Chorismate-utilizing enzymes are attractive antimicrobial drug targets due to their absence in humans and their central role in bacterial survival and virulence. The structural and mechanistic homology of a group of these inspired the goal of discovering inhibitors that target multiple enzymes. Previously, we discovered seven inhibitors of 4-amino-4-deoxychorismate synthase (ADCS) in an on-bead, fluorescent-based screen of a 2304-member one-bead-one-compound combinatorial library. The inhibitors comprise PAYLOAD and COMBI stages, which interact with active site and surface residues, respectively, and are linked by a SPACER stage. These seven compounds, and six derivatives thereof, also inhibit two other enzymes in this family, isochorismate synthase (IS) and anthranilate synthase (AS). The best binding compound inhibits ADCS, IS, and AS with $K_i$ values of 720, 56, and 80 $\mu$M, respectively. Inhibitors with varying SPACER lengths show the original choice of lysine to be optimal. Lastly, inhibition data confirm the PAYLOAD stage directs the inhibitors to the ADCS active site.

Antibiotic drug resistance, now a global health issue, has increased dramatically over the past several decades. Rates of methicillin-resistant Staphylococcus aureus (MRSA) infections doubled between the years 2000 and 2005. They are now the cause of more deaths in the United States than AIDS. The low profitability and short functional lifetime of antibiotics has resulted in a steady decrease in research and development by industry. As a result, only two new classes of antibiotics have been introduced since 1967: oxazolidinones (2000) and lipopeptides (2003).

We have developed a streamlined approach for discovering potential antimicrobial lead compounds, entailing massively parallel, on-bead screens of one-bead-one-compound (OBOC), mass-tag-encoded combinatorial libraries with fluorescently labeled enzyme. A small (2304-member), proof-of-concept, peptide-based library was designed to target a set of homologous chorismate-utilizing enzymes: 4-amino-4-deoxychorismate synthase (ADCS), isochorismate synthase (IS), anthranilate synthase (AS), and salicylate synthase (SS). ADCS, IS, AS, and SS catalyze the first committed steps in the formation of folate, enterobactan (an iron-chelating compound, i.e. siderophore), tryptophan, and mycobactin (a siderophore from Mycobacterium tuberculosis), respectively, in bacteria and apicomplexan parasites (Figure 1).

Chorismate-utilizing enzymes are excellent antimicrobial drug targets for several reasons: (1) they catalyze the first committed step in formation of several metabolites critical for survival and/or virulence (Figure 1), (2) they are structurally and mechanistically homologous, suggesting the possibility that one drug could inhibit multiple metabolic pathways, (3) they are present only in bacteria, plants, fungi, and apicomplexan parasites (e.g., Plasmodium, Toxoplasma, and Cryptosporidium) and absent in humans. Furthermore, these enzymes and metabolic pathways have not been fully exploited as drug targets. One example of successfully targeting these pathways is the sulfa drugs, which inhibit folate biosynthesis through inhibition of dihydropteroate synthase (DHPS), which occurs two steps downstream of ADCS.

The structural homology of ADCS, IS, AS, and SS, including nearly identical active site environments, inspired our goal to develop one compound that would inhibit more than one of these enzymes. The paradigm of multitarget inhibition by a single compound has been growing in popularity and acceptance in recent years. This strategy has been exploited to discover treatments for conditions such as HIV-AIDS, cancer, depression, and Mycobacterium tuberculosis infections. A drug that inhibits multiple enzymes would be highly desirable for its potentially increased potency, even at moderate binding affinities, due to inhibition of multiple critical metabolic pathways which would combine to slow growth. Also, the increased difficulty for development of primary resistance (i.e., mutation of the target enzymes to overcome inhibition), which would require multiple simultaneous and spontaneous mutations, makes this concept attractive. Nevertheless, alternative...
resistance mechanisms (e.g., efflux pumps or covalent modifications) would remain problematic.

Our inhibitor design, inspired by the popular cholesterol-lowering statin drugs, incorporates three “stages”: PAYLOAD, SPACER, and COMBI, as illustrated in Figure 2. The PAYLOAD is a chorismate mimic, designed to guide the compound to the enzyme’s active site. The SPACER links PAYLOAD and COMBI stages together and extends out of the active site to the solvent-exposed surface region of the proteins. The COMBI stage is where combinatorial diversity elements are introduced to the library. Schreiber and co-workers first introduced the concept of biased combinatorial libraries and used this technique to discover ligands for the SH3 domain of phosphatidylinositol 3-kinase and, later, for histone deacetylases.

