Binding isotope effects for *para*-aminobenzoic acid with dihydropteroate synthase from *Staphylococcus aureus* and *Plasmodium falciparum*

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Supporting Information

A. SUPPORTING INFORMATION FIGURE S1

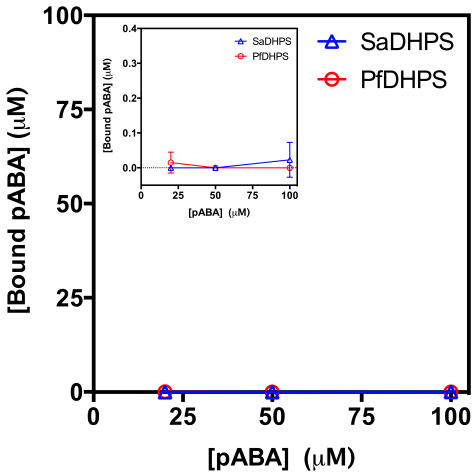


Figure S1. Binding curve for the formation of the *p*ABA·DHPS binary complex. Binding of *p*ABA to the apo form of SaDHPS and PfHPPK-DHPS was measured at 20 μM, 50 μM, and 100 μM [7-^{14}C]*p*ABA using a fixed concentration of enzyme (10 μM). The concentration of bound *p*ABA is presented as a function of the concentration of *p*ABA used in each assay. Error bars were generated using the standard deviation between two replicates for each concentration of *p*ABA tested.

Binding of *p*ABA to apo-SaDHPS and apo-PfHPPK-DHPS was measured using increasing concentrations of [7-^{14}C]*p*ABA in a rapid equilibrium assay analogous to the approach used for the BIE measurements. In this experiment, 10 μM SaDHPS or PfHPPK-DHPS was incubated with 20 μM, 50 μM, or 100 μM *p*ABA ([7-^{14}C]*p*ABA + unlabeled carrier) in 40 mM Tris-HCl, 5 mM $MgCl_2$ and 5 mM DTT using a rapid equilibrium dialysis (RED) device with inserts containing 8 kD MW cutoff membranes (Thermo Fisher Scientific). The total solution volume in the well containing enzyme ("enzyme well") was 100 μL and the total solution volume in the well containing buffer only ("buffer well") was 300 μL. The dialysis inserts were covered with Crystal Clear Sealing Film (Hampton Research, Aliso Viejo, CA) and the RED device was shaken at 275 rpm for 4 hr at 25 ºC. Equivolume samples (50 μL) were then taken from the buffer and enzyme wells of each dialysis insert, diluted in 450 μL H_2O, and mixed with 10 mL of scintillation fluid. Scintillation counting and spectral deconvolution of the raw count data was performed as described for the BIE measurements. The total concentration of bound *p*ABA was calculated from the difference in ^{14}C counts in the enzyme well relative to the buffer well.

No significant binding of [7-^{14}C]*p*ABA was observed for apo-SaDHPS or apo-PfHPPK-DHPS, up to 100 μM *p*ABA (**Figure S1**).

B. SUPPORTING INFORMATION TABLES S1-S3

Solvent model in bound state	*Dielectric constant*	*Calculated BIE*
carbon tetrachloride	2.228	1.0650
chlorobenzene	5.697	1.0306
dichloroethane	10.125	1.0175
acetone	20.493	1.0077
methanol	32.613	1.0039
water	78.355	1.0000

Table S1. Calculated BIEs for [3,5-^{3}H]*p*ABA in solvents with varying dielectric constants.[1]

H3-C3-C4-C5 dihedral in unbound structure	*Change relative to unbound structure*	*H3-C3-C4-C5 dihedral in bound structure*	*H5-C5-C4-C3 dihedral in unbound structure*	*Change relative to unbound structure*	*H5-C5-C4-C3 dihedral in bound structure*	*Calculated BIE*
179.940°	–1°	178.940°	–179.943°	1°	–178.943°	1.0082
179.940°	–2°	177.940°	–179.943°	2°	–177.943°	1.0088
179.940°	–3°	176.940°	–179.943°	3°	–176.943°	1.0097
179.940°	–4°	175.940°	–179.943°	4°	–175.943°	1.0108
179.940°	–5°	174.940°	–179.943°	5°	–174.943°	1.0121
179.940°	–6°	173.940°	–179.943°	6°	–173.943°	1.0135
179.940°	–7°	172.940°	–179.943°	7°	–172.943°	1.0152
179.940°	–8°	171.940°	–179.943°	8°	–171.943°	1.0173
179.940°	–9°	170.940°	–179.943°	9°	–170.943°	1.0198
179.940°	–10°	169.940°	–179.943°	10°	–169.943°	1.0229

Table S2. Calculated BIEs for [3,5-^{3}H]*p*ABA with symmetrical restriction of the H3–C3–C4–C5 and H5–C5–C4–C3 dihedral angles. Fixed values for the H3–C3–C4–C5 and H5–C5–C4–C3 dihedral angles in each of the structures used to calculate BIEs for Figure 3c in the main text. The dihedral angles were fixed at the indicated values prior to optimization in acetone to generate the bound state structure.

C3–H3 and C5–H5 bond length in unbound structure	***Change relative to unbound structure***	***Bond length in bound structure***	***Calculated BIE***
1.0887 Å	0.990	1.0778 Å	0.949
1.0887 Å	0.980	1.0670 Å	0.906
1.0887 Å	0.970	1.0561 Å	0.876
1.0887 Å	0.960	1.0452 Å	0.859
1.0887 Å	0.950	1.0343 Å	0.831
1.0887 Å	0.940	1.0234 Å	0.812
1.0887 Å	0.930	1.0125 Å	0.745

Table S3. Calculated BIEs for [3,5-^{3}H]*p*ABA with compression of the C3–H3 and C5–H5 bond lengths. Fixed values for the C3–H3 and C5–H5 bond lengths in the bound state are shown relative to the equilibrium bond lengths in the free state. The C3–H3 and C5–H5 bonds were fixed at the indicated lengths prior to optimization in acetone to generate the bound state structure.

