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Rational Optimization of Diphenyl Ether Binding Kinetics to the Enoyl-ACP Reductase FabI1 from *Burkholderia pseudomallei*

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Abstract

There is growing awareness of the link between drug-target residence time and *in vivo* drug activity, and there are increasing efforts to determine the molecular factors that control the lifetime of a drug-target complex. Rational alterations in drug-target residence time require knowledge of both the ground and transition states on the inhibition reaction coordinate, and we have determined the structure-kinetic relationship for 22 ethyl or hexyl substituted diphenyl ethers that are slow-binding inhibitors of bpFabI1, the enoyl-ACP reductase FabI1 from *Burkholderia pseudomallei*. Analysis of enzyme inhibition using a 2D-kinetic map demonstrates that the ethyl and hexyl diphenyl ethers fall into two distinct clusters. Modifications to the ethyl diphenyl ether B ring result in changes to both on and off-rates, where residence times of up to ~700 min (~11 h) are achieved by either ground state stabilization (**PT444**) or transition state destabilization (slower on-rate) (**PT404**). By contrast, modifications to the hexyl diphenyl ether B ring result in residence times of 300 min (~5 h) through changes in only ground state stabilization (**PT119**). Structural analysis of 9 enzyme:inhibitor complexes reveal that the variation in structure-kinetic relationships can be rationalized by structural rearrangements of bpFabI1 and subtle changes to the orientation of the inhibitor in the binding pocket. Finally, we demonstrate that three compounds with residence times on bpFabI1 of between 118 min (~2 h) and 670 min (~11 h) have *in vivo* efficacy

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SUPPORTING INFORMATION

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in an acute *B. pseudomallei* murine infection model using the virulent *B. pseudomallei* strain Bp400.

Graphical Abstract

Keywords

MUT056399; diphenyl ethers; enoyl-ACP reductase; *Burkholderia pseudomallei*; slow binding inhibitors

INTRODUCTION

Melioidosis is a complex disease to treat due to its rapid progression and tendency to generate latent infections.¹ One of the most severe manifestations of this disease is melioidosis septic shock, which is often associated with pneumonia and bacterial dissemination to distant sites.² The etiologic agent of melioidosis is the Gram-negative soil-dwelling organism *Burkholderia pseudomallei*. Whilst *B. pseudomallei* is susceptible to therapeutics such as ceftazidime, chloramphenicol, doxycycline, amoxicillin-clavulanate, trimethoprim-sulphamethoxazole, ureidopenicillins and carbapenems, mortality is high because relapse often occurs.^{3–5} Moreover, antibiotic resistance mechanisms have been identified in *B. pseudomallei* including efflux from the cell, target mutation, target redundancy, exclusion from the cell and enzymatic inactivation.⁶ In addition, *B. pseudomallei* can potentially be used as a biowarfare agent, and is thus now classified as a Tier 1 Biological Select Agent or Toxin (BSAT) by the Centers for Disease Control and Prevention (CDC).^{5, 7} Consequently, there is a need to develop chemotherapeutics that can be used to treat *B. pseudomallei* infections.

Enzymes in the bacterial fatty acid biosynthesis (FAS) pathway are attractive antimicrobial targets because this pathway produces metabolic precursors for the bacterial phospholipid membrane that are essential for Gram-positive and Gram-negative bacteria survival, and also because there is low sequence homology and fundamental structural differences between the mammalian FAS (FAS-I) and bacterial FAS (FAS-II) systems.^{8, 9} In this regard, inhibitor discovery programs have focused primarily on the NAD(P)H-dependent enoyl-ACP reductase (ENR) which catalyzes the last reaction in the FAS-II elongation cycle and which is targeted by antibacterial agents including the diazaborines, triclosan and isoniazid (Scheme 1).^{10–14}

We have previously characterized the ENR isoforms FabI1, FabI2 and FabV in *B. pseudomallei* and demonstrated that FabI1 (herein bpFabI1) was the transcriptionally active and clinically relevant FabI isoform.^{15–17} We also showed that diphenyl ethers, analogues of

the broad-spectrum inhibitor triclosan (Figure 1A), were potent low nM slow binding inhibitors of bpFabI1 with antibacterial activity against *B. pseudomallei*. These diphenyl ethers were found to preferentially bind to the enzyme-NAD⁺ product complex, as observed for the inhibition of FabI enzymes from other species by similar analogues.^{11, 15, 18, 19} In a second study, we confirmed that the inhibition of bpFabI1 with the diphenyl ether **PT01** reduced bacterial burden and achieved efficacy in an acute *B. pseudomallei* murine model of infection.¹⁶

Although we previously identified time dependent diphenyl ether inhibitors of bpFabI1, we did not determine the precise mechanism of inhibition. Diphenyl ether inhibitors of the FabI enzymes from other pathogens follow either a one-step mechanism (Figure 1, **Mechanism A**), or an induced-fit, two-step slow binding mechanism (Figure 1, **Mechanism B**).^{20–23} Since the inhibitor concentration is not constant *in vivo*, the rates of formation and breakdown of the enzyme-inhibitor complex are critical factors that may influence *in vivo* activity.²⁴ Thus, there is a strong need to understand enzyme-inhibitor association and dissociation kinetics at the molecular level so that this information can be used to optimize target engagement under fluctuating drug concentrations.

In this study, we performed an extensive analysis of bpFabI1-diphenyl ether binding kinetics and extended our structure-kinetic relationship (SKR) to include a potent anti-staphylococcal clinical candidate developed by Mutabilis, **MUT056399**, which is a FabI-specific diphenyl ether inhibitor (Figure 1B).^{25, 26} Using kinetic and structural studies, we identified substituents on the inhibitor B-ring that affect the transition and ground state energies on the inhibition reaction coordinate. Using this information, we were able to increase the residence time of diphenyl ethers on bpFabI1 from 12 to ~700 min. Many of the diphenyl ethers in this study with a 5-ethyl substituent had antibacterial activity in the virulent efflux-compromised *B. pseudomallei* strain Bp400, and three diphenyl ethers reduced bacterial burden in an acute *B. pseudomallei* mouse model of infection.

METHODS

Materials

Luria-Bertani (LB) Agar Miller and Mueller-Hinton broth were purchased from BD. BALB/c female mice were obtained from Charles River Laboratories. His-bind Ni²⁺-NTA resin was purchased from Invitrogen. Crotonyl coenzyme A (crot-CoA) was purchased from Sigma-Aldrich. **MUT056399** was a kind gift from Anacor Pharmaceuticals. All other chemical reagents were obtained from Fisher.

Expression and purification of bpFabI1

An expression plasmid for the *B. pseudomallei* FabI1 enoyl-ACP reductase was available from previous studies, in which a His-tag was encoded at the C terminus of bpFabI1.¹⁵ Protein expression and purification for bpFabI1 were performed as previously reported.^{15, 17, 28} The protein purity was verified by a 15 % SDS-PAGE gel, which indicated an apparent molecular weight of ~28 kDa. The concentration of bpFabI1 was spectrophotometrically determined using an extinction coefficient of 13,490 M⁻¹ cm⁻¹. This

value was calculated from the primary sequence of the protein using the ExPASy ProtParam tool.