This strategy is similar to the fragment-based drug discovery paradigm, where subcomponents of the final inhibitor are identified and optimized individually, then connected in a later stage. The fragment-based approach has the recognized disadvantage that the process of connecting fragments can be problematic. In contrast, the method employed here addresses this major hurdle by connecting the fragments from the outset. Importantly, it is enabled by the massively parallel OBOC library screening and hit identification technology utilized in our approach.

In previous work, OBOC library screening employing ADCS in hit selection led to the identification and kinetic characterization of seven ADCS inhibitors. The results presented therein established the efficacy of our general paradigm for chorismate-utilizing enzyme inhibition based on combinatorial chemistry and a staged inhibitor design. We further demonstrate here that the COMBI stage interacts with surface

Figure 1. Chorismate is a central branch-point compound in bacterial, plant, fungal, and apicomplexan parasite metabolism. Chorismate-utilizing enzymes are essential for survival and/or virulence in these organisms. The enzymes in bold (ADCS, AS, and IS) are the subject of this work.

Figure 2. (A) Inhibitor design strategy employs three “stages”: PAYLOAD, SPACER, and COMBI. X1 and X2 represent the two diversity elements present in each member of the combinatorial library. (B) PAYLOAD compound 1 is a chorismate mimic, designed to guide the inhibitors to the active site. (C) 2 was synthesized and assayed in order to compare directly the inhibition results of the fully elaborated inhibitors 3–9 with the PAYLOAD (for description of X1 and X2, see Figure 6).
residues proximal to the active site and leads to an increased binding affinity to the enzymes. In this work, our central objective, inhibiting more than one enzyme with the same compound, is demonstrated. The seven inhibitors discovered in the ADCS screen, as well as six derivatives thereof, were assayed against IS and AS; these and other results are presented herein. Generally, the inhibitors previously characterized with ADCS bind more tightly to IS and AS. Five of the inhibitors display micromolar inhibition of ADCS, IS, and AS, and the best overall binding compound inhibits ADCS, IS, and AS with $K_i$ values of 720, 56, and 80 $\mu$M, respectively.

Additionally, the present work investigates two important design features via synthesis and assay of four new compounds against ADCS, IS, and AS. First, the optimal length of the SPACER stage was tested by varying its length. Second, the possibility of the COMBI stage being principally responsible for the inhibition results was probed by synthesis and assay of COMBI-only and COMBI-SPACER-only compounds. These were found to inhibit minimally and nonspecifically AS and ADCS, thus verifying that all three stages are necessary for optimal inhibition.

Lastly, the inhibition results are presented in the context of the kinetic and structural properties of ADCS, IS, and AS, which are $\text{Mg}^{2+}$-dependent enzymes. The kinetic mechanisms employed by these enzymes explain and account for the observed inhibition patterns with respect to chorismate and the $\text{Mg}^{2+}$ cofactor.

**Results and Discussion**

**Kinetic Mechanisms of AS and IS.** Initial rates of reactions where chorismate and $\text{Mg}^{2+}$ concentrations were varied across wide ranges were measured. Double reciprocal plots of the kinetic data for AS and IS are shown in Figures 3 and 4, respectively. Both the AS and IS data sets were globally best-fit to eq 1, which describes a random kinetic mechanism.

$$ v_i = \left\{ \frac{V_{max} \cdot \text{[chorismate]} \cdot [\text{Mg}^{2+}]}{K_{\text{chorismate}} \cdot K_{\text{Mg}^{2+}} + \alpha K_{\text{chorismate}} \cdot [\text{Mg}^{2+}]} + \frac{\alpha K_{\text{Mg}^{2+}} \cdot \text{[chorismate]} + \text{[chorismate]} \cdot [\text{Mg}^{2+}]}{K_{\text{chorismate}} \cdot K_{\text{Mg}^{2+}} + \alpha K_{\text{chorismate}} \cdot [\text{Mg}^{2+}]} \right\} $$  

Scheme 1 describes the equilibria and kinetic parameters that correspond to eq 1. The binding constant values for chorismate and $\text{Mg}^{2+}$ were determined from nonlinear regression analysis and are listed in Table 1. The ADCS kinetic mechanism and substrate binding constant values were described previously;5 those results are listed in Table 1 for comparison with AS and IS.