C. SUPPORTING METHODS

Expression and purification of wild-type SaDHPS. A synthetic gene was designed for SaDHPS (NCBI GenBank: Z84573) and purchased from DNA2.0 Inc. (Menlo Park, CA) in a pJexpress404 expression vector. The encoded protein has 19 additional amino acids at the *N*-terminus, including a His_6 tag and a thrombin cleavage site. Heterologous expression of SaDHPS was achieved in *E. coli* using the One Shot® BL21 Star™ (DE3) cell line. A 10 mL starter culture in LB-ampicillin (100 μg/mL) medium was incubated overnight at 37 °C. The next day, 6 mL of the starter culture was diluted in 1 L of fresh LB-ampicillin (100 μg/mL) and incubated at 37 °C to OD_{600} = 0.7. Induction was initiated by the addition of 1 mM isopropyl-D-thiogalactoside (IPTG) and the culture was incubated overnight at 28 °C.

The next day, cells were harvested via centrifugation and resuspended in 40 mL of Buffer A (20 mM Tris-HCl, 500 mM NaCl, 1 mM DTT, 10 mM imidazole, pH = 8.0). Lysozyme and DNAse were added to the cell suspension and the mixture was incubated at 4 °C for 40 min. Cells were lysed via sonication and cell debris was removed by centrifugation. Cleared cell lysate was poured over a column of Ni-NTA resin (Qiagen), which had been pre-equilibrated with Buffer A. The resin was washed with five column volumes of Buffer A and the protein was eluted with a gradient of 30 mM to 500 mM imidazole in the same buffer system. Fractions containing SaDHPS were identified via SDS-PAGE, pooled, and exchanged into 20 mM Tris-HCl, 300 mM NaCl, 1 mM DTT, pH = 8.0 via dialysis. The pure protein was concentrated to 100 μM and stored at –80 °C.

Expression and purification of wild-type PfHPPK–DHPS. A synthetic gene was designed for PfHPPK-DHPS (NCBI GenBank: AAA19963.1) and purchased from DNA2.0 Inc. in a pJexpress404 expression vector. The encoded protein had a His_6 tag included at the *C*-terminus. Heterologous expression of PfHPPK–DHPS was achieved in *E. coli* using the One Shot® BL21(DE3)pLysE cell line. A 10 mL starter culture in LB with ampicillin (100 μg/mL) and chloramphenicol (100 μg/mL) was incubated overnight at 37 °C. The next day, 6 mL of the starter culture was diluted in 1 L of fresh LB-ampicillin (100 μg/mL) and incubated at 37 °C to OD_{600} = 0.7. Induction was initiated by the addition of 1 mM IPTG and the culture was incubated overnight at 28 °C.

The next day, cells were harvested via centrifugation and resuspended in 40 mL of Buffer B (20 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole pH = 7.4). Lysozyme, DNAse, and protease inhibitor were added to the cell suspension and the mixture was incubated at room temperature for 20 min. Cells were lysed via sonication and cell debris was removed by centrifugation. Cleared cell lysate was poured over a column of Ni-NTA resin, which had been pre-equilibrated with Buffer B. The resin was washed with five column volumes of Buffer B and the protein was eluted with a gradient of 30 mM to 500 mM imidazole in the same buffer system. Fractions containing PfHPPK–DHPS were identified via SDS-PAGE, pooled, and exchanged into 20 mM Tris-HCl, 300 mM NaCl, 10% glycerol v/v, pH = 7.4 via dialysis. The pure protein was concentrated to 50 μM and stored at –80 °C.

Expression and purification of Δ628–668PfHPPK–DHPS. The Δ628-668PfHPPK-DHPS construct was generated using a Q5 Site-directed Mutagenesis Kit (New England Biolabs, Ipswich, MA). Primers used for deletion were Δ628-668 forward, 5'-GGT GGC TTG GCT ATT GCA-3' and Δ628-668 reverse, 5'-TTG TTG CGT GTT AAT CAC AAC-3'. The full gene coding for wild-type PfHPPK-DHPS was used as a template and the resulting plasmid was verified by sequencing. Heterologous expression of Δ628-668PfHPPK-DHPS was achieved in *E. coli* using the One Shot® BL21(DE3) cell line. A 10 mL starter culture in LB with ampicillin (100 μg/mL) was incubated overnight at 37 °C. The next day, 6 mL of the starter culture was diluted in 1 L of fresh LB-ampicillin (100 μg/mL) and incubated at 37 °C to OD_{600} = 0.6. Induction was initiated by the addition of 0.5 mM IPTG and the culture was incubated overnight at 28 °C.

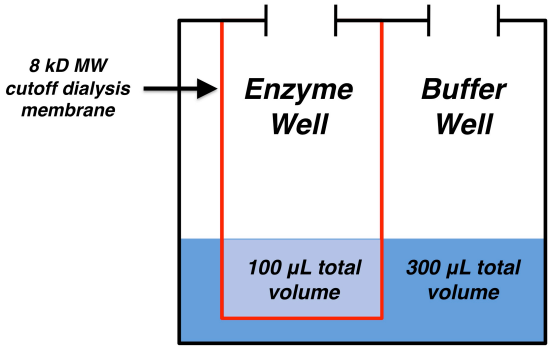
The next day, cells were harvested via centrifugation and resuspended in 40 mL of Buffer B. Lysozyme, DNAse, and protease inhibitor were added to the cell suspension and the mixture was incubated at rt for 20 min. Cells were lysed via sonication and cell debris was removed by centrifugation. Cleared cell lysate was poured over a column of Ni-NTA resin, which had been pre-equilibrated with Buffer B. The resin was washed with five column volumes of Buffer B and the protein was eluted with a gradient of 10 mM to 250 mM imidazole in the same buffer system. Fractions containing Δ628–668PfHPPK–DHPS were identified via SDS-PAGE, pooled, and exchanged into 20 mM Tris-HCl, 300 mM NaCl, 10% glycerol v/v, pH = 7.4 via dialysis. The pure protein was concentrated to 30.3 μM and stored at –80 °C.

