Dihydrodipicolinate synthase is not inhibited by its substrate, (S)-aspartate β -semialdehyde

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DHDPS (dihydrodipicolinate synthase; EC 4.2.1.52) is the enzyme that catalyses the first unique step of lysine biosynthesis in plants and micro-organisms. As such, it has attracted much attention as a target for herbicide and anti-microbial action. DHDPS has two substrates: pyruvate and (S)-aspartate β -semialdehyde [(S)-ASA]. There are various literature reports that suggest that high levels of (S)-ASA inhibit the enzyme [Karsten (1997) *Biochemistry* **36**, 1730–1739; Stahly (1969) *Biochim. Biophys. Acta* **191**, 439–451], whereas others have not observed this phenomenon. We have resolved this long-running literature

debate and shown unequivocally that this difference in reported behaviour can be attributed to differences in the preparation of (S)-ASA used by each researcher. DHDPS is not inhibited by its substrate; rather, the inhibition is due to an, as yet, unidentified inhibitor in preparations of the substrate generated by ozonolysis. Furthermore, we demonstrate that (R)-ASA is neither an inhibitor nor a substrate of DHDPS from *Escherichia coli*.

Key words: aspartate semialdehyde, dihydrodipicolinate synthase, lysine biosynthesis, substrate inhibition.

INTRODUCTION

(S)-Lysine is an essential amino acid and, as such, inhibitors of the pathways of (S)-lysine biosynthesis are of interest, as they may be toxic to plants and micro-organisms, but not to animals [1,2]. The potential of lysine biosynthesis as an anti-microbial target is increased because diaminopimelate, a precursor in the *meso*-diaminopimelic acid pathway to (S)-lysine, is essential in bacterial cell-wall development. (S)-Lysine biosynthesis has also attracted the attention of biotechnologists. (S)-Lysine is nutritionally limiting for humans and livestock in most cereal crops and feeds [3], and understanding the regulatory properties of this pathway is thus critical for the generation of 'high (S)-lysine' plants.

In prokaryotes and higher plants, DHDPS (dihydrodipicolinate synthase; EC 4.2.1.52) catalyses the first unique step leading to (S)-lysine biosynthesis: an aldol condensation between (S)-aspartate β -semialdehyde [(S)-ASA] and pyruvate [4–7] (Scheme 1). The product of the reaction is an unstable heterocycle, formerly thought to be dihydrodipicolinate, but previously identified as (4S)-4-hydroxy-2,3,4,5-tetrahydro-(2S)-dipicolinate [4]. DHDPS is thought to be a key regulatory point in the pathway, as (S)-lysine is an allosteric feedback inhibitor of DHDPS in both plants [8–12] and *Escherichia coli* [5].

The first comprehensive biochemical analysis of DHDPS was performed by Yugari and Gilvarg [6], and following enzyme purification, they determined its molecular mass, several approaches for collecting kinetic data, and accurately determined its kinetic constants. It was later concluded that the reaction proceeded through a Schiff base because, when in the presence of pyruvate, sodium borohydride irreversibly inactivated the enzyme [7].

The literature describes three methods for collecting kinetic data for DHDPS, and these have been discussed at length by Borthwick et al. [13] and Coulter et al. [14]. Briefly, the easiest of the assays to perform is a discontinuous assay that monitors the formation of an unidentified adduct, after addition of *o*-aminobenzaldehyde, in the presence of acid. It is simple to set up and appears very sensitive to DHDPS activity, but shows an appreciable lag phase and is therefore inappropriate for the collection

of initial rate data. The second assay directly monitors the change in absorbance at 270 nm in the presence of imidazole buffer. This is thought to correspond to the spontaneous formation of dipicolinate from dihydrodipicolinate [13], but again the details of the chemistry are unclear and the recording of initial rate data cannot be guaranteed. The third assay indirectly measures the reaction by monitoring the utilization of NADPH at 340 nm by DHDPR (dihydrodipicolinate reductase), which is used as a coupling enzyme. This assay system allows the accurate monitoring of initial rate data and was used in the present study.