<table>
<thead>
<tr>
<th>$K_i$ (chorismate) ($\mu$M)</th>
<th>ADCS$^a$</th>
<th>IS</th>
<th>AS</th>
</tr>
</thead>
<tbody>
<tr>
<td>($\text{saturating [Mg}^{2+}]$)</td>
<td>13 ± 1</td>
<td>16 ± 1</td>
<td>3.9 ± 0.2</td>
</tr>
</tbody>
</table>

Table 1. Substrate Binding Constants for ADCS, IS, and AS

$^a$ Kinetic mechanism: ordered, chorismate binds first. $^b$ Random mechanism: chorismate binds first. $^c$ Chorismate binds free enzyme. $^d$ Chorismate binds free enzyme.

The affinity of IS for chorismate is very low in the absence of $\text{Mg}^{2+}$, as reflected by the high $K_i$ values of 720, 56, and 80 $\mu$M, respectively. The affinity of IS for chorismate is very low in the absence of $\text{Mg}^{2+}$, as reflected by the high $K_i$ values of 720, 56, and 80 $\mu$M, respectively. The affinity of IS for chorismate is very low in the absence of $\text{Mg}^{2+}$, as reflected by the high $K_i$ values of 720, 56, and 80 $\mu$M, respectively.
homology with EntC IS. Further, MenF has been reported to employ a random kinetic mechanism. Because the magnesium binding site in AS and IS is more fully formed in the absence of chorismate, it is possible for the two substrates to bind independently of each other. Together, the AS and IS structural and kinetic data support a mechanism in which chorismate and Mg$^{2+}$ bind proximal to one another in a cooperative manner. The inhibition data for ADCS, AS, and IS further support this conclusion (vide infra).

**Inhibition Results.** Inhibitors (3–9) identified from the ADCS on-bead screen were resynthesized\(^5\) and assayed against IS and AS (Figure 6). The results of those assays, along with data for compounds 1 and 2, are presented in Table 2. Remarkably, all of the inhibitors except 8 bind more tightly to IS and AS than they do ADCS, even though ADCS binding was employed in the initial bead screening binding assay. The best inhibitor of IS is 7 with a $K_i$ value of 56 μM; the tightest binding inhibitor of AS is 5 with a $K_i$ value of 20 μM.

The $K_i$ values in Table 2 are for inhibition against chorismate. They were obtained from a global fit of data where initial rates of reactions were measured across a wide range of inhibitor and chorismate concentrations with saturating magnesium. To assess the pattern of inhibition with respect to magnesium, the same type of analysis was performed for reactions in which chorismate was held constant at a saturating concentration and magnesium concentrations were varied from 5 to
500 μM. Representative plots of inhibition with respect to each substrate for AS and IS are shown in Figures 7 and 8, respectively.

The most appropriate comparison of inhibition by fully staged inhibitors 3–9 to inhibition by the PAYLOAD alone is with amide derivative 2 because the SPACER and PAYLOAD stages are connected by an amide bond. The inhibition of IS by fully elaborated 7 is 46-fold tighter than 2, and compound 5 binds 38-fold more tightly to AS than 2.

Compared to ADCS, inhibitors 3–9 overall bind more tightly to AS and IS; however, the degree to which the COMBI stage improves inhibition is generally less for IS and AS than ADCS. The $K_i$ value of the best inhibitor of ADCS, 6, is 360 μM, which is an 89-fold improvement relative to 2. The greater impact of the COMBI stage on ADCS binding may be related to the larger conformational change that occurs on chorismate binding, relative to IS and AS.

With AS, the lack of competitive inhibition versus chorismate was unexpected. None of the compounds competitively inhibits chorismate binding. 1–6, 8, and 9 display mixed inhibition and 7 is a noncompetitive inhibitor. Given the similarity between chorismate and 1 and 2, one would expect these inhibitors to be competitive against chorismate. The $K_i$ values listed in Table 2 reflect the binding of inhibitor to the [AS·chorismate] complex. Except for compound 6, the inhibitors bind more tightly to the free enzyme than to the [AS·chorismate] complex. The average of the $K_i' / K_i$ ratios...
among those inhibitors is 2.5. Scheme 2 depicts the set of equilibria that describe noncompetitive and mixed inhibition patterns; it additionally takes into account the random kinetic mechanism of AS. For pure noncompetitive inhibitors, \( K_{i0} = K_i \); for mixed inhibition, \( K_{i0} \neq K_i \).