Purification of radiolabeled *p*ABAs. [7-^{14}C]*p*ABA and [3,5-^{3}H]*p*ABA were purchased from Moravek Biochemicals Inc., Brea, CA. Stock solutions were made in water with the addition of unlabeled *p*ABA (TCI America) to reach a final *p*ABA concentration of 0.5 mM at 45,800 cpm/μL for [7-^{14}C]*p*ABA and 0.5 mM at 53,200 cpm/μL for [3,5-^{3}H]*p*ABA. Mixtures of [7-^{14}C]*p*ABA and [3,5-^{3}H]*p*ABA were prepared for KIE and BIE measurements and purified via HPLC on a C-18 column (Phenomenex Gemini, 4.6 x 250 mm). The buffers used in this purification were 10 mM NH_4HCO_3 (Buffer C) and 10 mM NH_4HCO_3 in 30% acetonitrile (Buffer D). The method used was 99% Buffer C at 1 mL/min for 1 min followed by a linear gradient of 1-100% Buffer D over 25 min (Method 1). The peak corresponding to the mixture of [7-^{14}C]*p*ABA and [3,5-^{3}H]*p*ABA was collected, evaporated to dryness via Speed Vac, dissolved in reaction buffer, and used directly in the KIE or BIE measurements.

Synthesis and purification of 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPP). DHPP was synthesized from 6-hydroxymethylpterin pyrophosphate (HMPP) using a method adapted from Shiota, T. *et al.*[2] For a typical preparative synthesis, HMPP (~1.0 mg) was dissolved in 200 μL sodium ascorbate solution (100 mg/mL; degassed with Ar) and 50 μL $Na_2S_2O_4$ solution (20 mg/mL; degassed with Ar). The reaction was allowed to sit at rt for 45 min under an Ar atmosphere. The reaction was then directly loaded onto a column of Q Sepharose Fast Flow resin (GE Healthcare) pre-equilibrated with 50 mM NH_4HCO_3. A gradient of 50 mM–250 mM NH_4HCO_3 over 25 min was then applied to the column and elution of the DHPP product was monitored by UV absorbance as 330 nm. The solution containing pure DHPP was frozen and solvent was removed by lyophilization. The resulting light-yellow solid was dissolved in reaction buffer and used directly in the KIE measurements. Concentration of the DHPP substrate was quantified by UV absorbance using an extinction coefficient of 6,200 M^{-1} cm^{-1} at 330 nm.[2]

Equilibrium BIE measurements. *General.* All BIE measurements were performed using a rapid equilibrium dialysis (RED) device (Thermo Fisher Scientific). The RED device consists of a polypropylene base with a standard 96-well plate footprint (9 mm x 9 mm) that holds 48 dialysis inserts. Each dialysis insert is constructed as two wells separated by a vertical cylinder of

dialysis membrane (8 kD MW cutoff). In the following experimental details, the well contained within the cylinder of dialysis membrane will be referred to as the "enzyme well" and the outer volume surrounding the dialysis membrane will be referred to as the "buffer well" as illustrated below.



Schematic of RED device inserts used for equilibrium BIE measurements

BIE measurements for SaDHPS. For measurement of BIEs on the SaDHPS•*p*ABA•HMPP ternary complex, the enzyme well contained 40 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTT, 32.5 µM SaDHPS, 14.1 µM *p*ABA (56.5 µM *p*ABA starting concentration prior to equilibration; [7-^{14}C]*p*ABA + [3,5-^{3}H]*p*ABA + unlabeled carrier), and 88 µM HMPP at pH = 8.3 in 100 µL. The buffer well contained only 40 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTT, and 88 µM HMPP at pH = 8.3 in 300 µL. For attempted measurement of BIEs on the SaDHPS•*p*ABA binary complex, conditions for the enzyme and buffer wells were identical to those used for measurements with the ternary complex except that HMPP was not added.

For a typical measurement, a 2× master mix was prepared for the enzyme well containing all reaction components (except SaDHPS) required for seven measurements in 350 µL. From this master mix, five reactions were removed in a single 250 µL aliquot and mixed with 250 µL of SaDHPS. This mixture was then split into five 100 µL aliquots and served as the experimental replicates. The remaining 100 µL of the master mix was combined with 100 µL of buffer and split into two 100 µL aliquots; these samples served as 'no-enzyme controls' to monitor equilibration of [7-^{14}C]*p*ABA and [3,5-^{3}H]*p*ABA across the dialysis membrane.

The dialysis inserts were covered with Crystal Clear Sealing Film (Hampton Research, Aliso Viejo, CA) and the RED device was shaken at 300 rpm for 3 hr at 25-27 ºC. Equivolume

samples (50 μL) were then taken from the enzyme and buffer wells of each dialysis insert. Samples were diluted in 450 μL H_2O and mixed with 10 mL of scintillation fluid. Scintillation counting was carried out using a Tri-carb 2910TR scintillation counter (Perkin-Elmer, Gaithersburg, MD), which is a dual-channel instrument that registers the signal for ^3H in Channel A and the signal for ^{14}C in both Channel A and Channel B. As such, the raw data must be deconvoluted to determine total counts for [3,5-^{3}H]*p*ABA and [7-^{14}C]*p*ABA. The control sample of [7-^{14}C]*p*ABA was used to determine the proportion of signal overlap between Channel A and Channel B for ^{14}C, as defined by Eq. 1

$$r = \text{Channel A / Channel B} \quad (1)$$

Spectral deconvolution of the BIE data was achieved for ^3H and ^{14}C using Eq. 2 and Eq. 3, respectively:

$$^3\text{H} = \text{Channel A} - (\text{Channel B} \times r) \quad (2)$$

$$^{14}\text{C} = \text{Channel B} + (\text{Channel B} \times r) \quad (3)$$

BIEs were calculated from Eq. 4 where $^{14}C_{BW}$ and $^{14}C_{EW}$ are the total counts of [7-^{14}C]*p*ABA in the buffer well and enzyme well (respectively), and $^3H_{BW}$ and $^3H_{EW}$ are the total counts of [3,5-^{3}H]*p*ABA in the buffer well and enzyme well (respectively).

$$\text{BIE} = [(^{14}C_{EW} / {}^{14}C_{BW}) - 1] / [(^3H_{EW} / {}^3H_{BW}) - 1] \quad (4)$$

BIE measurements for PfHPPK–DHPS. For measurement of BIEs on the PfHPPK-DHPS•*p*ABA•HMPP ternary complex, the enzyme well contained 20 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, 5 μM PfHPPK-DHPS, 6.3 μM *p*ABA (25 μM *p*ABA starting concentration prior to equilibration; [7-^{14}C]*p*ABA + [3,5-^{3}H]*p*ABA + unlabeled carrier), and 6.3 μM HMPP at pH = 9.0 in 100 μL. The buffer well contained only 20 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM DTT at pH = 9.0 in 300 μL. For attempted measurement of BIEs on the PfHPPK–DHPS•*p*ABA binary complex, conditions for the enzyme and buffer wells were identical to those used for measurements with the ternary complex except that HMPP was not added.