Crystallization and structure determination of the bpFabI1 ternary inhibitor complexes

Solutions of bpFabI1 (0.34 to 1.02 mM) were incubated on ice for 1 h with a 10-fold molar excess of cofactor and a 10- to 100-fold molar excess of inhibitor from a 100 mg/mL stock solution in DMSO (TCL, PT01, PT12, PT405, PT412), resulting in a final DMSO content of $\approx 5 - 15\%$; or the inhibitor was directly added as a pure solid (PT02, PT401, PT404, PT408). Subsequently the protein solution was centrifuged for 20 min at $16000 \times g$ and 4°C . The bpFabI-NAD⁺-inhibitor complexes were then crystallized by hanging-drop vapor diffusion at 20°C . Crystals of the [bpFabI-NAD⁺-PT12], [bpFabI-NAD⁺-PT404], [bpFabI-NAD⁺-PT405] and [bpFabI-NAD⁺-PT408] complexes were obtained in 0.1 M Bis-Tris/HCl, pH 6.5 and 20 to 30 % PEG 400 (*v/v*). The [bpFabI-NAD⁺-PT01], [bpFabI-NAD⁺-PT401] and [bpFabI-NAD⁺-PT412] crystals were obtained from 0.1 M Bis-Tris/HCl, pH 6.5 and 26 to 36 % PEG 300 (*v/v*) as precipitant. Crystals of both the bpFabI-PT02 and triclosan (TCL) complexes grew in a mixture of 8 % PEG 1000 (*w/v*) and 8 % PEG 8000 (*w/v*). All crystals were then transferred to a cryoprotectant containing the mother liquor with elevated PEG concentrations and 10 – 25 % of glycerol.

Data sets were collected at beamline ID29 at the ESRF,²⁹ at beamline MX 14.1 at the BESSY,³⁰ or at an in-house X-ray facility. The data sets were integrated with XDS³¹ or Mosflm³² and scaled in Scala^{33, 34} or Aimless.³⁵ The structures were solved by molecular replacement with Phaser³⁶ utilizing either the apo structure of bpFabI1 (PDB entry 3EK2), or the complex structure of bpFabI1 with PT155 (PDB entry 4BKU) as search model. Models were initially revised and adapted in Coot,³⁷ and subsequently refined using Refmac³⁸ or Phenix.^{39, 40} Data collection and refinement statistics are given in Table S1. Figures were prepared using the program PyMOL.⁴¹ The structure factors and coordinates for apo bpFabI1 and the bpFabI-NAD⁺-inhibitor ternary complexes have been deposited in the Protein Data Bank with the codes 5I7E (apo), 5I7S (TCL), 5I7V (PT01), 5I7V (PT02), 5I8Z (PT12), 5I8W (PT401), 5I9L (PT404), 5I7F (PT405), 5I9M (PT408), and 5I9N (PT412).

Synthesis of diphenyl ethers

The diphenyl ether compounds PT04, PT12, PT70, PT91, PT113, PT119, PT403, PT404, PT411, PT412, PT417, and PT443 were available from former studies and PT405 was synthesized as previously described.^{20, 25, 28, 42–44} PT400, PT401, PT406, PT407, PT408, PT409, and PT444 were synthesized as described in the supplement.

Inhibition kinetics

Slow-onset inhibition kinetics were monitored at 340 nm on a Cary 100 spectrophotometer (Varian) at 25°C in 30 mM PIPES buffer pH 8.0 containing 150 mM NaCl and 1.0 mM EDTA. The reactions were initiated by the addition of enzyme (8 nM) to a mixture containing glycerol (8 % *v/v*), bovine serum albumin (0.1 mg/mL), DMSO (2 % *v/v*), crot-CoA (750 μM), NADH (250 μM), NAD⁺ (200 μM) and inhibitor (0–8000 nM). All reactions were monitored until the steady-state was reached, indicated by the linearity of the progress

curve. Low enzyme and high substrate concentrations ensured substrate depletion would not significantly affect the reaction rates, such that in the absence of inhibitor the progress curves were linear for over a period of 30 min.^{21, 45, 46}

Data were globally fit to the Morrison and Walsh integrated rate equation (Equation 1),⁴⁷ where A_t and A_0 are the absorbance at time t and 0, v_i and v_s are the initial and steady-state velocities, and k_{obs} is the observed pseudo-first rate order constant for the approach to steady-state. Inhibition dissociation constants (K_i^{app} and K_i^{*app}) were subsequently obtained from Equations 2–3 where $v_i = v_0$ for a one-step slow binding model (Figure 1, Mechanism A) and Equations 4–6 for a two-step slow binding model (Figure 1, Mechanism B). The parameters v_0 and $[I]$ are the uninhibited reaction velocity and the inhibitor concentration, respectively. The reverse rate constant for enzyme inhibition is k_4 for a one-step model and the rate constant for conversion of EI^* to EI is k_6 for the two-step model. The parameters k_4 or k_6 are assumed to be the rate limiting step for recovery of active enzyme, and thus the residence time of the inhibitor on the enzyme (t_R) is the reciprocal of k_4 or k_6 .

$$A_t = A_0 - v_s t - (v_i - v_s) \times \frac{1 - e^{-k_{obs}t}}{k_{obs}} \quad (\text{Equation 1})$$

$$v_s = \frac{v_0}{1 + \frac{[I]}{K_i^{app}}} \quad (\text{Equation 2})$$

$$k_{obs} = k_4 \times \frac{v_0}{v_s} \quad (\text{Equation 3})$$

$$v_s = \frac{v_0}{1 + \frac{[I]}{K_i^{*app}}} \quad (\text{Equation 4})$$

$$v_i = \frac{v_0}{1 + \frac{[I]}{K_i^{app}}} \quad (\text{Equation 5})$$

$$k_{obs} = k_6 \times \frac{v_i}{v_s} \quad (\text{Equation 6})$$

Minimum inhibitory concentration (MIC) determination

B. pseudomallei 1026b (efflux-proficient) and *B. pseudomallei* Bp400 (1026b [bpeAB-oprB] [amrAB-oprA]) were grown to an OD₆₀₀ of 0.6. Samples were then stored at -80 °C in 10 % glycerol and used as standard bacterial stocks. For each analysis, bacteria were freshly prepared by growth from the standard stocks on Luria-Bertani (LB) Agar Miller plates for 48–72 h at 37 °C. Bacteria recovered from the LB plates were used to inoculate 10 mL of LB Broth. Broth cultures were then incubated for 18 h at 37 °C, diluted 1:100 and incubated for an additional 6 h at 37 °C. Bacteria were further diluted to a concentration of 1×10^6 colony forming units (CFU) per mL in cation-adjusted Mueller-Hinton broth (CAMHB) and 50 µL aliquots were added to each well in the test plate. For MIC determinations, compounds were added to a 96-well plate starting at 256 µg/mL and serially diluted 1:2 until a final concentration of 0.125 µg/mL in CAMHB was obtained. The plates were incubated at 37 °C for 18 h and the MIC was determined by the lowest inhibitor concentration to inhibit visible bacterial growth.