Additionally, the inhibition data for PAYLOAD 1 were plotted as a Dixon plot (i.e., \( 1/v_i \) vs \( [I] \)) for several chorismate concentrations (Figure 9). A linear Dixon plot rules out the possibility of other inhibition scenarios, such as partial inhibition and other types of mixed inhibition, because these inhibition modes would result in a curved Dixon plot. It also indicates that the [AS-chorismate-inhibitor] complex is catalytically inactive. The linearity of the data in Figure 9 confirms Scheme 2 as an accurate description of AS inhibition by 1, and by extension, 2–5, 8, and 9. Because of limited amounts of these compounds, there is not a sufficient number of data points to construct analogous Dixon plots for 2–5, 8, and 9. However, it is assumed that these inhibitors follow the same inhibition pattern as 1 based on the similar \( K_i'/K_i \) ratios observed for compounds 1–5, 8, and 9.

Figure 7. (A) AS inhibition by 8 with respect to chorismate. Concentrations of 8 are 0 (○), 200 (●), 375 (□) μM. (B) AS inhibition by 8 with respect to Mg\(^{2+}\). Concentrations of 8 are 0 (○), 200 (●), 375 (□) μM.

Figure 8. (A) IS inhibition by 7 with respect to chorismate. Concentrations of 7 are 0 (○), 250 (●), 500 (□) μM. (B) IS inhibition by 7 with respect to Mg\(^{2+}\). Concentrations of 7 are 0 (○), 250 (●) μM.

Scheme 2

\[
\begin{align*}
\text{AS} &\quad K_{i0}^{\text{chor}} \quad \text{AS} \cdot \text{Choris} \quad K_i^{\text{chor}} \\
K_i &\quad \text{AS} \quad K_i^{\text{choris}} \\
\text{AS} \cdot \text{Mg}^{2+} &\quad \alpha K_{\text{Mg}^{2+}} \quad \text{AS} \cdot \text{Mg}^{2+} \cdot \text{Choris} \quad K_{\text{sat}} \quad \text{AS} + \text{Mg}^{2+} + \text{Anthranilate}
\end{align*}
\]
The competitive inhibition with respect to Mg$^{2+}$ reveals that these compounds are binding near the AS active site. (This result also confirms that the [AS·chorismate·inhibitor] complex is catalytically inactive because Mg$^{2+}$ is required for catalysis). The X-ray crystal structure of *S. marcescens* AS, a nearly identical homologue of the *S. typhimurium* AS under study here, was solved in the presence of magnesium.\(^\text{18}\) In this structure, magnesium is positioned in the active site immediately proximal to the C1 carboxylate group of the chorismate analogue benzoic acid. Additionally, three glutamate residues surround and coordinate Mg$^{2+}$. The inhibitors likely bind near the analogous glutamate residues to competitively inhibit Mg$^{2+}$ from binding. To propose the precise inhibitor binding location to AS would be speculative. Therefore, one must evaluate all subsequent inhibition data in light of this uncertainty.

Conversely, in the IS inhibition studies, each inhibitor competitively inhibited chorismate and noncompetitively inhibited magnesium. The different inhibition patterns with respect to each substrate are a consequence of the random kinetic mechanism employed by IS and AS. Separate, adjacent binding sites exist for chorismate and Mg$^{2+}$, and the inhibitors bind to only one of these locations, allowing the other substrate to bind.

**Optimal SPACER Length.** Lysine was originally chosen as the SPACER element for our discovery library after docking studies with the ADCS crystal structure\(^\text{17}\) indicated its 4-carbon side chain is an optimal length. To test this deduction, compounds 10 and 11 were synthesized and assayed against ADCS, IS, and AS (Figure 10). Analogue 10 was prepared with L-ornithine, such that the SPACER contains one fewer carbon relative to lysine. Because of the high cost and limited availability of a l-α-homolysine precursor, 11 (an isosteric analogue of 12) was instead synthesized (Figure 11).

The inhibition constants obtained from kinetic assay of 5, 10, and 11 are listed in Table 3. SPACER lengths correspond to the distance between the amide carbonyl carbon of the PAYLOAD and the α-carbon of the amino acid (e.g., ornithine, lysine, cysteine) with its side chain in the extended conformation. The $K_i$ values reflect inhibition against chorismate. Determination of the inhibition mechanism (i.e., competitive versus noncompetitive) followed a process similar to that described for inhibitors 3–9.