For a typical measurement, a master mix was prepared for the enzyme well containing all reaction components (except PfHPPK-DHPS) required for seven measurements in 700 μL. This

mixture was then split into two 300 μL aliquots and served as the experimental replicates. The remaining 100 μL of the master mix was used as 'no-enzyme control' to monitor equilibration of [7-^{14}C]*p*ABA and [3,5-^{3}H]*p*ABA across the dialysis membrane. The BIEs were then measured and calculated in the same manner as SaDHPS above.

Measurement of *V/K* KIEs for SaDHPS and PfHPPK–DHPS. KIEs on the DHPS reaction were measured at 37 °C using the competitive radiolabel approach.[3,4] Typical reaction conditions for the KIE measurements were: 40 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM DTT, 800 μM DHPP, 400 μM *p*ABA ([7-^{14}C]*p*ABA + [3,5-^{3}H]*p*ABA + unlabeled carrier), 20 nM DHPS, pH = 8.3. For a typical KIE measurement, a master mix containing all reaction components (except DHPS) was prepared and distributed into equivolume aliquots. Then, DHPS was added to aliquots designated as experimental reactions and an equivalent volume of buffer was added to aliquots designated as no-enzyme controls. The DHPS reaction was allowed to proceed to ~50% completion and then quenched by the addition of ethylenediaminetetraacetic acid (EDTA) to a final concentration of 20 mM. HPLC Method 1 was used to purify remaining *p*ABA substrate and the DHP product from the quenched reactions. A sample of [7-^{14}C]*p*ABA was also purified in this manner for the purposes of spectral deconvolution.

Purified *p*ABA and DHP samples from the KIE reactions were evaporated to dryness in 20 mL scintillation vials via Speed Vac and then dissolved in 500 uL H_2O. To each vial was then added 10 mL of scintillation fluid and scintillation counting was performed on each sample over 10 cycles at 10 min/cycle. Scintillation counting and spectral deconvolution of the resulting data was performed as described for the BIE measurements.

V/K KIE values were calculated from Eq. 5, where R_0 and R_f are the ratio of [3,5-^{3}H]*p*ABA to [7-^{14}C]*p*ABA prior to the reaction (*i.e.*, no-enzyme control) and at partial conversion, respectively, and *f* is the fraction of substrate conversion.

$$KIE_{V/K} = \ln(1 - f) / \ln[(1 - f) \times (R_f / R_0)] \quad (5)$$

Computational Methods. *General.* DFT calculations were carried out using the B3LYP functional and 6-31g(d) basis set as implemented in Gaussian 09.[1] Equilibrium BIEs were calculated from the scaled vibrational frequencies of optimized structures of *p*ABA in the free and bound states using the ISOEFF98 program.[5] The optimized structure of *p*ABA in the free state was generated using water as an implicit solvent model and was identical for all BIE calculations. Bound states of *p*ABA were generated by optimizing structures using the solvent models and/or geometric constraints outlined below.

Calculations for the free state of pABA. The free state of *p*ABA used for all BIE calculations was the energy-minimized structure using water as an implicit solvent (polarizable continuum model).

Atomic coordinates for optimized structure of *p*ABA in water:

O	-2.84687400	1.13002900	0.00560600
C	-2.28158500	0.00000000	0.00353700
O	-2.84685200	-1.13004200	0.00597200
C	-0.75008100	0.00004100	-0.00246700
C	-0.02173800	-1.19761500	-0.00360700
C	1.37118100	-1.20785000	-0.00606200
C	2.09353400	-0.00001800	-0.00808000
N	3.49351200	-0.00000900	-0.07745300
C	1.37123300	1.20782600	-0.00591900
C	-0.02169500	1.19765100	-0.00347400
H	-0.57498100	-2.13220100	0.00119200
H	1.91291400	-2.15223100	-0.00674500
H	3.92707100	0.83188200	0.30827000
H	1.91300200	2.15219000	-0.00643700
H	-0.57490400	2.13226600	0.00147900
H	3.92703300	-0.83194000	0.30821800

Influence of bound-state dielectric constant on calculated BIEs. BIEs in **Figure 2a** and **Table S1** were calculated from energy-minimized structures of *p*ABA using a series of implicit solvent models with decreasing dielectric constants: water (78.4), methanol (32.6), acetone (20.5), dichloroethane (10.1), chlorobenzene (5.7), and carbon tetrachloride (2.2).[1] No geometric or bond length constraints were imposed on the bound structures.