Evaluation of efficacy in an acute *B. pseudomallei* mouse model of infection

Five to six week old BALB/c female mice were challenged by intranasal infection with 5,000 CFU/mouse of *B. pseudomallei* Bp400 (1026b [amrAB-oprA] [bpeAB-oprB]).⁴⁸ Animals were anesthetized with a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine delivered intraperitoneally. Bacteria were diluted in PBS to achieve an inoculum concentration of 2.5×10^5 CFU/mL. This inoculum was then delivered dropwise in alternating nostrils. Ceftazidime was formulated in PBS and test compounds in a lipid-based delivery system as previously described.¹⁷ Compounds were delivered intraperitoneally, b.i.d. (twice daily) starting at the time of infection. The number of viable bacteria in the lung and spleen was determined at 60 h post-infection by plating serial 10-fold dilutions of homogenates on LB agar and incubation for 48 h at 37 °C. The bacterial burden was assessed and difference in group means was determined using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. The significance was determined by a P value < 0.05.

RESULTS

FabI was initially revealed to be a target for the broad spectrum biocide triclosan by McMurry *et al* who discovered that triclosan-resistant *E. coli* strains had mutations in the *fabI* gene.⁴⁹ Shortly thereafter, Heath *et al* demonstrated that 2-hydroxydiphenyl ethers, including triclosan, directly inhibited FabI enzyme activity,⁵⁰ and triclosan was then shown to be a slow, tight binding inhibitor of *E. coli* FabI (ecFabI), binding preferentially to the E-NAD⁺ complex (K_i 7 pM).^{13, 18, 27, 45, 51} Subsequently, a number of groups including our own have synthesized series of diphenyl ether-based compounds to explore the structural determinants of FabI inhibition in *E. coli*^{19, 52} and in other bacteria, such as *Plasmodium falciparum*,⁵² *S. aureus*,^{21, 53, 54} *M. tuberculosis*,^{20, 55, 56} and *F. tularensis*.⁵⁷

Previously we demonstrated that the diphenyl ether class of compounds also inhibited the bpFabI1 homolog in *B. pseudomallei* and that selected compounds had nM K_i values and displayed time-dependent enzyme inhibition.^{15, 17} The bpFabI1 inhibitors were shown to

have antibacterial activity against *B. pseudomallei* both *in vitro* (0.5–4 µg/mL) and *in vivo*, consistent with the knowledge that bpFabI1 is essential.¹⁶ In the present work, we have performed a detailed structure-kinetic analysis to identify substituents on the diphenyl ether skeleton that modulate time-dependent inhibition. This work has concentrated primarily on two compound series, one based on the Mutabilis clinical candidate with fluoro and ethyl substituents on the A ring, and the second with a hexyl substituent on the A ring. We also report additional *in vivo* efficacy studies with a subset of the diphenyl ethers.

Mechanism of inhibition

The inhibition constants and kinetic parameters for 22 triclosan analogues are given in Table 1. The progress curve data have been analyzed by Equation 1 to give values for k_{obs} , v_i , and v_s , which have subsequently been analyzed by Equations 2–3. All progress curves resulting from the inhibition studies displayed curvature characteristic of slow binding kinetics (Figure 2A).

In principal, slow-onset inhibition can arise from one of three kinetic mechanisms (Figure 1),^{47, 58, 59} a one-step model in which formation of the final EI inhibitor complex occurs slowly without any stable intermediate (Figure 1, **Mechanism A**), and the induced-fit or conformational selection two-step models. For the induced-fit model, a slow conformational rearrangement occurs to form a more stable EI* complex after the rapid formation of EI (Figure 1, **Mechanism B**). In contrast, the conformational selection model involves the slow interconversion of two forms of the enzyme, only one of which is capable of binding the inhibitor (Figure 1, **Mechanism C**). To determine the mechanism of slow binding inhibition for this subset of diphenyl ethers, k_{obs} was plotted as a function of inhibitor concentration (Figure 2B). A positive linear fit was observed for all the compounds, which is typically a characteristic of a one-step mechanism, and the increasing k_{obs} at higher ligand concentrations unambiguously rules out the possibility of a conformational selection mechanism (Figure 1, **Mechanism C**).⁶⁰ We subsequently analyzed the inhibition data using both the one-step and two-step induced fit models and found that all datasets fit globally to the one-step model (Table S2). In some cases, we obtained a better fit for the two-step model ($P < 0.0001$), however, $K_i^{\text{app}} \gg K_i^{*,\text{app}}$ by 15 to >100 fold with the exception of **PT411** and **MUT056399** (6-fold). To further analyze the mechanism of inhibition, we studied X-ray structural data for 9 enzyme:inhibitor complexes. As we show below, bpFabI1 inhibitor complexes can populate structures in which the substrate binding loop (SBL) is either in an open or a closed conformation. Such structural rearrangements have been observed previously for InhA, the FabI from *M. tuberculosis*, where there is clear evidence for a two-step binding mechanism (**Mechanism B**) in which the open conformation is EI and closed conformation is EI*.^{20, 22, 61} Thus, our working hypothesis for bpFabI1 is that inhibition follows a special case of two-step mechanism that is kinetically indistinguishable from the one-step mechanism, similar to saFabI,^{21, 23} in which EI* is much lower in free energy than EI and the initial formation of EI cannot be detected at low inhibitor concentrations. Moreover, our results suggest that the detailed underlying mechanism for enzyme inhibition may be more complicated and suggest that the association and dissociation rates that are shown in Table 1 can be thought of as macroscopic rate constants of a multistep process. Therefore, we give values for both the residence time ($1/k_4$) and the overall on-rate (k_3) in

Table 1, and as the discussion unfolds, we find that the on-rate can be used to formulate explicit structure-kinetic relationships for bpFabI1 inhibition.