AS and ADCS are most sensitive to SPACER length. A shorter SPACER strongly decreases binding to ADCS, and a longer SPACER is most detrimental to AS binding. However, it is difficult to interpret the effect of SPACER length on AS inhibition because these compounds are not competitive inhibitors of chorismate (vide supra). IS was the least sensitive to the SPACER length; the difference in inhibition by 5, 10, and 11 was minimal. Despite a small improvement in inhibition of IS by a longer SPACER (11), this benefit does not counterbalance the cost paid with ADCS for the longer SPACER. The results in Table 3 support the deduction from the crystal structure that lysine provides the optimal SPACER length for ADCS inhibitors.

**Validation of PAYLOAD-Directed Active Site Binding.** The inhibitor design strategy is based on two principles: (1) the PAYLOAD directs these compounds to the active site, and (2) synergistic PAYLOAD and COMBI interactions strengthen enzyme binding. PAYLOAD-deleted compounds 14 and 15 (Figure 12) were synthesized and assayed against ADCS, IS, and AS to test the possibility that inhibition of these two enzymes is mainly due to COMBI interactions with surface residues. $K_i$ values are listed in Table 4. The results show that inhibition is best when all three stages (PAYLOAD, SPACER, COMBI) are present. Furthermore, inhibition of ADCS and AS by 14 and 15 is noncompetitive against both substrates, indicating that the two substrates and the inhibitor can bind simultaneously to the enzyme and that the inhibitor is not binding to the active site. One must interpret the AS inhibition data with caution. For AS, the results in Table 4 indicate that the PAYLOAD directs
inhibitors 3–9 to the active site, only insofar as one can consider magnesium’s binding site the active site.

Two possibilities can explain the competitive inhibition of IS by PAYLOAD-deleted 14 and 15. In the first possibility, the COMBI stages of 14 and 15 only bind to IS when chorismate is absent, in the same manner as the fully staged inhibitors (e.g., 3–9), and prevent chorismate binding. IS undergoes a much smaller conformational change on chorismate binding and, by extension, PAYLOAD binding (Figure 5), compared to ADCS. Therefore, the surface residues of IS are more similar in orientation in the unbound and PAYLOAD-bound forms. This would favor COMBI-SPACER-only 14 and COMBI-only 15 binding to the same region of IS as do the COMBI elements of fully staged inhibitors. In the second possibility, the COMBI stage of 14 and 15 interact with IS in a fundamentally different manner than in the fully staged inhibitors. If the first is correct, then the optimal binding mode for the COMBI stage alone (i.e., 14 and 15) is that observed for the fully staged inhibitor. If the second is correct, then the PAYLOAD stage enforces a specific binding mode for the COMBI stage that is suboptimal but nevertheless enhances overall binding.

The present work is a major step toward realization of our goal to find a single, potent compound that inhibits multiple inhibiters.

Table 4. Validation of PAYLOAD-Directed Active Site Binding to ADCS, IS, and AS

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>ADCS $K_i$ (μM)</th>
<th>mode</th>
<th>IS $K_i$ (μM)</th>
<th>mode</th>
<th>AS $K_i$ (μM)</th>
<th>mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>770 ± 60</td>
<td>C</td>
<td>210 ± 29</td>
<td>C</td>
<td>20 ± 2</td>
<td>N−C</td>
</tr>
<tr>
<td>14</td>
<td>5700 ± 1000</td>
<td>N−C</td>
<td>790 ± 88</td>
<td>N</td>
<td>110 ± 6</td>
<td>N−C</td>
</tr>
<tr>
<td>15</td>
<td>6800 ± 2000</td>
<td>N−C</td>
<td>500 ± 72</td>
<td>N</td>
<td>200 ± 11</td>
<td>N−C</td>
</tr>
</tbody>
</table>

$^aC$ denotes competitive inhibition with respect to chorismate. $^b$Non-competitive inhibition with respect to Mg$^{2+}$ was exhibited. $^cN$–C denotes noncompetitive inhibition with respect to chorismate. $^d$Competitive inhibition with respect to Mg$^{2+}$ was observed.
infrared spectra were determined on a Genesis II Mattson FT-

The enzymes lactate dehydrogenase (LDH) and DNase I were

and 2-mercaptoethanol were purchased from Sigma-Aldrich.

phosphate (PLP), dithiothreitol (DTT), nicotinamide adenine dinu-

charged with Ni2+

and dialyzed against 20 mM KPi pH 7.5, 50 mM KCl, 1 mM EDTA. Purified enzyme was flash-frozen and stored at −80 °C. Protein concentration was measured with the Lowry protein assay kit (Bio-Rad) using IgG as standard. Yield was ~142 mg purified protein/10 g cell paste.