Atomic coordinates for optimized structure of *p*ABA in carbon tetrachloride:

O	2.84106900	1.13360900	-0.01538000
C	2.29273800	-0.00020600	0.00704100
O	2.83882200	-1.13483300	0.04267400
C	0.74959600	0.00054500	-0.00876400
C	0.02393200	-1.19627600	-0.02302000
C	-1.37047900	-1.20576000	-0.02723900
C	-2.08921800	0.00020700	-0.01305200
N	-3.50333500	0.00057100	-0.06788200
C	-1.37076400	1.20635600	-0.00159600
C	0.02373000	1.19730800	-0.00151100
H	0.58902700	-2.12423300	-0.02479500
H	-1.91496100	-2.15011600	-0.04077800
H	-3.91003300	0.82245600	0.36760800
H	-1.91551000	2.15061400	0.00589600
H	0.58808400	2.12576300	0.00997800
H	-3.90960400	-0.83173200	0.34776100

Atomic coordinates for optimized structure of *p*ABA in chlorobenzene:

O	2.84312500	1.13148000	0.04929000
C	2.28616000	0.00016500	0.00505700
O	2.84449700	-1.13070000	-0.03128900
C	0.74980200	-0.00030200	-0.00491200
C	0.02271200	-1.19739300	0.00777500
C	-1.37094200	-1.20702600	0.00796000
C	-2.09155600	-0.00020900	-0.01024700
N	-3.49778000	-0.00104000	-0.07356600
C	-1.37080700	1.20658400	-0.02714100
C	0.02276900	1.19680800	-0.02190000
H	0.58071500	-2.12935700	0.02273700
H	-1.91376700	-2.15136900	0.02049800
H	-3.91985600	0.83502800	0.31668800
H	-1.91357600	2.15091200	-0.04376700
H	0.58130100	2.12849000	-0.02722400
H	-3.92015900	-0.82442800	0.34247100

Atomic coordinates for optimized structure of *p*ABA in dichloroethane:

O	2.84529900	-1.13047000	-0.02265300
C	2.28405200	0.00008900	0.00433800
O	2.84494400	1.13066700	0.03793100
C	0.74991000	0.00005500	-0.00391100
C	0.02229700	1.19728400	-0.01671100
C	-1.37105600	1.20709800	-0.02107700
C	-2.09241200	-0.00013300	-0.00926800
N	-3.49584600	-0.00090700	-0.07523500
C	-1.37092900	-1.20735200	0.00509700
C	0.02235600	-1.19736200	0.00561600
H	0.57826500	2.13043400	-0.01984800
H	-1.91336300	2.15143200	-0.03359600
H	-3.92320700	-0.82724700	0.32934500
H	-1.91323800	-2.15172100	0.01482400
H	0.57850100	-2.13035000	0.01827100
H	-3.92328000	0.83416100	0.31091900

Atomic coordinates for optimized structure of *p*ABA in acetone:

O	-2.84616300	-1.13038400	-0.00056400
C	-2.28270200	0.00002100	0.00397800
O	-2.84607200	1.13039000	0.01418700
C	-0.75000700	0.00001000	-0.00334700
C	-0.02198700	1.19752900	-0.00741600
C	1.37113200	1.20760000	-0.01020200
C	2.09306900	-0.00000100	-0.00854700
N	3.49452600	-0.00017700	-0.07611700
C	1.37112300	-1.20760400	-0.00377300
C	-0.02198600	-1.19752400	-0.00197600
H	-0.57631000	2.13157700	-0.00473200
H	1.91306500	2.15201000	-0.01385600
H	3.92537400	-0.83093800	0.31519600
H	1.91307500	-2.15201200	-0.00197100
H	-0.57631500	-2.13156600	0.00468300
H	3.92546500	0.83193400	0.31221700

Atomic coordinates for optimized structure of *p*ABA in methanol:

O	2.84652600	1.13021000	0.00576500
C	2.28212100	0.00000700	0.00379200
O	2.84646600	-1.13024500	0.00681300
C	0.75009200	0.00002100	-0.00290100
C	0.02185200	-1.19755700	-0.00429900
C	-1.37113700	-1.20768800	-0.00674400
C	-2.09332300	0.00003100	-0.00833300
N	-3.49405500	-0.00003800	-0.07676800
C	-1.37116800	1.20770500	-0.00625800
C	0.02185600	1.19756000	-0.00391600
H	0.57561200	-2.13189700	0.00032200
H	-1.91300400	-2.15207200	-0.00776600
H	-3.92632000	0.83167300	0.31088200
H	-1.91297200	2.15212800	-0.00685700
H	0.57560100	2.13191200	0.00099700
H	-3.92623600	-0.83167200	0.31112800

Influence of H3–C3–C4–C5 and H5–C5–C4–C3 dihedral restriction on calculated BIEs. BIEs in **Figure 2b** and **Table S2** were calculated using bound structures with symmetric restriction of the H3–C3–C4–C5 and H5–C5–C4–C3 dihedral angles. A total of 10 structures were generated by fixing the H3–C3–C4–C5 and H5–C5–C4–C3 dihedral angles at the values listed in **Table S2** prior to energy minimization. Acetone was used as an implicit solvent model in the optimization of each structure.

Atomic coordinates for optimized structure of *p*ABA with fixed dihedral angles H3–C3–C4–C5 = 178.940º and H5–C5–C4–C3 = –178.943º:

O	-2.8462400000	1.1304100000	0.0044670000
C	-2.2826260000	-0.0000010000	0.0023920000
O	-2.8462330000	-1.1304140000	0.0047400000
C	-0.7501330000	0.0000020000	-0.0035630000
C	-0.0219380000	-1.1974350000	-0.0018760000
C	1.3711500000	-1.2075480000	-0.0040370000
C	2.0932440000	0.0000030000	-0.0116980000
N	3.4942230000	0.0000010000	-0.0812830000
C	1.3711500000	1.2075500000	-0.0039250000
C	-0.0219390000	1.1974370000	-0.0017780000
H	-0.5762000000	-2.1314940000	0.0070220000
H	1.9129930000	-2.1519740000	0.0076450000
H	3.9265980000	0.8316710000	0.3062670000
H	1.9129900000	2.1519760000	0.0078990000
H	-0.5761960000	2.1314990000	0.0071930000
H	3.9265940000	-0.8317020000	0.3062020000

Atomic coordinates for optimized structure of *p*ABA with fixed dihedral angles H3–C3–C4–C5 = 177.940º and H5–C5–C4–C3 = –177.943º:

