on the same face of the cyclohexenol ring. (For comparison the cross-peak between H₄ and H_{9a} in Figure 5B is weaker. The cross-peak between H₄ and H₇ in Figures 5B and 5C can be used to normalize the spectra to each other.) Also in Figure 5C, almost equivalent cross-peak intensities are seen between H₇ and H_{8b} as between H₇ and H_{9b}. For this to be possible, H_{8b} would need to be on the opposite face of H₇, while H_{9b} would need to be on the same face as H₇. This correlation agrees with the determination made above that H₄ and H_{9b} are on the same face of the cyclohexenol ring (Figure 6C). Combined, these determinations imply that H₄, H₇, H_{8a}, and H_{9b} are on one ring face, while H_{8b} and H_{9a} are on the opposing face (as hinted in Figure 5B by the strong correlation between H_{8b} and H_{9a}). Additional support for these analyses comes from the ¹H-¹H NOESY spectra of protonated 4*R*-H₄HPP spectra

(Figure S9) where H_{8a} is a distinct resonance. In this spectrum, strong correlations are seen between H_{8a} and the protons on C₄ and C₇ (same face) while little or no correlations are seen between H_{9a} and either H₄ or H₇ (opposite face).

Stereochemical Insights at C₈ and C₉ in Deuterated Samples of H₄HPP and H₄Tyr

A bonus from NMR analyses of the deuterated 4*R*- and 4*S*-H₄Tyr samples (as seen in Figures 5C, S5B, S6C, and S7C) is information about the placement of deuterium at C₈ (from BacA action) and C₉ (from BacB action). We know the stereochemistry at C₂ is 2*S* from BacF transaminase action data cited above. We have not tried to determine stereochemistry at the C₃-HD center. One deuterium is picked up at that site during BacG action. But there is also solvent exchange at C₃ when BacB carries out the equilibration between the 3*E* and 3*Z* isomers of *ex*-H₂HPP, via dienolate intermediates⁸. The extent of that exchange may well have differed during different incubations, leading to partial deuterium incorporations at C₃.

At C₈ the deuterium in both the 4*R*- and 4*S*-H₄Tyr products is on the same face as H₇; therefore the stereochemistry in the C₈-HD samples is 8*S*. The stereochemical outcome at C₉ in the C₉-HD samples is more complex. As shown in Figure 5 and S5B in the 4*R*-H₄Tyr sample from simultaneous BacABGF D₂O incubations with prephenate, the deuterium at C₉ is on the ring face opposite to both H₄ and H₇, and therefore is the 9*S* mono-deutero isomer.

The results with 4*S*-H₄Tyr samples from D₂O incubations were less clear-cut. Under some conditions (Figure S7C), where BacB had been present, there was partial deuterium substitution at both the 9*S* and 9*R* positions. In experiments where the BacA product *en*-H₂HPP was allowed to isomerize nonenzymatically (in solutions estimated at 98% D₂O and then carried through the BacGF reactions in D₂O), there was very little deuterium at C₉ (Figure S8A), perhaps reflecting a large deuterium isotope effect in the nonenzymic isomerization of *en*-H₂HPP solely to the 3*E*-*ex*-H₂HPP isomer. Because of these vagaries due to BacB-mediated exchange and the nonenzymatic isomerization giving solely the 3*E*-*ex*-H₂HPP product⁸, we have not conducted a full interpretation of the C₉ results in the 4*S*-H₄Tyr diastereomer.

DISCUSSION

Prephenate is the immediate precursor to both phenylpyruvate (via prephenate dehydratase^{16, 17}) and hydroxyphenylpyruvate (via prephenate dehydrogenase^{18, 19}) on the way to L-Phe, L-Tyr, and many phenylpropanoid metabolites^{20, 21}. In the anticapsin/bacilysin pathway, prephenate is instead shunted down a newly appreciated metabolic branch. BacA, the first enzyme in this and related pathways to dihydro- and tetrahydroaromatic metabolite scaffolds⁹, is a remarkable catalyst, engineering the decarboxylation without aromatization of the 1,4-cyclohexadiene ring of prephenate⁷. It is a regiospecific isomerization catalyst, working only on the pro-*R*, not the pro-*S*, double bond of the prephenate diene and delivering a proton only to C₆ not C_{6'}, a carbon that becomes C₈ in the *en*-H₂HPP product⁸. Three enzymes later, by tandem action of BacBGF, H₄Tyr is formed.