Structure-kinetic analysis of bpFabI1 inhibition

Fluoro-ethyl A ring Diphenyl Ethers—**MUT056399** is a diphenyl ether with potent activity towards several pathogens including *S. aureus*.^{25, 26} We first quantified the inhibition of bpFabI1 by **MUT056399** and found that it had a K_i^{app} of 405 nM and t_R of 12 min (Table 1). Compared to the inhibition of bpFabI1 by triclosan, **MUT056399** binds significantly (~13-fold) less potently (K_i^{app} 405 nM v 32 nM for triclosan), but with a t_R value that is only ~1.3-fold smaller (12 v 16 min for triclosan), despite the significant differences in diphenyl ether substitution pattern for the two compounds: **MUT056399** has both fluoro and ethyl-substituents on the A-ring, and o-fluoro and p-amide substituents on the B-ring whereas triclosan has an A-ring p-chloro and B-ring o- and p-chloro substituents. We thus set out to evaluate the contributions of the **MUT056399** substituents to bpFabI1 binding and have analyzed enzyme inhibition by a series of ethyl diphenyl ethers with a variety of substitution patterns. **PT01**, which has only an ethyl substituent on the A ring, inhibits bpFabI1 with a K_i^{app} of 32 nM and t_R of 19 min. Addition of a fluoro group to the A ring of **PT01** results in **PT411**, in which the inhibition parameters K_i^{app} , k_3^{app} and k_4 were unaffected. Addition of an o-fluoro group to the B ring of **PT411** gave **PT405**, which has a higher affinity (K_i^{app} 4 nM) and longer residence time (t_R 250 min). The B ring of **PT411** was further modified to give **PT406** (o-chloro), **PT412** (o-nitro), **PT407** (p-nitro), **PT403** (o-chloro and p-amino), **PT404** (o-chloro and p-nitro) and **PT408** (methyl-pyridine ring). Compounds with the highest affinity contained less bulky or medium sized substituents at the ortho position (i.e. fluoro or chloro). Also, the k_3^{app} was notably reduced by the addition of both ortho and para substituents to the B ring. Therefore, we conclude that retaining or slightly increasing the halogen size of the ortho substituent and replacing the para amide substituent with a nitro group can increase the residence time of **MUT056399** on bpFabI1.

Hexyl A ring—Previously we reported that the hexyl diphenyl ether **PT70** was a slow tight-binding inhibitor of InhA, the FabI from *M. tuberculosis*.²⁰ To examine the ability of hexyl diphenyl ethers to inhibit bpFabI1 we compared the binding of several analogs to the enzyme. This included **PT04**, which lacks substituents on the B ring, as well as **PT113** (o-F), **PT91** (o-Cl), **PT119** (o-CN), **PT70** (o-CH₃) and **PT12** (p-NO₂). We found that the compounds with the highest affinities had the lowest dissociation rates and little on-rate variability. For example, the B ring cyano (**PT119**) has the longest residence time of ~300 min and a K_i^{app} of 15 nM. This effect is also seen when the same substituent is added to **PT01** (t_R 19 min) to generate **PT444** (t_R ~670 min).

We also compared the impact of introducing an A-ring fluoro substituent into the hexyl-substituted diphenyl ethers. Examination of the data reveals that **PT400** (empty B-ring), **PT401** (B ring o-CH₃) and **PT417** (B ring o-Cl) have different kinetic parameters for bpFabI1 inhibition compared to the corresponding analogs that lack an A ring fluorine (**PT04**, **PT70** and **PT91**). In general, the introduction of a fluoro group on the A ring led to slower association rates from 10^3 to 10^2 M⁻¹s⁻¹. The slower association rate for **PT400** led to a weaker affinity for bpFabI1 compared to **PT04** yet the rate of dissociation was unaffected.

Similarly, the dissociation rates for **PT417** and **PT91** were comparable although the A ring fluoro substituent reduced the association rate and weakened the affinity for the enzyme. In contrast, the *o*-CH₃ group on the B ring compensated for the slower association rate, so that **PT401** bound to the enzyme with similar affinity to the parent compound. Therefore, we conclude that the A ring fluoro substituent plays a significant role in governing enzyme affinity as well as the on rate for the hexyl-substituted diphenyl ethers, however B ring modifications may offset the changes in binding resulting in comparable dissociation rates.

Diphenyl Ether Kinetic Optimization—The overall association and dissociation kinetics for the ethyl and hexyl diphenyl ethers were analyzed using a 2D-kinetic map, in which the combinations of these rates that result in the same K_i values are represented as diagonal lines (Figure 3). Inhibitors with the highest affinity and longest residence time, such as **PT444**, are found in the lower right quadrant (IV), having high association and low dissociation rate constants. However, it is also possible to obtain long residence time inhibitors, such as **PT404**, by reduction in the association rate constant and without changing thermodynamic affinity. Such compounds are found in the lower left quadrant (III) of the plot with k_3 values ranging between 10^2 and 10^4 M⁻¹s⁻¹. These results reveal that we are able to moderately shift the on-rate kinetics of the analogs into the ideal ‘sweet spot’ (10^3 and 10^5 M⁻¹s⁻¹) recently reported by Schoop and Dey to be the most attractive range to generate residence times of multiple hours without requiring sub-nM affinity of the inhibitors for the enzyme.⁶²

Interestingly, the kinetic map revealed that the off-rate correlates better with affinity for the hexyl-substituted diphenyl ethers, while the on-rates remain almost constant. By contrast, a broader range of on-rates was observed for the ethyl-substituted diphenyl ethers. Both trends were observed whether or not the fluoro group was present on the A-ring. These results indicate that an increase in residence time within a compound series can occur either by increase in affinity *or by reduction in k_3* .

Structural elements and key residues found in bpFabI1-inhibitor ternary complexes

Induced-fit ligand binding and the role of F203 in inhibitor binding—In a search for a molecular understanding of enzyme inhibition, ternary inhibitor complexes of bpFabI1 (bpFabI-NAD⁺-inhibitor) were determined for 9 substituted diphenyl ethers as well as the apo bpFabI1 structure. Structural studies revealed that α -helix 6 (residues T¹⁹⁴-G¹⁹⁹) of the bpFabI1 substrate binding loop (SBL) becomes ordered and undergoes closure upon ligand binding. This loop is found in a similar closed conformation for all bpFabI1-inhibitor complexes including those formed by triclosan (**TCL**), **PT01**, **PT02**, **PT12**, **PT401**, **PT404**, **PT405**, **PT408**, and **PT412**. In crystal structures containing more than one monomer in the asymmetric unit (e.g. **PT02**, **PT405**, **PT408**, **TCL**) the inhibitor is present in each subunit and the corresponding SBL displays an identical conformation in all of the subunits. The closed conformation for all bpFabI1-inhibitor complexes is distinctly different from the open SBL conformation found in the apo bpFabI1 structure and the recently published structure of 4-pyridone inhibitor **PT155** in complex with bpFabI1 (PDB entry 4BKU) (Figure 4A). **PT155** is a rapid reversible inhibitor of bpFabI1 displaying mixed inhibition with respect to *trans*-2-octenoyl-CoA (K_i 130 nM, α 0.48, Figure S1). Thus, these results are consistent

with previous studies in which a closed SBL conformation has been observed for structures of ENRs from other bacteria in complex with slow binding inhibitors.^{20, 23, 63–65}