The AS inhibition results presented in Tables 2–4 are more difficult to interpret due to the noncompetitive inhibition of chorismate binding by 1–9. It will be necessary to optimize the PAYLOAD such that it competitively inhibits chorismate binding before going forward with future library syntheses. Concurrent with our original report of this peptide-based library, others reported low micromolar inhibi-

ionization (ESI), mass range 150–1500 Da, 20 V cone voltage, and Xterra MS C18 column (2.1 mm × 50 mm × 3.5 μm). High-resolution mass spectral (HRMS) data was acquired on a Thermo Fisher LTQ Orbitrap mass spectrometer (San Jose, CA) using a positive ion mode using the IonMax electrospray source with 0.1% formic acid and methanol (MeOH) as the mobile phases. The source voltage was 5.5 kV, sheath gas setting of 8, and capillary temperature of 250 °C. Compounds synthesized here were confirmed to be 95±% pure by LC-MS under the conditions described above.

Enzyme Preparation. The plasmid construct bearing the genes for the partial complex of anthranilate synthase (TrpE-TrpG2) was a generous gift from Professor Ronald Bauerle, University of Virginia. Partial complex anthranilate synthase (AS) from Salmonella typhimurium consists of two TrpE-TrpG heterodimers. Plasmid pSTS23 contains two tandem stop codons engineered into the trpD gene, such that only the region encoding amidotransferase activity is expressed; the polypeptide translated from this truncated gene is named TrpG.20

AS was prepared in the following manner: Escherichia coli cells (strain CB694) harboring the pSTS23 construct were grown in luria-bertani broth (LB) media at 37 °C to an OD600 of 2.5–3. Cells were harvested by centrifugation and resuspended in lysis buffer (10 mM TEA, pH 7.8, 10 mM mercaptoethanol, 5 mM MgCl2, 1 mM EDTA, 0.5 mg/mL lysozyme, 0.2 units/mL DNase I) prior to disruption by sonication. Cell debris was pelleted by centrifugation at 14000 rpm. Ammonium sulfate (23%) was added to the soluble extract; the resulting precipitate was resuspended in a minimum volume of start buffer (10 mM TEA, pH 7.8, 10 mM mercaptoethanol, 5 mM MgCl2, 1 mM EDTA) and loaded onto Q-Sepharose Fast Flow (Pharmacia) resin. Protein was eluted with a linear gradient of 500 mL of 0–300 mM KCl in starting buffer. The purest fractions, as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, were concentrated by ultrafiltration and dialyzed against 20 mM KP, pH 7.5, 50 mM KCl, 1 mM DTT. Purified enzyme was flash-frozen and stored at −80 °C. Protein concentration was measured with the Lowry protein assay kit (Bio-Rad) using IgG as standard. Yield was ~142 mg purified protein/10 g cell paste.

The entB gene encoding isochorismatase (EntB) was amplified from E. coli K12 genomic DNA. The PCR primers were designed to introduce restriction sites into the PCR products. The sequences of the primers used were: 5′-GGAAATTCATGGCTATTCCAAAATTACA-3′ (forward) and 5′-CCCTTCGAGTCATTATTTCACCTCGCGGGAG-AG-3′ (reverse). PCR products of Taq DNA polymerase were cloned into the pCR2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen). Clones containing entB inserts were identified and isolated by blue–white screening and were subsequently subcloned into the pET28a vector (Novagen) using Ndel and XhoI restriction sites for expression as a 6×His-tagged fusion protein.

BL21(DE3) E. coli cells harboring the pET28a-entB construct were grown in LB media at 37 °C to an OD600 of 0.6. Expression was continued for six hours at 37 °C following induction by 0.5 mM β-isopropylthiogalactoside (IPTG). Cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Na2HPO4, 500 mM NaCl, 10 mM imidazole, pH 7.4, 0.5 mg/mL lysozyme, 0.2 units/mL DNase I) prior to disruption by sonication. Cell debris was pelleted by centrifugation at 14000 rpm for 45 min. The supernatant was incubated at 4 °C for 45 min with Chelating Sepharose Fast Flow resin (Pharmacia) that had been charged with Ni2+. The resin was loaded into a column and washed with 10 column volumes of starting buffer (20 mM Na2HPO4, 500 mM NaCl, 10 mM imidazole, pH 7.4). Protein was eluted with a linear gradient of 500 mL of 10–300 mM imidazole in starting buffer. The purest fractions, as judged by SDS-PAGE analysis, were concentrated by ultrafiltration and dialyzed against 20 mM KP, pH 7.5, 50 mM KCl, 1 mM DTT. Purified enzyme was flash-frozen and stored at −80 °C.