O	-2.8462880000	1.1303940000	0.0025090000
C	-2.2826020000	-0.0000010000	0.0009170000
O	-2.8462830000	-1.1303980000	0.0027760000
C	-0.7501140000	0.0000030000	-0.0038700000
C	-0.0219130000	-1.1974280000	0.0006890000
C	1.3711890000	-1.2075630000	-0.0013210000
C	2.0932210000	0.0000020000	-0.0145970000
N	3.4940510000	0.0000010000	-0.0863040000
C	1.3711900000	1.2075650000	-0.0012120000
C	-0.0219140000	1.1974310000	0.0007840000
H	-0.5761640000	-2.1314470000	0.0134910000
H	1.9129160000	-2.1518230000	0.0227240000
H	3.9271810000	0.8317260000	0.3003660000
H	1.9129140000	2.1518230000	0.0229720000
H	-0.5761590000	2.1314520000	0.0136570000
H	3.9271770000	-0.8317550000	0.3003040000

Atomic coordinates for optimized structure of *p*ABA with fixed dihedral angles H3–C3–C4–C5 = 176.940º and H5–C5–C4–C3 = –176.943º:

O	2.8463460000	-1.1303760000	0.0004710000
C	2.2825860000	0.0000010000	-0.0005870000
O	2.8463410000	1.1303790000	0.0007250000
C	0.7500810000	-0.0000030000	-0.0040840000
C	0.0218750000	1.1974190000	0.0033580000
C	-1.3712530000	1.2075830000	0.0014530000
C	-2.0931710000	-0.0000020000	-0.0174990000
N	-3.4938560000	-0.0000010000	-0.0914570000
C	-1.3712540000	-1.2075840000	0.0015580000
C	0.0218760000	-1.1974200000	0.0034480000
H	0.5761250000	2.1313760000	0.0201010000
H	-1.9128350000	2.1515350000	0.0378120000
H	-3.9277640000	-0.8317840000	0.2942930000
H	-1.9128330000	-2.1515330000	0.0380510000
H	0.5761210000	-2.1313800000	0.0202580000
H	-3.9277610000	0.8318110000	0.2942350000

Atomic coordinates for optimized structure of *p*ABA with fixed dihedral angles H3–C3–C4–C5 = 175.940º and H5–C5–C4–C3 = –175.943º:

O	2.8459190000	-1.1304890000	-0.0021010000
C	2.2825100000	0.0000000000	-0.0021680000
O	2.8459220000	1.1304880000	-0.0019210000
C	0.7500160000	0.0000000000	-0.0036090000
C	0.0219570000	1.1974680000	0.0068920000
C	-1.3711560000	1.2076310000	0.0042410000
C	-2.0928300000	-0.0000020000	-0.0203670000
N	-3.4933750000	-0.0000020000	-0.0973160000
C	-1.3711560000	-1.2076310000	0.0043140000
C	0.0219580000	-1.1974660000	0.0069550000
H	0.5763030000	2.1313040000	0.0273910000
H	-1.9125930000	2.1510960000	0.0529360000
H	-3.9281610000	-0.8318320000	0.2874600000
H	-1.9125890000	-2.1510920000	0.0531180000
H	0.5763040000	-2.1313020000	0.0275020000
H	-3.9281590000	0.8318460000	0.2874230000

Atomic coordinates for optimized structure of *p*ABA with fixed dihedral angles H3–C3–C4–C5 = 174.940º and H5–C5–C4–C3 = –174.943º:

O	2.8458180000	-1.1305050000	-0.0041270000
C	2.2824300000	0.0000000000	-0.0036680000
O	2.8458210000	1.1305040000	-0.0039810000
C	0.7499700000	0.0000000000	-0.0038320000
C	0.0219400000	1.1974600000	0.0096070000
C	-1.3711930000	1.2076640000	0.0069640000
C	-2.0926550000	-0.0000020000	-0.0232460000
N	-3.4930140000	-0.0000020000	-0.1024140000
C	-1.3711930000	-1.2076640000	0.0070220000
C	0.0219410000	-1.1974580000	0.0096570000
H	0.5763390000	2.1311720000	0.0339470000
H	-1.9124370000	2.1505190000	0.0680160000
H	-3.9286320000	-0.8319170000	0.2812980000
H	-1.9124330000	-2.1505150000	0.0681730000
H	0.5763410000	-2.1311690000	0.0340340000
H	-3.9286310000	0.8319270000	0.2812710000

Atomic coordinates for optimized structure of *p*ABA with fixed dihedral angles H3–C3–C4–C5 = 173.940º and H5–C5–C4–C3 = –173.943º:

O	2.8457180000	-1.1305150000	-0.0060880000
C	2.2823400000	0.0000000000	-0.0051560000
O	2.8457210000	1.1305130000	-0.0059730000
C	0.7499100000	0.0000000000	-0.0041310000
C	0.0219100000	1.1974490000	0.0122390000
C	-1.3712490000	1.2077040000	0.0096500000
C	-2.0924540000	-0.0000010000	-0.0261100000
N	-3.4926230000	-0.0000010000	-0.1074130000
C	-1.3712490000	-1.2077040000	0.0096950000
C	0.0219120000	-1.1974470000	0.0122770000
H	0.5763780000	2.1310130000	0.0403850000
H	-1.9122670000	2.1498040000	0.0830850000
H	-3.9290570000	-0.8320090000	0.2752340000
H	-1.9122620000	-2.1498000000	0.0832180000
H	0.5763790000	-2.1310100000	0.0404500000
H	-3.9290560000	0.8320140000	0.2752170000

Atomic coordinates for optimized structure of *p*ABA with fixed dihedral angles H3–C3–C4–C5 = 172.940º and H5–C5–C4–C3 = –172.943º:

O	2.8456290000	-1.1305150000	-0.0079790000
C	2.2822420000	0.0000000000	-0.0066300000
O	2.8456320000	1.1305130000	-0.0078900000
C	0.7498340000	0.0000010000	-0.0045090000
C	0.0218660000	1.1974350000	0.0147800000
C	-1.3713260000	1.2077510000	0.0122970000
C	-2.0922300000	-0.0000010000	-0.0289550000
N	-3.4922060000	-0.0000010000	-0.1123080000
C	-1.3713250000	-1.2077510000	0.0123320000
C	0.0218670000	-1.1974330000	0.0148080000
H	0.5764150000	2.1308270000	0.0466930000
H	-1.9120820000	2.1489500000	0.0981410000
H	-3.9294410000	-0.8321070000	0.2692720000
H	-1.9120770000	-2.1489450000	0.0982560000
H	0.5764160000	-2.1308240000	0.0467410000
H	-3.9294390000	0.8321090000	0.2692630000