F203 plays a key role in stabilizing the ternary inhibitor complexes of bpFabI1 for both rapid reversible and slow binding inhibitors, in which its steric bulk may influence the open and closed SBL conformations. F203 was found to occupy the space in the apo-form of bpFabI1 where A197 of α -helix 6 in the inhibitor-bound (closed) form is located. Ligand binding causes a rotation of the ring plane of F203 towards L207, thus flanking the hydrophobic pocket to accommodate the 5-alkyl substituents. For the 4-F diphenyl ethers, potential C-H...F halogen-bonding interactions can be observed between the 4-F substituent and the hydrogen of the aromatic system of F203 with $C_{e,F203} - F_{C4,PT}$ distances ranging from ≈ 3.0 Å to 3.5 Å (Figure 4B). However, the interactions between F203 and the A-ring display a larger hydrophobic component for inhibitors lacking a substituent at the 4-position, with $C_{e,F203} - C_{4,inhibitor}$ distances of about 4 Å, and in which the side chain of F203 is slightly more oriented towards the interior of the substrate-binding pocket (Figure 4C). In contrast, the 4-CH₃ group of **PT155** causes the ring plane of F203 to be perpendicular relative to the planes observed in the other bpFabI-NAD⁺-inhibitor structures, generating C-H... π interaction distances of 3.4 Å to 4.1 Å from the center of the ring plane to the 4-CH₃ group of **PT155** (Figure 4D). As a consequence, A197 is forced out of the binding pocket to avoid a steric clash with F203, leading to a more open SBL conformation when **PT155** is bound. These results indicate the enzyme inhibition modulates the position of the SBL, and providing direct insight into the structural changes that accompany the induced-fit slow binding mechanism.

Flexibility of the closed SBL ENREF 39—The 2D-kinetic plot indicates that ethyl-substituted diphenyl ethers have a broader range of on-rates compared to the hexyl-substituted diphenyl ethers. To provide a structural basis for the modulation in on-rate, we analyzed the precise conformation of the SBL in the enzyme:inhibitor complexes. Comparative analysis revealed that residues S¹⁹⁸ – K²⁰¹, located in the loop between α -helix 6 and α -helix 7 of the SBL, have moved slightly further away from the substrate binding pocket in the ternary **PT12** and **PT401** complexes, reaching a maximum C_{α} -distance at the position of L201 ranging from 1.9 to 4.1 Å, compared to the other inhibitor structures. Owing to the fact that both inhibitors contain a 5-hexyl substituent on the inhibitor A-ring, this movement is related to the spatial requirements to accommodate longer alkyl chains and I200 shifts out of the substrate-binding pocket by $1.8 - 3.0$ Å (Figure 4E). This observation coincides with previous structural studies of ecFabI in complex with a 2-pyridone inhibitor, CG400549, in which I200 in ecFabI (corresponding to bpFabI1 position 200) was shown to restrict the available space for compounds containing large 5-position substituents.⁶⁶ Thus, longer alkyl (hexyl-) chains require a slightly more open SBL conformation compared to inhibitors containing only a short alkyl (ethyl- or propyl-) substituent at this position. These results indicate a less flexible binding mode for the hexyl-substituted diphenyl ethers within the substrate-binding pocket, coinciding with the observed kinetic behavior of low on-rate variability on the 2D-kinetic map.

Interaction patterns between the B ring substituents and the substrate-binding pocket

Modifications on the B ring of diphenyl ethers may also result in changes to either the on or off-rates, which can lead to long residence times by ground state stabilization or transition state destabilization. To explore structural elements that can help distinguish between these mechanisms, we evaluated the orientation of the diphenyl ether B ring, and interaction patterns between the B-ring substituents and the substrate-binding pocket. In general, the B-ring of 4'-unsubstituted inhibitors forms van-der-Waals contacts with M159 and is oriented away from the SBL residues, with $C4'_{\text{inhibitor}} - C7_{\text{agr.}}, A196^-$ distances ranging from 5.7 to 6.2 Å (Figure 5A). In contrast, the B-ring planes for 4'-substituted inhibitors shift slightly closer to the side chains of the SBL residues (5.1 to 5.4 Å) in order to accommodate spatial requirements within the upper part of the substrate-binding pocket created by interactions between the SBL and residues F94/A95. Taken together with the kinetic data, the movement of the B-ring plane in the 4'-substituted inhibitor:enzyme complexes correlates with the slower on-rates and higher/lower affinities for the ethyl-substituted diphenyl ethers (Table 1). Although the 4'-substituted inhibitors have lower affinities compared to the 4'-unsubstituted inhibitors, there are well-defined subtle differences in interaction patterns to stabilize inhibitor-binding. For instance, the 4'-NO₂ group of **PT404** may participate in a water-bridged hydrogen-bond network with the side chain of R97 and the carbonyl of G93, and an even more extensive network can also be observed for **PT12** (Figure 5B–C). Due to this network, R97 can only be oriented towards the inhibitor leading to a structure that resembling a lid on top of the binding-pocket if the SBL is fully closed, whereas it would clash in the apo-state and ternary **PT155** complex. These interactions of the 4'-NO₂ group may contribute significantly to the stabilization of inhibitor-binding and the structural elements provide insights into the observed kinetic behavior of slower on-rates.

Compounds with substituents at the 2'-position display higher affinities for bpFabI1, unlike the 4'-substituted inhibitors, through favorable interactions with A196 of the bpFabI1 SBL. The orientation of the 2'-Cl substituents of **PT404** and **TCL** are more likely to form favorable hydrophobic contacts with A196 of the SBL. Inhibitor **PT405** (2'-F) assumes a similar orientation as the compounds with a 2'-Cl substituent, and due to its smaller van-der-Waals radius it fills the cavity between the SBL residue A196 and the opposing G93 to a lesser extent (Figure 5D–E). However, the nature of interaction between A196 and the 2'-F may differ compared to compounds with a 2'-Cl because the electronegativity of F is higher than that of Cl and the F – C_β distance in the **PT405** structure ranges between 3.3 and 3.5 Å. Thus, the 2'-F group may have characteristics of a CH---F hydrogen bond in contrast to the rather hydrophobic nature which it would have with a 2'-Cl substituent. Nevertheless, a halogen group at the 2'-position is thermodynamically favorable with no significant change in on-rate, as observed for **PT411**, **PT405**, and **PT406** where K_i^{app} is reduced from 31 to 3 nM. In contrast, the nitro group of **PT412** at the 2'-position is faced with a less advantageous, rather hydrophobic surrounding. Although a hydrogen-bond with the 2'-OH group of the nicotinamide ribose can be formed by one of the oxygens from **PT412**, no hydrogen-bond donor from bpFabI1 is available in a favorable position (Figure 5F). This structural observation coincides with kinetic data in which little to no difference in K_i^{app} was observed between **PT411** and **PT412** (31 to 59 nM), however K_i^{app} can be significantly

improved by replacement with a 2'-CN group as observed for **PT443** and **PT444** (0.5 to 1 nM). The interaction patterns that are observed between substituents at the 2'-position and bpFabI1 residues or NAD⁺ supports ground state stabilization effects.