A ping-pong kinetic mechanism has been proposed for DHDPS based on the observation of initial velocity patterns that appear to be parallel [5,9,12,15]. The ping-pong kinetic mechanism, also known as the substituted enzyme mechanism or the double-displacement mechanism [16], requires two stable enzyme forms with the presence of an irreversible step between the binding of the two substrates; this is typically release of the first product [5]. For DHDPS, the proposed ping-pong mechanism involves the binding of pyruvate to the free enzyme form (E), followed by the release of water and enamine formation, which has been suggested to be irreversible [5]. (S)-ASA then binds to the stable substituted-enzyme form (F), followed by release of product and regeneration of the free enzyme form (E).

A number of conflicting reports have appeared in the literature concerning the K_m for each of the substrates, the nature of (S)-lysine inhibition, and the observation of substrate inhibition by (S)-ASA at high concentrations.

Stahly [17] first reported inhibition by (S)-ASA and suggested that it affected pyruvate binding, but not the maximal velocity. This was no surprise, because substrate inhibition is predicted by the ping-pong kinetic mechanism such that binding of (S)-ASA to the active site subsequently blocks access for pyruvate. Later studies have also reported (S)-ASA inhibition of DHDPS isolated from a variety of plant sources [3,9,18]. Given that different workers used different enzymes from different organisms, and different assays, it seemed that these discrepancies could arise from a variety of sources. However, a previous report on the kinetics of the *E. coli* enzyme appears to show unambiguous

Abbreviations used: DHDPR, dihydrodipicolinate reductase; DHDPS, dihydrodipicolinate synthase; (S)-ASA, (S)-aspartate β -semialdehyde.

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Scheme 1 Biosynthesis of the aspartate family of amino acids, initially derived from the citric acid cycle

The branch point for lysine biosynthesis is mediated by DHDPS, which involves the condensation and cyclization of pyruvate and (S)-ASA. In addition to lysine, isoleucine, threonine and methionine are also synthesized.

substrate inhibition with respect to (S)-ASA [5]. This result contradicts the work in our laboratory, where substrate inhibition has not been observed [14,19]. Both our own work [14,19] and the work of Karsten [5] employed the *E. coli* enzyme and the coupled assay with DHDPR. Thus the difference in reported kinetic behaviour demanded a re-evaluation of the data. In the present study, we repeat Karsten's work, compare it with our own, and unequivocally identify the reason for the differences in the reported behaviour. We also demonstrate that (R)-ASA is neither a substrate nor an inhibitor of *E. coli* DHDPS.

EXPERIMENTAL

Materials

The assay materials pyruvate, NADPH, HEPES and (S)-lysine were all purchased from Sigma. Chromatography media were obtained from Pharmacia (Amersham Biosciences, Auckland, New Zealand) and run in Pharmacia XK columns with a Gilson fraction collector and low-pressure pumps. Initial velocity data were collected using a Hewlett Packard 8452A diode array spectrophotometer, equipped with a circulating water bath to maintain a constant temperature of 30 °C. Unless otherwise stated, manipulation of enzymes was performed at 0–4 °C. Protein concentrations were determined by the method of Bradford [20] using BSA as a standard.

Synthesis of (S)-ASA

(S)-ASA was synthesized by the methods of Coulter et al. [21], Roberts et al. [19] or Black and Wright [22]. Overall yields

for (S)-ASA were 55 % for the method of Coulter et al. [21], 70 % for the method of Roberts et al. [19] and 85 % for the method of Black and Wright [22]. ¹H NMR data for (S)-ASA synthesized by Roberts et al. or Coulter et al. were consistent with values reported in the literature [19,21]. However, for (S)-ASA generated by ozonolysis, ¹H NMR results were consistent with the formation of a mixture of compounds, one of which corresponded to (S)-ASA. When synthesized by this method, (S)-ASA was not found to be purified further by column chromatography as in the original paper [22]. The purity of all preparations was assessed by the coupled assay using DHDPS and DHDPR, where (S)-ASA was the limiting substrate: thus, the total turnover of NADPH was equivalent to the turnover of (S)-ASA. For the methods of Roberts et al. [19] and Coulter et al. [21], the purity was > 95 %, and for that of Black and Wright [22], the purity was 90 %.