Atomic coordinates for optimized structure of *p*ABA with fixed dihedral angles H3–C3–C4–C5 = 171.940º and H5–C5–C4–C3 = –171.943º:

O	2.8455550000	-1.1305030000	-0.0098100000
C	2.2821380000	0.0000000000	-0.0080910000
O	2.8455580000	1.1305010000	-0.0097400000
C	0.7497420000	0.0000010000	-0.0049560000
C	0.0218040000	1.1974180000	0.0172380000
C	-1.3714250000	1.2078060000	0.0149100000
C	-2.0919870000	-0.0000010000	-0.0317780000
N	-3.4917680000	-0.0000010000	-0.1171120000
C	-1.3714250000	-1.2078060000	0.0149370000
C	0.0218060000	-1.1974160000	0.0172590000
H	0.5764470000	2.1306150000	0.0528860000
H	-1.9118850000	2.1479560000	0.1131860000
H	-3.9297880000	-0.8322120000	0.2633980000
H	-1.9118790000	-2.1479520000	0.1132860000
H	0.5764490000	-2.1306120000	0.0529210000
H	-3.9297870000	0.8322120000	0.2633950000

Atomic coordinates for optimized structure of *p*ABA with fixed dihedral angles H3–C3–C4–C5 = 170.940º and H5–C5–C4–C3 = –170.943º:

O	2.8454950000	-1.1304790000	-0.0115890000
C	2.2820280000	0.0000000000	-0.0095380000
O	2.8454980000	1.1304780000	-0.0115330000
C	0.7496350000	0.0000000000	-0.0054590000
C	0.0217260000	1.1973980000	0.0196190000
C	-1.3715480000	1.2078670000	0.0174960000
C	-2.0917240000	-0.0000010000	-0.0345780000
N	-3.4913060000	0.0000000000	-0.1218380000
C	-1.3715480000	-1.2078670000	0.0175160000
C	0.0217280000	-1.1973960000	0.0196360000
H	0.5764740000	2.1303790000	0.0589750000
H	-1.9116710000	2.1468230000	0.1282200000
H	-3.9301050000	-0.8323230000	0.2575890000
H	-1.9116660000	-2.1468180000	0.1283080000
H	0.5764760000	-2.1303760000	0.0590030000
H	-3.9301040000	0.8323220000	0.2575920000

Atomic coordinates for optimized structure of *p*ABA with fixed dihedral angles H3–C3–C4–C5 = 169.940º and H5–C5–C4–C3 = –169.943º:

O	2.8456510000	-1.1303490000	-0.0133380000
C	2.2819410000	0.0000000000	-0.0109670000
O	2.8456540000	1.1303480000	-0.0132930000
C	0.7494790000	0.0000010000	-0.0059590000
C	0.0215630000	1.1973670000	0.0219000000
C	-1.3717640000	1.2079270000	0.0200960000
C	-2.0915160000	-0.0000010000	-0.0373240000
N	-3.4908970000	0.0000000000	-0.1265380000
C	-1.3717640000	-1.2079270000	0.0201110000
C	0.0215650000	-1.1973640000	0.0219130000
H	0.5764200000	2.1301030000	0.0650090000
H	-1.9115220000	2.1455360000	0.1432760000
H	-3.9304960000	-0.8324430000	0.2517570000
H	-1.9115170000	-2.1455320000	0.1433560000
H	0.5764210000	-2.1301000000	0.0650300000
H	-3.9304950000	0.8324400000	0.2517630000

Influence of C3–H3 and C5–H5 bond length compression on calculated BIEs. BIEs shown in **Figure 2c** and **Table S3** were calculated from bound structures with symmetric restriction of the C3–H3 and C5–H5 bond lengths. A total of seven structures were calculated by freezing the C3–H3 and C5–H5 bonds at decreasing lengths (99 to 93%), relative to their equilibrium bond lengths, prior to energy optimization. Acetone was used as an implicit solvent in the optimization of each structure.

Atomic coordinates for optimized structure of *p*ABA with C3–H3 and C5–H5 bond lengths fixed at 1.0778 Å:

O	-2.84602200	1.13044300	0.00688800
C	-2.28262800	-0.00000100	0.00404900
O	-2.84601800	-1.13044500	0.00712900
C	-0.74994500	-0.00000600	-0.00349100
C	-0.02167400	-1.19727500	-0.00496700
C	1.37165600	-1.20821000	-0.00723600
C	2.09282600	0.00000600	-0.00861500
N	3.49435600	0.00000300	-0.07548000
C	1.37164700	1.20821800	-0.00712700
C	-0.02168400	1.19727000	-0.00487800
H	-0.57626100	-2.13125700	-0.00040300
H	1.90785100	-2.14321400	-0.00833600
H	3.92574400	0.83146900	0.31352100
H	1.90783200	2.14322800	-0.00814700
H	-0.57627100	2.13125000	-0.00026500
H	3.92574700	-0.83149600	0.31345000

Atomic coordinates for optimized structure of *p*ABA with C3–H3 and C5–H5 bond lengths fixed at 1.0670 Å:

O	-2.84598900	1.13037900	0.00685200
C	-2.28236000	-0.00000100	0.00403800
O	-2.84598600	-1.13038100	0.00709900
C	-0.74975500	-0.00000400	-0.00345400
C	-0.02137200	-1.19716500	-0.00493900
C	1.37213000	-1.20884500	-0.00720600
C	2.09253800	0.00000500	-0.00854000
N	3.49415600	0.00000300	-0.07553100
C	1.37212500	1.20885100	-0.00709600
C	-0.02137900	1.19716200	-0.00484700
H	-0.57597600	-2.13118900	-0.00039900
H	1.90269500	-2.13453600	-0.00832800
H	3.92585800	0.83140200	0.31327800
H	1.90268300	2.13454600	-0.00813400
H	-0.57598000	2.13118600	-0.00025100
H	3.92585900	-0.83142900	0.31320900