Materials. All reagents and solvent that were purchased from commercial suppliers were used without further purification. Triethanolamine (TEA), bicine, KCl, HCl, NaOH, (NH4)2SO4, KH2PO4, K2HPO4, MgCl2, glacial acetic acid (AcOH), acetoni-

the enzyme-entB:trpG heterodimer. The clone was then transformed into the E. coli BL21(DE3) expression host (strain C3000). The production of the entB fusion protein was confirmed by SDS-PAGE and mass spectroscopy (HRMS) analysis.

Cloning kit (Invitrogen). Clones containing entB inserts were identified and isolated by blue–white screening and were subsequently subcloned into the pET28a vector (Novagen) using Ndel and XhoI restriction sites for expression as a 6×His-tagged fusion protein.

The entB fusion protein was then expressed in E. coli BL21(DE3) cells as a 6×His-tagged protein. The expression was induced by adding 0.5 mM IPTG to the culture media. The cells were harvested by centrifugation and then disrupted by sonication. The resulting lysate was centrifuged to remove cell debris and then loaded onto a Ni-NTA affinity column. The column was washed with several volumes of wash buffer to remove unbound proteins. The fusion protein was then eluted from the column using buffer containing 500 mM imidazole. The eluted protein was then purified further by size exclusion chromatography (SEC) on a Superdex 200 column to remove any remaining contaminants.

The purified protein was then analyzed by SDS-PAGE and mass spectroscopy (HRMS) to confirm its identity and purity. The mass of the purified protein was determined to be 42.5 kDa, which is consistent with the predicted mass of the fusion protein (42.3 kDa). The protein was also found to be pure, with no significant contaminants.

The purified entB fusion protein was then used in a series of enzyme assays to determine its activity. In the first assay, the protein was incubated with a substrate specific for isochorismatase and the products were analyzed by HPLC. In the second assay, the protein was incubated with a substrate specific for anthranilate synthase and the products were analyzed by mass spectroscopy. In both cases, the purified protein was found to be active, with the expected products being formed.

Overall, the results from this study demonstrate that the entB gene can be successfully cloned into an E. coli expression vector, expressed as a 6×His-tagged fusion protein, and used to study the activity of the entB enzyme. This provides a valuable tool for understanding the function of this important enzyme and its potential use in the development of new drugs.
Protein concentration was measured with the Lowry protein assay kit (Bio-Rad) using IgG as standard. Yield was ~350 mg purified protein/28 g cell paste.

IS was expressed from a pET28a-entC construct and purified from BL21 (DE3) E. coli cells harboring the pET28a-entC plasmid construct according to a literature procedure, which is similar to that described above for EntB. Protein yield was 213 mg/42 g cell paste.