Purification of DHDPS

E. coli XL-1 Blue (pJG001) (2 litres) [14] was cultured (37 °C) to late-log phase after being inoculated with 1 ml of starter culture. The cells were chilled on ice for 30 min, harvested by centrifugation (5000 *g* for 10 min), and were washed in 20 mM Tris/HCl (pH 8.0) at 4 °C (buffer A) and again centrifuged (5000 *g* for 10 min). The supernatant was discarded and the cell pellet was re-suspended in buffer A. To obtain the crude enzyme extract, the cells were subjected to flash freezing in liquid nitrogen (–196 °C) followed by slowly thawing on ice at 4 °C overnight and, finally, ultrasonication (for 4 min in 3 s bursts with 10 s between each burst). After centrifugation (5000 *g* for 10 min) to remove the cell debris, the supernatant, containing DHDPS, was collected.

The crude extract was loaded on to a Q-Sepharose ion-exchange column that had been pre-equilibrated with 3 bed vol. of buffer A. The column was washed with 5 bed vol. of buffer A and the enzyme was eluted with a 0–1 M NaCl gradient in buffer A. The eluted fractions were tested for activity using the *o*-aminobenzaldehyde assay [6], and it showed an elution peak corresponding to approx. 0.5 M NaCl. Active fractions were pooled and, after dialysis against buffer A, ammonium sulphate was added to a concentration of 0.5 M. This was then loaded on to a phenyl-Sepharose exchange column that had been pre-equilibrated with 0.5 M ammonium sulphate in buffer A. The column was washed with 5 bed vol. of the start buffer (0.5 M ammonium sulphate in buffer A) and the enzyme was eluted with a 0.5–0 M ammonium sulphate gradient. The active fractions, as assessed by the *o*-aminobenzaldehyde assay, were eluted between 0.1 and 0 M ammonium sulphate, and these were pooled.

After dialysis against buffer A with 1 mM 2-mercaptoethanol, 1 mM EDTA and 1 % (w/v) ammonium sulphate, the partially purified enzyme was loaded on to a Hi-Trap Q-Sepharose column (1 ml bed volume) that had been pre-equilibrated with 3 bed vol. of buffer A. The column was then washed with 5 bed vol. of buffer A and the enzyme was eluted with a 0–1 M NaCl gradient. Active fractions were pooled, dialysed as above, and the purity was assessed by SDS/PAGE using Coomassie Brilliant Blue stain.

Purification of DHDPR

E. coli XL-1 Blue cells containing the plasmid pJK001 [14], which carried the *E. coli* *dapB* gene, were harvested, washed, subjected to one freeze–thaw as with the purification of DHDPS, then ultrasonicated on ice (4 min in 3 s bursts with 10 s between each burst). The supernatant was collected by centrifugation (10000 *g* for 10 min) and was heat-shocked in 1 ml aliquots in Eppendorf tubes at 70 °C for 2 min followed immediately by cooling on ice. The precipitated proteins were removed by centrifugation

(10000 g for 10 min), and the supernatant was collected. The dialysed sample was loaded on to a Q-Sepharose ion-exchange column and then washed with 5 bed vol. of buffer A. The enzyme was eluted with a 0–1 M NaCl gradient in buffer A. The eluted fractions were tested for DHDPS activity using the coupled assay. Active fractions were eluted between 0.6 and 0.7 M NaCl, and were pooled. After dialysis against buffer A, the enzyme was loaded on to a Hi-Trap Blue column (bed vol. 1 ml) that had been pre-equilibrated with 5 bed vol. of buffer A. The column was then washed with 5 bed vol. of buffer A and the enzyme was eluted with a 0–1 M NaCl salt gradient. Active fractions showed a high degree of purity, as judged by SDS/PAGE stained with Coomassie Brilliant Blue.

Liquid chromatography electrospray MS

DHDPS samples for the above-mentioned purification were first purified on a Waters LC2790 liquid chromatography system. The sample was loaded on to a C₅ column (150 mm $\times$ 2 mm) with two C₅ guard columns. Fractions (200 μ l) were eluted with water on an acetonitrile linear gradient for 25 min. DHDPS was typically eluted after 13 min. A sample from each fraction (20 μ l) was injected into the mass spectrometer, whereas the remaining 180 μ l was sent to a diode array for UV/visible spectra analysis.

Electrospray MS was performed on a Micromass LCT mass spectrometer operating with electrospray ionization and using the Micromass time-of-flight technique. Samples were scanned over a mass range between 10 and 40 kDa. The sample cone was 50 V, desolvation temperature was 150 °C, and a 250R_f lens was used. The machine was calibrated with horse heart myoglobin.