Most diphenyl ethers displayed a resistance index against Bp400

The antimicrobial activity was evaluated for each compound against the efflux competent strain *B. pseudomallei* 1026b and the efflux mutant strain *B. pseudomallei* Bp400 (1026b [*bpeAB-oprB*] [*amrAB-oprA*]) (Table 1). In general, the minimum inhibitory concentration (MIC) values for triclosan-based analogues ranged between 64 and >128 mg/L in Bp1026b. These MIC values were at least 2-4 fold higher in comparison to triclosan (30 mg/L).¹⁵ In contrast, only compounds with 5-ethyl substituents displayed improved antimicrobial activity against an efflux-compromised *B. pseudomallei* strain, ranging between 4 and 16 mg/L with an average resistance index of ~12-fold. **PT404** had the maximum resistance index against Bp400 of 32-fold and **PT01** had the minimum resistance index against Bp400 of 4-fold. The MIC values for **PT408** and all diphenyl ethers with 5-hexyl substituents remained >128 mg/L against Bp400.

Selected diphenyl ethers demonstrate *in vivo* efficacy in acute *B. pseudomallei* infection animal model

The efficacies of long residence time inhibitors **PT443** (t_R 118 min), **PT405** (t_R 250 min), and **PT444** (t_R 670 min) were evaluated in the acute *B. pseudomallei* animal model using the virulent *B. pseudomallei* strain Bp400 (Figure 6). Mice were challenged with 5000 CFU and the bacterial burden was assessed at 60 h post infection in lung and spleen homogenate.

The efficacies of these compounds were measured against an untreated control group and a positive control treated with 200 mg/kg ceftazidime. Tested compounds showed a significant decrease in bacterial burden in the spleen, which is used to determine efficacy in the acute model of disease because it provides information about disease progression and dissemination—both factors of disease relapse. All three inhibitors demonstrated better efficacy than previous generations of diphenyl ethers such as **PT01**, **PT52** and **PT68** that were found to reduce bacterial burden in the spleen between 1.1–1.4 Log₁₀ CFU/mL.^{16, 17} **PT405** showed a modest improvement in reduction ($P < 0.001$) of 1.69 Log₁₀ CFU/mL, and **PT443** and **PT444** significantly ($P < 0.001$) reduced the bacterial burden even more by a factor of 2.48 and 2.26 Log₁₀ CFU/mL.

DISCUSSION

Previously we demonstrated that diphenyl ether inhibitors of bpFabI1 had *in vitro* and *in vivo* antibacterial activity against *B. pseudomallei*, consistent with the observation that bpFabI1 was essential for bacterial growth.^{16, 17} Although these initial studies revealed that some diphenyl ethers displayed slow binding behavior, the mechanism of bpFabI1 inhibition was not reported at that time. Given the potential importance of both on and off-rates for governing *in vivo* drug pharmacology, we have now expanded our analysis of bpFabI1 inhibition in an attempt to provide a foundation for the rational optimization of drug-target kinetics in this system.^{60, 67, 68}

The diphenyl ethers generally bind to the E-NAD⁺ FabI product complex. In Figure 7A, we show the free energy profile of the binding reaction coordinate for the interaction of inhibitors with the bpFabI-NAD⁺ complex. This analysis enables us to distinguish ground and transition state contributions to the residence time observed for each compound. For the 5-ethyl substituted diphenyl ethers, we can see that residence time can be modulated either by effects on the ground state, transition state or both relative to **PT01** (Figure 7B). Similarly, modulations were also observed for the ground and transition state contributions for each compound with a 5-hexyl substituent relative to **PT04** (Figure 7C). In general, little to no difference in residence time was observed for compounds that exhibited transition and ground state destabilization to the same extent, such as **MUT056399** and **PT407** compared to **PT01**. In contrast, differential effects on the transition and ground states were observed for the other compounds and their contributions to residence time were driven predominantly by either ground state destabilization, ground state stabilization or transition state destabilization. Long residence times can therefore be rationalized by improvement of affinity through ground state stabilization, as seen with compounds **PT444** and **PT119**, or by transition state destabilization, as seen with compounds **PT404** and **PT417**.

Structural studies suggest that stabilization of the ground state can occur through the introduction of nonpolar substituents at the 2'-position, which can interact with nonpolar amino acids nearby the SBL. For instance, the introduction of halogen groups at the 2'-position (2'-F, **PT405** and 2'-Cl, **PT406** and **PT91**) can partially or completely fill a cavity between A196 and G93 (Figure 5D-E). The replacement of the halogen group at this position with a nitrile (**PT119**, **PT443** and **PT444**) can also stabilize the ground state, presumably because the 2'-CN substituent may form a hydrogen-bond with the 2'-OH group of the nicotinamide ribose. Nevertheless, due to the relatively long distances between the 2'-substituents and the 2'-OH of the nicotinamide ribose observed in the structures, ranging from 3.5 – 3.8 Å, and unfavorable angles, this interaction might have a rather weak character. Interestingly, the distances and angles between the 2'-substituent and the 2'-OH of the nicotinamide ribose improve for 4'-substituted inhibitors, e.g. **TCL** and **PT404**, since the B-ring planes are oriented differently within the substrate-binding pocket. By contrast, although one of the oxygens from the 2'-NO₂ found in **PT409** and **PT412** can form a hydrogen-bond with the 2'-OH group of the nicotinamide ribose, there are no available hydrogen-bond donors from bpFabI to interact with the other oxygen from the NO₂ group (Figure 5F). Thus, this less-than-optimal interaction does not significantly affect the ground state compared to **PT01**, yet it results in a destabilization of the transition state plausibly because the 2'-NO₂ is positioned in a less favorable, hydrophobic environment. Moreover, replacement of the 2'-NO₂ with a 2'-CH₃ (**PT70**, **PT401** and **PT408**) may compliment this hydrophobic environment, however, the CH₃ group is still more sterically demanding than the favored small halogen groups and thus the transition state is destabilized.

While the substituents at the 2'-position may contribute to ground and transition state stabilization, substituents at the 4'-position predominantly alter the transition state. Structural studies revealed that the B-ring planes for 4'-substituted inhibitors shift closer to the side chains of the SBL residues in order to accommodate spatial requirements within the upper part of the substrate-binding pocket created between the SBL and residues F94/A95.

These differences in the diphenyl ether skeleton are relevant to the B ring planes for **PT12**, **PT403** and **PT404** and such structural rearrangements may also result in a destabilization in transition state.

Owing to the fact that k_3 is related to the difference in free energy between the ground and transition states on the binding coordinate, the evaluation of k_3 values can be used to further understand how transition state energies can be modulated to optimize residence time. The relationship between incremental changes to the diphenyl ether skeleton and its kinetic behavior was evaluated using a 2D-kinetic map (Figure 3).^{62, 69} The 2D-kinetic plot revealed that the overall improvement of residence time for the hexyl-substituted diphenyl ethers was achieved by affinity gain while there was a low variability in the corresponding on-rates. This may be due to a lack of flexible binding modes for compounds with longer alkyl groups, in which hexyl diphenyl ethers require a slightly more open SBL conformation in the loop region between α -helices 6 and 7 compared to diphenyl ethers with shorter alkyl substituents at this position (Figure 4A). These results are also consistent with the observation that ethyl-substituted diphenyl ethers exhibited a broader range of on-rate variability, in which k_3 was a much more important contributor to residence time.