Unlike its familiar aromatic congener L-tyrosine that has one chiral center (2*S*), H₄Tyr has three chiral centers: C₂, the ring junction C₄, and the original alcoholic carbon of prephenate, C₇. One expects enzymes to act as chiral catalysts such that of the eight possible H₄Tyr diastereomers, only one is anticipated. The 2*S*,4*S*-diastereomer of H₄Tyr is indeed a known nonproteinogenic building block of the cyanobacterial nonribosomal peptides aeruginoside 126A and aeruginoside 126B²². In the initial structure assignment of anticapsin, the C₄ stereochemistry was initially incorrectly assigned 1 but corrected

subsequent to total synthesis²³ as the *2S,4S* diastereomer, consistent with *2S,4S*-H₄Tyr being on pathway. The stereochemistry at C₇ of anticapsin has become moot because of the oxidation of the C₇ alcohol in prephenate to the ketone in anticapsin. Given that we have recently proven that BacA, by isomerizing the pro-*R* olefin in prephenate, generates the *7R*- Δ^3,Δ^5 -diene, we therefore anticipated the H₄Tyr emerging from tandem action of BacABGF would thus be the *2S, 4S, 7R* diastereomer.

As we have reported here this expectation is met, but the *2S,4R,7R* diastereomer also emerges from the tandem action of the four Bac enzymes. Perhaps most intriguing is the action of the second enzyme, BacB, to generate a mix of *3Z* and *3E* isomers of the *ex*-H₂HPP. Both are then substrates for conjugate hydride reduction by BacG with delivery of a (pro-*S*) hydride to C₄ and a solvent-derived hydrogen at C₃. At the level of these H₄HPP keto acid products, and after sweeping them through to the more stable H₄-tyrosines via BacF, it is clear that opposite chirality results at C₄. The *3Z* isomer yields *2S,4S,7R*-H₄Tyr from BacG action while the *3E* isomer gives *2S,4R,7R*-H₄Tyr (Figure 6D). We expect the *4S* diastereomer is on pathway to anticapsin (*4S*) but not the *4R* diastereomer. However, this proposition must await study of the last two missing enzymatic steps in anticapsin biogenesis.

The relative orientation of the dihydronicotinamide ring of the hydride donor NADH and the conjugated dienyl keto acid of the *ex*-H₂HPP must switch between the *3Z* and *3E* isomers as they are recognized as substrates. Assuming that NADH orientation stays the same within its BacG binding site, as it transfers the pro-*S* hydrogen to both geometric isomers of substrate, the different faces of the olefin would be presented to the incoming hydride as shown in Figure 6AB, assuming the C₃-C₁ chain of the geometric *ex*-H₂HPP isomers stay in the same orientation.

The formation of C₄ diastereomers of H₄HPP (and then of H₄Tyr from subsequent BacF action) gives further insight into the possibly corrective role of BacB in the pathway. We have previously noted that the *7R-en*-H₂HPP product of BacA can undergo nonenzymatic conversion to the more conjugated *7R-ex*-H₂HPP isomer. We wondered why the producing cell would expend energy to make a protein (BacB) that would accelerate a reaction that would occur otherwise. Certainly matching the kinetics of metabolite flux and prevention of off pathway decomposition and aromatization are reasonable rationales, but most telling is the finding that the nonenzymatic route gives 98% *3E* geometric isomer. The work reported here shows that the *E*-isomer will lead to, via BacG action as reductase, the *4R* diastereomer of H₄Tyr, which is presumably the wrong chirality to go on to the anticapsin antibiotic. BacB instead equilibrates the more stable *3E* and less stable *3Z* geometric isomers to give up to 25% of the *3Z* isomer, which on subsequent reduction by BacG gives the desired *4S* stereochemistry. If there is enough BacB in producer cells coupled to the BacG catalytic efficiency preference of *3Z/E* (64/1 found in this study) then flux could be routed selectively to the productive *4S* cyclohexenol scaffold. It may be that the *4R* stereochemistry is useful for one or more alternate downstream metabolites in this or related microbes (i.e. the *diepi* form of 2-carboxy-6-hydroxyoctahydroindole (*diepi*-Choi) (*2R,4R*-stereochemistry) residues found as constituents of nonribosomal peptides in cyanobacteria²⁴.

While ascertainment of the C₄ stereochemistry from action of the NADH-utilizing BacG at the stage of *ex*-H₂HPP reduction to H₄HPP was a main objective of this study, we have also

¹Abbreviations: H₂HPP, dihydro-4-hydroxyphenylpyruvate; H₄HPP, tetrahydro-4-hydroxyphenylpyruvate; H₄Tyr, tetrahydrotyrosine; HPP, 4-hydroxyphenylpyruvate; LC/MS, liquid chromatography/mass spectrometry; NMR, nuclear magnetic resonance; gCOSY, gradient homonuclear correlation spectroscopy; gHSQC, gradient heteronuclear single-quantum coherence; gHMBC, gradient heteronuclear multiple bond coherence; NOESY, nuclear Overhauser effect spectroscopy; HPLC, high performance liquid chromatography; D₂O, deuterium oxide; GlcNAc, *N*-acetylglucosamine; *B. subtilis*, *Bacillus subtilis*.