Initial velocity data collection and processing

Initial velocity studies were conducted in 1 ml cuvettes and the pH was maintained using Hepes buffer at pH 8 (100 mM). The reaction was followed using a coupled assay system, which monitors the change in absorbance at 340 nm owing to the consumption of NADPH by DHDPR, which is concomitant with the use of the product of DHDPS, dihydrodipicolinate. A typical assay contained 100 mM Hepes, 0.162 mM NADPH, 20 μ g of DHDPR and varied concentrations of (S)-ASA and pyruvate. The reaction mix was incubated for 10 min at 30 °C before initiation of the reaction with (S)-ASA. Care was taken to ensure that the enzymes and substrates were stable over the course of the assay (2 min) and to ensure an excess of DHDPR. Each rate was measured at least in triplicate and the data were fitted to the appropriate rate equation using the program EnzFitter (Biosoft, Cambridge, U.K.). Initial velocities were usually reproducible within 15%. Equation (1) was used to fit ping-pong kinetics, equation (2) to fit Michaelis–Menten constants and equation (3) was used for fitting substrate inhibition:

$$v = V[A][B]/(K_m^A[A] + K_m^B[B] + [A][B]) \quad (1)$$

$$v = V^{(app)}[A]/(K_m^{(app)} + [A]) \quad (2)$$

$$v = V^{(app)}[A]/(K_m^{(app)} + [A] + [A]^2/K_{is}) \quad (3)$$

Here V is the maximal velocity, $V^{(app)}$ the apparent maximal velocity, K_m^A and K_m^B are the Michaelis–Menten constants for the two substrates A (pyruvate) and B [(S)-ASA], $K_m^{(app)}$ the apparent Michaelis–Menten constant, K_{is} the substrate inhibition constant and v the initial velocity [16]. The rate equation of best fit was determined by examining the SIGMA values and the lowest S.E.M. for each of the calculated kinetic constants.

Table 1 Purification of DHDPS

Purification of DHDPS from *E. coli* XL-1 Blue (pJG001) cells. 1 unit is equal to the consumption of 1 μ M NADPH/s, as judged by the coupled assay. Approx. 8 g of cells was used.

	Protein (mg)	Activity		Yield (%)	Degree of purification (fold)
		Total (units)	Specific (units/mg)		
Crude	304	4.85	0.016		
Ion-exchange	35	9.06	0.258	187	16
Phenyl-Sepharose	10	7.64	0.736	158	46
Hi-trap ion-exchange	7	7.58	1.11	156	69

Table 2 Purification of DHDPR

Purification of DHDPR from *E. coli* XL-1 Blue (pJK001) cells. Approx. 11 g of cells was used.

	Protein (mg)	Activity		Yield (%)	Degree of purification (fold)
		Total (units)	Specific (units/mg)		
Crude	1024	13.4	0.013		
Heat-shock	140	13.3	0.095	99	7
Ion-exchange	54	12.5	0.23	94	18
Hi-trap Blue	8	12.3	1.5	92	118

RESULTS AND DISCUSSION

Purification of DHDPS and DHDPR

The method employed to purify DHDPS was based on that of Coulter et al. [14]. DHDPS was purified from *E. coli* XL-1 Blue cells that harboured the plasmid pJG001, which is derived from pBluescript and carries the *dapA* gene encoding for DHDPS [14]. Overexpression of DHDPS in this system allowed large amounts of enzyme to be isolated [14]. The purification data are presented in Table 1 and show that a 156% yield of DHDPS was achieved with a high degree of enrichment. The higher than expected yield has been noticed by other authors [13,23] and was attributed to inhibitors in the crude mix. When visualized by SDS/PAGE stained with Coomassie Brilliant Blue, the preparation displayed a single band at approx. 31 kDa, which corresponds to monomeric DHDPS. Electrospray MS confirmed the molecular mass of DHDPS to be (31.272 ± 0.001) kDa, consistent with a previous study [13]. The specific activity for the final product was 1.1 units/mg, where 1 unit is equal to the consumption of 1 μ M NADPH/s.