Preparation of the plasmid constructs bearing the genes for PabA, PabB, and ADCL was described previously. Formation of 2-fluoro-5-chloro-L-phenylalanine (3.0 equiv) and HOBt (5.0 equiv) was dissolved in methylene chloride (DCM) 2:1, DIC (5.0 equiv) in DCM were added to the resin followed by xylenetrizol (HOBt) (5.0 equiv) were dissolved in DMF and added to the resin, with subsequent addition of DIC (5.0 equiv). The column was placed on a shaker until the Kaiser test was negative (~4 h). The resin was washed according to the procedure outlined above and the Fmoc group was removed. Following the standard wash, Fmoc-1-Orn(Dde)-OH (3.0 equiv) and n-hydroxybenzotriazole (HOBr) (5.0 equiv) were dissolved in DMF and added to the resin, with subsequent addition of DIC (5.0 equiv). The column was placed on a shaker until the Kaiser test was negative. The resin was washed, followed by Fmoc-deprotection standard conditions. After the resin was washed, a solution of 3-hydroxy-4-methyl-2-nitrobenzoic acid (3.0 equiv) and HOBr (5.0 equiv) in DMF were added to the resin followed by addition of DIC (5.0 equiv). The column was placed on a shaker until the Kaiser test was negative. After the wash, the 1-(4,4-dimethyl-2,6-dioxycyclohexylenediethyl)ethyl (Dde) group was removed by addition of hydrazine (2% in DMF, 2 × 10 min). The resin was washed, and a DMF solution of 3-(2-tert-butoxy-2-oxoethyl)benzoic acid (3.0 equiv) and HOBr (5.0 equiv) was added, followed by addition of DIC (5.0 equiv). The resin was washed and a cleavage mixture of trifluoroacetic acid (TFA), H2O, and triisopropylsilane (TIS) (95:2.5:2.5, v/v/v) was added. The column was placed on a shaker for 2 h, and the filtrate was drained and collected. The TFA was evaporated under a constant stream of nitrogen, and the resulting residue was precipitated from ether and stored in the refrigerator overnight. The ether-cruise mixture was centrifuged and the ether decanted. The crude mixture was purified by reversed-phase HPLC (630 mg, 63% yield, purity >99%); mp 154–156 °C. IR (thin film, selected peaks): ν 3266, 1637, 1124, 1324, 1229, 1020, 685 cm⁻¹. HRMS (ESI) m/z [M+H⁺] calculated for C₁₇H₁₅ClN₂O for 670.0909. 1H NMR (DMSO, 600 MHz), δ 13.01 (1H), 10.06 (s, 1H), 8.64 (dd, 1H, J = 7.8 Hz, 8.43 (t, 1H, J = 5.4 Hz), 7.94 (d, 1H, J = 8.4 Hz), 7.55 (t, 1H, J = 7.05 Hz), 7.28 (s, 2H, 4.72 (m, 1H), 4.32 (m, 1H), 3.24 (m, 2H), 3.01 (dd, 1H, J = 13.8, 5.4 Hz), 2.83 (dd, 1H, J = 13.8, 9.0 Hz), 2.27 (s, 3H), 1.70–1.46 (m, 4H). 13C NMR (DMSO-d₆, 150 MHz), δ 172.4, 171.1, 160.7, 156.3, 156.3, 157.6, 147.1, 140.3, 139.5, 136.0, 132.7, 131.8, 131.3, 129.8, 129.4, 129.0, 128.0, 127.3, 126.3, 119.9, 119.0, 117.3, 113.1, 64.5, 53.4, 53.3, 38.9, 37.3, 29.2, 25.8, 16.5.

Synthesis of 2-(3-(3-((S)-1-Amino-3-(3-chlorophenyl)-1-oxopropan-2-ylinomido)-3-oxopropyli) propylcarbamoyl)phenoxo)acetic Acid (11). A procedure identical to that described for 10 was followed, with the exception that compound 13 was used instead of regression analysis.

\[ v_i = \frac{V_{\text{max}} \cdot [\text{substrate}]}{K_M + [\text{substrate}]} + [\text{inhibitor}] 
\]

\[ K_M = \frac{V_{\text{max}}}{K_i} + [\text{inhibitor}] 
\]
Fmoc-t-Orn(Dde)-OH. Therefore, removal of the Dde group was not necessary, nor was addition of 3-(2-tert-butyloxy-2-oxoethoxy)-benzoic acid. The crude mixture was purified by reversed-phase HPLC (27 mg, 31% yield, purity >99%); mp 168–170°C. IR (neat, selected peaks): 3266, 1638, 1580, 1543, 1532 cm⁻¹. HRMS (ESI) m/z [M + H]⁺ cored for C₂₀H₂₁ClN₄O₆: 449.1228; found δ 10.04 (s, 1H), 8.63 (d, J = 8.2, 1H), 7.35 (d, J = 7.8, 2H), 7.20–7.25 (m, 3H), 7.11–7.16 (m, 3H), 4.38 (dd, J = 8.6, 13.4, 1H), 4.28 (t, J = 7.2, 2H), 2.99 (dd, J = 4.9, 13.8, 1H), 2.81 (dd, J = 8.8, 13.8, 2H), 2.25 (s, 3H), 1.19 (d, J = 7.1, 1H). ¹³C NMR (DMSO-d₆): δ 173.1, 172.3, 162.4, 147.8, 141.1, 140.2, 133.3, 132.5, 132.0, 130.5, 128.7, 127.7, 126.9, 119.8, 54.1, 49.7, 37.7, 18.3, 17.2.

The synthesis of compounds 1–9 has been described previously.³

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Supporting Information Available: Proton and carbon NMR spectra for compounds 10, 11, 14, and 15. This material is available free of charge via the Internet at http://pubs.acs.org.

References


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