To provide a starting point for correlating drug-target kinetics with antibacterial activity, we determined the *in vitro* activity of all compounds against wild-type and a pump mutant strain of *B. pseudomallei* and analyzed the *in vivo* efficacy of three compounds with significant residence times (**PT405**, **PT443**, and **PT444**) against efflux-compromised *B. pseudomallei* Bp400. Whilst none of the hexyl diphenyl ethers demonstrated antibacterial activity towards either Bp400 or the efflux competent strain Bp1026b, all the ethyl-substituted analogs except **PT408** had MIC values of 4–16 mg/L towards Bp400. The MIC values of the ethyl analogs increased to 128 mg/L for Bp1026b demonstrating that they were efficiently effluxed by this strain of *B. pseudomallei*. In those cases where circumvention of the efflux pumps showed improved antibacterial activity, there was, however, no observable correlation between MIC values and residence time. This is not surprising since MIC values are obtained at constant drug concentration whereas residence time is a kinetic parameter that is more likely to impact time-dependent antibacterial activity. We then analyzed the *in vivo* efficacy of three compounds with measurable MIC values against Bp400 in an animal model of infection, and treatment with **PT405**, **PT443**, and **PT444** was found to cause a 1.7–2.5 log₁₀ reduction of bacterial burden in the spleens of the infected animals. The observed efficacy is consistent with our previous reports with this series of compounds,^{16, 17} substantiating the importance of bpFabI as a clinically relevant molecular target for the development of novel agents to treat *B. pseudomallei* infection. Although **PT405**, **PT443**, and **PT444** have residence times that range from 2–11 h at 25°C, all had similar levels of activity in the animal model of infection. Thus, at this stage it is not possible to draw any conclusions about the relationship between residence time and efficacy in this system. Future studies will aim to explore this relationship by determining the time-dependent *in vivo* activity (*in vivo* post-antibiotic effect) of selected compounds and including drug pharmacokinetics in models that relate time-dependent target engagement to drug activity.^{70, 71}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. One and two-step kinetic mechanisms for enzyme inhibition and slow binding FabI inhibitors

A) The three possible mechanisms (**A**, **B** and **C**) for slow binding inhibition. B) Triclosan is a broad-spectrum inhibitor that targets the FabI enoyl-ACP reductase, C) The FabI-specific inhibitor MUT056399.^{25–27}

Figure 2. Representative data for the time-dependent inhibition of bpFabI1

(A) Progress curves for bpFabI1 inhibition by **PT12**. The experimental data (●) were globally fit to Equation 1 generating the solid lines ($R^2 = 0.98$). (B) Plot of the pseudo-first order rate constant (k_{obs}) as a function of inhibitor concentration (●). Curve fitting gave the solid line ($R^2 = 0.99$). Inhibition parameters for each compound are summarized in Table 1.

Figure 3. 2D-Kinetic map for the diphenyl ether inhibitors of bpFabI

The 2D plot enables the structure-kinetic relationship (SKR) for bpFabI to be visualized. On-rate (k_3) and off-rate (k_4) data are plotted for the A-ring ethyl (dark blue) and hexyl (light blue) diphenyl ethers. Combinations of k_3 and k_4 values that result in the same K_i value are represented as diagonal lines. **PT443** and triclosan are shown in green. The diphenyl ethers segregate into four distinct quadrants. Quadrant I: shorter residence time inhibitors with sub- to low nM affinity. Quadrant II: shorter residence time inhibitors with high to sub-nM affinity. Quadrant III: longer residence time inhibitors with high to sub-nM affinity. Quadrant IV: longer residence time inhibitors with low nM affinity.

Figure 4. Substrate-binding loop conformations for the bpFabII-inhibitor ternary complexes

The figure shows the influence of inhibitor substituents on the orientation of F203. (A) The substrate-binding loop (SBL) of bpFabII forms a closed conformation in the diphenyl ether inhibitor-bound bpFabII complexes, while this loop is found in an open conformation in the apo-form (purple) and in the complex with rapid reversible 4-pyridone inhibitor **PT155** (dark blue, PDB entry 4BKU). (B–D) Substituents at the 4-position alter the interaction of the diphenyl ethers with residue F203. The 4-F group of **PT404** (red), **PT405** (magenta), and **PT412** (orange) participate in halogen-bonding interactions (B). Diphenyl ethers lacking a substituent at the 4-position, such as triclosan (purple), **PT01** (light green) and **PT02** (cyan), participate in van-der-Waals interactions (C). The presence of a 4-methyl substituent on **PT155** (dark blue) leads to an approximate 90°-flip of the aromatic ring plane of F203, which consequently engages in C-H--- π interactions with the methyl group. The steric requirements of the flipped residue results in A197 being pushed further out of the binding pocket to avoid a steric clash, resulting in a more open SBL conformation compared to the **PT02** (cyan) complex (D). The orientation of I200 is influenced by the chain length of the 5-position A-ring acyl group, where longer chains require a slightly more open SBL conformation as shown in E for **PT401** (yellow), **PT155** (dark blue) and **PT12** (light pink) complexes compared to the **PT01** complex (light green). The loops in question are shown in colored cartoon representation. Interaction distances are represented by yellow dashes. Residues, inhibitors and cofactors are shown as sticks.

Figure 5. Binding modes of different 2' and/or 4'-substituted bpFabII inhibitors
 (A) The B ring planes for the 4'-substituted inhibitors were compared with 4'-unsubstituted inhibitors, here using the example of **PT01** (light green) and **PT404** (red). For the 4'-substituted inhibitors, the B ring planes shift closer to the side chains of the SBL residues (magenta) in which the $C4'_{\text{inhibitor}} - C_{\alpha, A196}$ -distances range from 5.1 to 5.4 Å. However, the $C4'_{\text{inhibitor}} - C_{\alpha, A196}$ -distances range increases (5.7 to 6.2 Å) for the B rings of 4'-unsubstituted inhibitors as the B rings establish van-der-Waals contacts with M159 (dark cyan). The 4'-NO₂ group may participate in a water-bridged hydrogen-bond network with the side chain of R97 and the carbonyl of G93, as observed for **PT404** (B) and **PT12** (C), which displays an additional water-bridged hydrogen-bond with the carbonyl of A196. (D–F) Substituents at the 2'-position can participate in both polar and nonpolar interactions. The 2'-Cl of **PT404** (D, red space filling) fills a cavity between the SBL residue A196 and the opposing G93, and this cavity is partially filled with a 2'-F substituent as observed for **PT405** (E, magenta, space filling). (F) One of the oxygens from the 2'-NO₂ group of **PT412** forms a hydrogen-bond with the 2'-OH group of the nicotinamide ribose and there is no available hydrogen-bond donor from bpFabII to interact with the other oxygen. Interaction distances are represented by dashes and residues, inhibitors and cofactors are shown as sticks.