Atomic coordinates for optimized structure of *p*ABA with C3–H3 and C5–H5 bond lengths fixed at 1.0561 Å:

O	-2.84591200	1.13033600	0.00681900
C	-2.28210000	-0.00000100	0.00403300
O	-2.84590900	-1.13033800	0.00707100
C	-0.74958100	-0.00000200	-0.00342000
C	-0.02109900	-1.19706200	-0.00491200
C	1.37255600	-1.20940000	-0.00718300
C	2.09228800	0.00000300	-0.00848300
N	3.49398800	0.00000200	-0.07556800
C	1.37255300	1.20940400	-0.00707100
C	-0.02110200	1.19706000	-0.00481900
H	-0.57571700	-2.13112600	-0.00038800
H	1.89752100	-2.12574800	-0.00831500
H	3.92598200	0.83134400	0.31304800
H	1.89751500	2.12575400	-0.00811500
H	-0.57571600	2.13112500	-0.00023300
H	3.92598000	-0.83137200	0.31298000

Atomic coordinates for optimized structure of *p*ABA with C3–H3 and C5–H5 bond lengths fixed at 1.0452 Å:

O	-2.84579900	1.13031200	0.00680500
C	-2.28186000	-0.00000100	0.00403600
O	-2.84579500	-1.13031400	0.00706200
C	-0.74941700	-0.00000100	-0.00340300
C	-0.02084000	-1.19695700	-0.00490600
C	1.37296000	-1.20992100	-0.00717900
C	2.09204400	0.00000300	-0.00844600
N	3.49383800	0.00000200	-0.07556600
C	1.37295900	1.20992400	-0.00706600
C	-0.02084200	1.19695600	-0.00481100
H	-0.57549000	-2.13105100	-0.00039400
H	1.89234200	-2.11691700	-0.00831800
H	3.92608500	0.83128600	0.31289600
H	1.89233900	2.11692200	-0.00811500
H	-0.57548700	2.13105200	-0.00023300
H	3.92608100	-0.83131500	0.31282900

Atomic coordinates for optimized structure of *p*ABA with C3–H3 and C5–H5 bond lengths fixed at 1.0343 Å:

O	-2.84566700	1.13029800	0.00680500
C	-2.28163300	-0.00000100	0.00404100
O	-2.84566400	-1.13029900	0.00706500
C	-0.74925700	0.00000000	-0.00339600
C	-0.02058800	-1.19684900	-0.00491200
C	1.37335500	-1.21042700	-0.00718700
C	2.09179900	0.00000200	-0.00842000
N	3.49369600	0.00000200	-0.07554000
C	1.37335600	1.21042900	-0.00707300
C	-0.02058900	1.19684900	-0.00481500
H	-0.57527900	-2.13096800	-0.00041200
H	1.88716100	-2.10806700	-0.00833200
H	3.92617500	0.83122700	0.31279200
H	1.88716000	2.10807100	-0.00812700
H	-0.57527400	2.13097100	-0.00024700
H	3.92617100	-0.83125700	0.31272500

Atomic coordinates for optimized structure of *p*ABA with C3–H3 and C5–H5 bond lengths fixed at 1.0234 Å:

O	-2.8455280000	1.1302880000	0.0068090000
C	-2.2814150000	-0.0000010000	0.0040470000
O	-2.8455240000	-1.1302900000	0.0070720000
C	-0.7490990000	0.0000010000	-0.0033930000
C	-0.0203370000	-1.1967400000	-0.0049220000
C	1.3737450000	-1.2109200000	-0.0071990000
C	2.0915560000	0.0000020000	-0.0084000000
N	3.4935600000	0.0000020000	-0.0755070000
C	1.3737460000	1.2109220000	-0.0070840000
C	-0.0203370000	1.1967400000	-0.0048240000
H	-0.5750720000	-2.1308830000	-0.0004320000
H	1.8819830000	-2.0992110000	-0.0083500000
H	3.9262600000	0.8311690000	0.3127040000
H	1.8819820000	2.0992140000	-0.0081440000
H	-0.5750670000	2.1308860000	-0.0002650000
H	3.9262550000	-0.8311990000	0.3126370000

Atomic coordinates for optimized structure of *p*ABA with C3–H3 and C5–H5 bond lengths fixed at 1.0125 Å:

O	-2.84542700	1.13025600	0.00678200
C	-2.28122000	0.00000000	0.00404800
O	-2.84542500	-1.13025700	0.00704600
C	-0.74894300	0.00000100	-0.00336900
C	-0.02009100	-1.19663300	-0.00490600
C	1.37410700	-1.21130500	-0.00716900
C	2.09136900	0.00000100	-0.00829800
N	3.49350000	0.00000200	-0.07556600
C	1.37411100	1.21130600	-0.00705300
C	-0.02008900	1.19663300	-0.00480800
H	-0.57484200	-2.13082100	-0.00045900
H	1.87682500	-2.09020800	-0.00836300
H	3.92643700	0.83111800	0.31250100
H	1.87682800	2.09020900	-0.00815500
H	-0.57483400	2.13082500	-0.00028700
H	3.92643000	-0.83114700	0.31243500

D. SUPPORTING INFORMATION REFERENCES

1. Gaussian 09, Revision D.01, Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, J. A., Jr.; Peralta, J. E.; Ogliaro, F.; Bearpark, M.; Heyd, J. J.; Brothers, E.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, J. M.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. Gaussian, Inc., Wallingford CT, 2009.
2. Shiota, T.; Baugh, C. M.; Jackson, R.; and Dillard, R. "The enzymatic synthesis of hydroxymethyldihydropteridine pyrophosphate and dihydrofolate." *Biochemistry* **1969**, *8*, 5022–5028.
3. Schramm, V. L. "Enzymatic transition-state analysis and transition-state analogs." *Methods Enzymol.* **1999**, *308*, 301–355.
4. Schramm, V. L. "Enzymatic transition states and transition state analog design." *Annu. Rev. Biochem.* **1998**, *67*, 693–720.
5. Anisimov, V. P., P. "ISOEFF98: A program for studies of isotope effects using Hessian modifications." *J. Math Chem.* **1999**, *26*, 75–86.