focused on the stereochemical outcomes at C₈ and C₉ from consecutive action of BacA and BacB in deuterated buffers. While C₈ and C₉ sequentially become CH₂ groups in *en*-H₂HPP and *ex*-H₂HPP, respectively, and the stereochemistry is thus cryptic, in principle the stereochemical course of each enzyme at those carbons could be revealed with deuterium in place of hydrogen. We have previously found ⁷ that one deuteron from solvent is fixed at C₈ and one at C₉, and in this study have shown that BacA introduces the solvent derived (H⁺/D⁺) from above the plane of the diene in prephenate, yielding 6*S*-*en*-H₂HPP-*d*₁. Because of the numbering priority changes this compound becomes 8*S*-*ex*-H₂HPP upon BacB action (Figure 6C). In turn, as gathered from the 4*R*-H₄Tyr result, BacB instead transfers a solvent-derived D⁺ from below the plane at C₉ (H_{9a}) and yields a 9*R*-deutero product, swept through to H₄Tyr. (BacB also removes one of the methylene hydrogens at C₃ as it equilibrates the *E*- and *Z*-geometric isomers of *ex*-H₂HPP ⁸). Interpretation of the ¹H-¹H-NOESY spectrum of protonated 3*E*-H₂HPP (Figure S8B) established that H_{9a} is on the ring face opposite of H₇.

While it will take structural studies of BacA in complex with substrate and/or product to identify candidate side chains of active site residues as such a BH⁺, it must be a very efficient process to derail what has been viewed as the default outcome in other members of the prephenate dehydratase superfamily ¹⁶. That default option is cleavage of the C₇-O bond as the cyclohexadiene ring aromatizes and there has been substantial debate over whether decarboxylation and loss of water is a concerted process ^{25, 26}.

Knowledge of the configuration of the H₄Tyr diastereomers generated by the first four enzymes of the Bac pathway, and the realization that 4*R* and 4*S* products emerge, will provide the appropriate substrates for evaluation and identification of the enzymes responsible for what should be the remaining two steps to obtain the anticapsin warhead: 7*R*-alcohol oxidation and olefin epoxidation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Ligation of L-alanine and anticapsin yields the dipeptide antibiotic bacilysin. The host cell further self-protects from anticapsin/bacilysin toxicity by transporting it into the extracellular environment, where it can be imported by neighboring cells. Peptide bond cleavage of bacilysin releases the anticapsin warhead, which blocks cell wall biosynthesis via inhibition of glucosamine-6-phosphate synthase.

Figure 2.

Proposed pathway for the biosynthesis of anticapsin from prephenate. YwfG and YwfH are hereby renamed BacF and BacG, respectively, as noted in the inset.

Figure 3. Michaelis-Menten kinetic traces for BacG reductase action on (A) 3Z-ex-H₂HPP ($k_{cat} = 71.3 \text{ min}^{-1}$; $K_m = 0.09 \text{ mM}$) and (B) 3E-ex-H₂HPP ($k_{cat} = 33.2 \text{ min}^{-1}$; $K_m = 2.7 \text{ mM}$) in the presence of saturating NADH (2mM).

Figure 4. ^1H NMR traces of purified (A) 4S-H₄HPP from BacG action on 3Z-ex-H₂HPP; (B) 4S-H₄Tyr from BacF action on (A); (C) 4R-H₄HPP from BacG action on 3E-ex-H₂HPP; (D) 4R-H₄Tyr from BacF action on (C).

Figure 5. ^1H - ^1H NOESY spectra and accompanying NOE correlations used to determine the C_4 stereochemistry of (A) $4\text{S-H}_4\text{Tyr}$ and (B) $4\text{R-H}_4\text{Tyr}$. Due to spectral overlap of protons 3a, 8a, and 9b in the spectra of (B) $4\text{R-H}_4\text{Tyr}$, NOE correlations of (C) $4\text{R-H}_4\text{Tyr-d}_4$ were also analyzed to allow analysis of correlations to and from H_{9b} to complete the stereochemical determination. Black cross-peaks in the NOESY spectra indicate positive resonances while red cross-peaks indicate negative resonances. Key NOE interactions have been notated on the structures with dashed lines, and the correlating cross-peaks were boxed on the NOESY spectra.

Figure 6.

BacG transfers the pro-*S* hydride of NADH to opposite faces of (A) 3*E*-*ex*-H₂HPP and (B) 3*Z*-*ex*-H₂HPP to produce 4*R*-H₄HPP and 4*S*-H₄HPP, respectively. (C) BacA decarboxylation of prephenate performed in D₂O solvent produces *en*-H₂HPP with “*S*” stereochemistry at C₆. This indicates that the incoming proton is delivered to the face of the cyclohexadiene ring opposite to the departing CO₂. (D) BacABGF action on prephenate produces 2*S*, 4*S*, 7*R*-H₄Tyr and 2*S*, 4*R*, 7*R*-H₄Tyr, with the relative amounts produced being determined by the action of BacB.