Figure 6. Bacterial burden in mouse lung (A) and spleen (B) at 60 h post infection
The mean of each group was plotted and error bars indicating $\pm$ standard deviation. Significance was determined by one-way ANOVA and Tukey's multiple comparison post-test (** $P < 0.001$; **** $P < 0.0001$). Dotted line represents limit of detection.

Figure 7. Free energy profile for bpFabI1-diphenyl ether interactions

(A) The diphenyl ether inhibitors of bpFabI1 display a special case of the two-step induced-fit slow binding mechanism that is kinetically indistinguishable from a one-step mechanism, and in which k_3 and k_4 define the respective overall apparent association and dissociation rate constants for the formation and dissociation of EI, and K_i is the dissociation constant of EI. A decrease in k_4 (increase in residence time) can occur either by an increase in barrier height (transition state (TS) destabilization) or an increase stability of EI (ground state (GS) stabilization) (red line). In order to depict the relative contribution that changes in GS and TS stability play to the residence time of each inhibitor, we calculated the change in GS and

TS free energy relative to either **PT01** or **PT04**. For compound x the change in GS free energy at 298 K is given by $G_{GS} = -RT \ln(K_{i(X)}/K_{i(PT01)})$ and the change in TS free energy is given by $G_{TS} = -RT(\ln(K_{i(PT01)}/K_{i(X)}) - \ln(k_{4(PT01)}/k_{4(X)}))$. **(B)** For the 5-ethyl substituted diphenyl ethers, the change in free energy was calculated relative to **PT01**. **(C)** For the 5-hexyl substituted diphenyl ethers, the change in free energy was calculated relative to **PT04**. GS contributions are shown in blue, in which positive values indicate GS stabilization relative to the standard state and negative values indicate GS destabilization. TS contributions are shown in red, in which positive values indicate TS destabilization and negative values indicate TS stabilization relative to the standard state.

Scheme 1.

Reaction catalyzed by the enoyl-ACP reductase (ENR).

Table 1Kinetic and thermodynamic parameters for inhibitors of bpFabII.^a

Compound	Structure	$K_i^{\text{app}}(\text{nM})^b$	$k_3^{\text{app}}(\text{M}^{-1}\text{s}^{-1})^c$	$k_4(\text{min}^{-1})^b$	$t_R(\text{min})^d$	MIC (mg/L)	
						Bp400	Bp1026b
Triclosan		32 ± 0	3.25 × 10 ⁴	0.062 ± 0.001	16 ± 0	0.09	1.12
PT443		1 ± 0	1.42 × 10 ⁵	0.0085 ± 0	118 ± 0	16	128
<i>Derivatives with 5-ethyl substituents</i>							
MUT056399		405 ± 2	3.33 × 10 ³	0.081 ± 0	12 ± 0	16	>128
PT01		32 ± 1	2.82 × 10 ⁴	0.054 ± 0.002	19 ± 1	16	64
PT411		31 ± 0	2.80 × 10 ⁴	0.052 ± 0	19 ± 1	16	128

Compound	Structure	K_i^{app} (nM) ^d	k_3^{app} ($\text{M}^{-1}\text{s}^{-1}$) ^e	k_4 (min^{-1}) ^f	t_R (min) ^d	MIC (mg/L)	
						Bp400	Bp1026b
PT405		4 ± 0	1.67×10^4	0.0040 ± 0	250 ± 1	16	>128
PT406		3 ± 0	1.56×10^4	0.0028 ± 0.0001	357 ± 6	8	128
PT412		58 ± 2	1.83×10^3	0.0064 ± 0.0003	156 ± 7	16	>128
PT407		156 ± 0	4.38×10^3	0.041 ± 0	24 ± 0	8	128
PT403		32 ± 0	1.30×10^4	0.025 ± 0	40 ± 0	16	>128

Compound	Structure	K_i^{app} (nM) ^b	k_3^{app} (M ⁻¹ s ⁻¹) ^c	k_4 (min ⁻¹) ^b	t_R (min) ^d	MIC (mg/L)	
						Bp400	Bp1026b
PT404		153 ± 0	1.53×10^2 e	0.0014 ± 0.0005 e	713 ± 25 e	4	>128
PT408		55 ± 0	2.78×10^3	0.0092 ± 0.0001	109 ± 1	>128	>128
PT444		0.51 ± 0.01	4.90×10^4	0.0015 ± 0	667 ± 9	8	128
PT409		44 ± 0	6.43×10^3	0.017 ± 0	59 ± 0	16	128
<i>Derivatives with 5-hexyl substituents</i>							
PT04		138 ± 3	6.65×10^3	0.055 ± 0	18 ± 0	>128	>128
PT113		105 ± 0	1.75×10^3	0.011 ± 0	91 ± 0	>128	>128

Compound	Structure	K_i^{app} (nM) ^b	k_3^{app} (M ⁻¹ s ⁻¹) ^c	k_4 (min ⁻¹) ^b	t_R (min) ^d	MIC (mg/L)	
						Bp400	Bp1026b
PT91		25 ± 0	3.73 × 10 ³	0.0056 ± 0.0001	179 ± 2	>128	>128
PT119		15 ± 0	3.67 × 10 ³	0.0033 ± 0	303 ± 1	>128	>128
PT70		48 ± 0	2.57 × 10 ³	0.0074 ± 0	135 ± 1	>128	>128
PT12		158 ± 0	3.07 × 10 ³	0.029 ± 0	34 ± 0	>128	>128
PT400		1134 ± 5	7.05 × 10 ²	0.048 ± 0.001	21 ± 0	>128	>128
PT401		55 ± 1	1.39 × 10 ³	0.0046 ± 0.0001	217 ± 2	>128	>128

Compound	Structure	K_i^{app} (nM) ^d	k_3^{app} (M ⁻¹ s ⁻¹) ^e	k_4 (min ⁻¹) ^b	t_R (min) ^d	MIC (mg/L)	
						Bp400	Bp1026b
PT417		130 ± 2	8.85×10^2	0.0069 ± 0.0001	145 ± 2	>128	>128

^aParameters were determined using 8 nM enzyme, 0.75 mM crotonyl-CoA, 0.25 mM NADH and 0.20 mM NAD⁺ at fixed inhibitor concentrations (0–8 μM).

^bAll datasets at fixed inhibitor concentrations were globally fit to Equation 1. v_s and k_{obs} are defined by Equations 2–3 with $v_i = v_0$.

^cCalculated using $k_3^{app} = k_4/K_i^{app}$.

^dCalculated using $t_R = 1/k_4$.

^eValues reported using a direct dissociation method developed by Yu *et al.*²⁸