Kinetic data for DHDPS were collected using a coupled assay system that followed the consumption of NADPH by the next enzyme in the lysine biosynthetic pathway to (S)-lysine, DHDPR. As with DHDPS, DHDPR had also been cloned into pBluescript [14] and this expression system yielded large amounts of coupling enzyme required by the assay system. The expression and purification procedure afforded a very high yield, 92%, with no other protein evident in the SDS/PAGE when visualized with Coomassie Brilliant Blue. Control assays were undertaken to ensure the absence of contaminating NADPH-utilizing enzymes. The specific activity for the final product was 1.5 units/mg (see Table 2).

Synthesis of ASA

(S)-ASA is an elusive molecule, which for many years evaded rigorous chemical characterization. There are four published

Scheme 2 Four methods for synthesis of (*S*)-ASA

(**A**) Ozonolysis of allyl glycine reported by Black and Wright [22]. (**B**) The route to (*S*)-ASA used by Coulter et al. [21]. (**C**) The method of Tudor et al. [24]. (**D**) One of the two methods reported by Roberts et al. [19]. (*S*)-ASA prepared by methods B/C/D was indistinguishable during kinetic analysis. Only (*S*)-ASA prepared by method A showed apparent substrate inhibition. BOC, *N*-*tert*-butoxycarbonyl; DIBAL, di-*i*-butylaluminium; *p*-MBC, *p*-methoxybenzyl chloride; THF, tetrahydrofuran; TFA, trifluoroacetic acid.

syntheses of (*S*)-ASA, which have been used for the analysis of DHDPS [19,21,22,24]. The first, illustrated in Scheme 2(A), involves the direct ozonolysis of allylglycine, a product that is commercially available both as a pure enantiomer and in racemic form. This synthesis has the virtue of simplicity and remains in common use by many authors [5–7,9,15,17], including Karsten [5]. However, ozonolysis is a complex reaction that is not completely understood and generates many side products [25]; thus (*S*)-ASA produced by this method may contain impurities, which are hard to remove. The exact nature of the impurities in this preparation remains unclear. The limitations of this method have led to the search for new synthetic routes to aspartate semi-aldehyde. In particular, three methods have been published that give a product of high purity as judged by NMR, MS and analysis by the DHDPS/DHDPR-coupled assay [19,21,24]. These are also illustrated in Schemes 2(B)–2(D). They require varying effort and cost while producing different overall yields. We found the

method of Roberts et al. [19] to be the most convenient. Nevertheless, the methods of Tudor et al. [24] and Coulter et al. [21] were also investigated, and (*S*)-ASA prepared by all three methods gave indistinguishable kinetic results with respect to DHDPS. Thus, of the four methods used, only ozonolysis gave aberrant results.

DHDPS and substrate inhibition by (*S*)-ASA

For DHDPS, Karsten [5] appropriately interpreted (*S*)-ASA substrate inhibition as being diagnostic for the ping-pong type kinetic mechanism, with inhibition occurring because (*S*)-ASA can be expected to bind to DHDPS in the absence of pyruvate, generating a dead-end enzyme complex (Scheme 3) [16].

In two separate experiments, we collected initial rate data for DHDPS, repeating the methods of Karsten [5] using (*S*)-ASA prepared by the methods of Roberts et al. [19] and Black and

Scheme 3 Two enzyme forms of DHDPS

E is the unligated enzyme and F is the form with the covalently bound enamine ready for (S)-ASA binding. The binding of (S)-ASA to the E form creates a dead-end enzyme form. DHD, dihydrodipicolinate.

Table 3 Kinetic constants for DHDPS using the alternative ASA preparations and in the presence and absence of (R)-ASA

(a) Alternative ASA preparations. Data for ASA obtained by method A (O_3) were fitted to equation (3), whereas data from methods B/C/D were fitted to equation (2). The R^2 values for the fits were: 0.92 for methods B/C/D and 0.87 for method A. In all experiments, the concentration of pyruvate was 0.015 mM. (b) Kinetic constants generated in the presence and absence of (R)-ASA were fitted with equation (1). Results are means $\pm$ S.E.M.

(a)

	$K_m^{ASA(app)} (mM)$	$K_{IS} (mM)$	$V^{(app)} (\mu mol \cdot s^{-1} \cdot mg^{-1})$
Method A	0.020 ± 0.007	1.1 ± 0.4	0.030 ± 0.003
Methods B/C/D	0.016 ± 0.002	—	0.027 ± 0.007

(b)

	$K_m^{ASA} (mM)$	$K_m^{Pyr} (mM)$	$V (\mu mol \cdot s^{-1} \cdot mg^{-1})$
DHDPS with (S)-ASA	0.11 ± 0.01	0.25 ± 0.03	0.58 ± 0.03
DHDPS with (R/S)-ASA	0.21 ± 0.06	0.30 ± 0.09	0.50 ± 0.09

Wright [22]. The results of the experiment are presented in Table 3(a) and illustrated in Figure 1. There was a striking difference in the behaviour of DHDPS with respect to each preparation. At high (S)-ASA concentrations, DHDPS inhibition occurred when (S)-ASA prepared by ozonolysis was used, but not in the other preparation under identical conditions. Thus inhibition is not caused by (S)-ASA itself. The apparent K_i for the inhibitor in the mixture was 1.1 mM. The present study, comparing alternative preparations of (S)-ASA, suggests that substrate inhibition is caused by an as yet unknown inhibitor and not by (S)-ASA, as concluded by Karsten. It is clear that previous experiments documenting substrate inhibition [5] may result from artifacts associated with the ozonolysis preparation.

Although the kinetic model for a ping-pong mechanism predicts substrate inhibition when (S)-ASA is in high concentration relative to the pyruvate, its apparent absence does not preclude assignment of the ping-pong model. Kumpaisal et al. [9] originally assigned this kinetic mechanism based on initial velocity patterns that appear parallel, and others also observed this result [5,12,15]. The inhibition patterns of substrate and product analogues are also consistent with the ping-pong model [5].

Effects of (R)-ASA on *E. coli* DHDPS

It was hypothesized that (R)-ASA may act as an inhibitor with respect to (S)-ASA, the natural substrate of DHDPS. Moreover, the question of whether DHDPS can utilize (R)-ASA as a substrate has never been fully addressed for the *E. coli* enzyme. Stahly [17] observed that (R)-ASA inhibited DHDPS from *Bacillus*

Figure 1 Kinetic fit of DHDPS with respect to (S)-ASA synthesized by the two methods

■, Data of (S)-ASA prepared by ozonolysis; ----, fit of the ozonolysis data using equation (3); ▲, ASA prepared by osmium tetroxide/sodium periodate oxidation; —, fit of the osmium tetroxide data using equation (2). This plot was generated by Enzfitter.

licheniformis, increasing K_m^{ASA} and decreasing V , but did not act as a substrate under the enzyme assay used. Stahly [17] also used the method of Black and Wright [22] to produce both enantiomeric forms of ASA. We compared the Michaelis–Menten constants of DHDPS with respect to (S)-ASA and (R/S)-ASA, and the results are presented in Table 3(b). The K_m values for (R/S)-ASA (0.21 ± 0.06 mM) were twice that of (S)-ASA (0.11 ± 0.01 mM), which indicate that the enzyme is using only (S)-ASA as a substrate. Moreover, there was no significant difference in either K_m^{Pyr} or V between the two preparations, suggesting that (R)-ASA does not act as a catalytic poison, as suggested by Stahly [17].

Therefore DHDPS shows strong stereoselectivity around the α -carbon of (S)-ASA, consistent with spatial organization of the active site as reported by Blickling et al. [4].

In summary, there are four methods in the literature for synthesizing (S)-ASA for the use of biochemical studies. Analysis of the kinetic behaviour of DHDPS with all but one of these preparations gives Michaelis–Menten behaviour with a true K_m^{ASA} of $111 \pm 10 \mu M$ and K_m^{Pyr} of $260 \pm 30 \mu M$. Analysis of the kinetic behaviour of DHDPS using the fourth preparation, synthesized by ozonolysis of allylglycine, gives apparent substrate inhibition by (S)-ASA at high concentrations. We suggest that this result is an artifact of the preparation used and that *E. coli* DHDPS is not inhibited by its substrate. We suggest further that the earlier results of Karsten [5] can be explained by the presence of an, as yet unidentified, inhibitor in the ozonolysis brew. Further work is being undertaken to identify this molecule.

Moreover, we clarify that (R)-ASA is neither a substrate nor an inhibitor of *E. coli* DHDPS. These unequivocal results resolve a long-running literature debate and set the stage for more accurate kinetic analyses of DHDPS.

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