Selective inhibition of Biotin Protein Ligase from *Staphylococcus aureus*

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**Running title:** Inhibition of *Staphylococcus aureus* biotin protein ligase

**Keywords:** biotin protein ligase; enzyme inhibitor; antibiotic; medicinal chemistry; X-ray crystallography.

**Background:** Inhibitors of biotin protein ligase potentially represent a new antibiotic class

**Results:** Biotin triazoles inhibit the BPL from *S. aureus*, but the human homologue.

**Conclusion:** Our most potent inhibitor shows cytotoxicity against *S. aureus*, but not cultured mammalian cells.

**Significance:** This is the first report demonstrating selective inhibition of BPL.

**SUMMARY (250 words)**

There is a well-documented need to replenish the antibiotic pipeline with new agents to combat the rise of drug resistant bacteria. One strategy to combat resistance is to discover new chemical classes, immune to current resistance mechanisms, which inhibit essential metabolic enzymes. Many of the obvious drug targets that have no homologous isozyme in the human host have now been investigated. Bacterial drug targets that have a closely related human homologue represent a new frontier in antibiotic discovery. However, to avoid potential toxicity to the host, these inhibitors must have very high selectivity for the bacterial enzyme over the human homolog. We have demonstrated that the essential enzyme biotin protein ligase (BPL) from the clinically important pathogen *Staphylococcus aureus* could be selectively inhibited. Linking biotin to adenosine via a 1,2,3 triazole yielded the first BPL inhibitor selective for *S. aureus* BPL over the human equivalent. The synthesis of new biotin 1,2,3 triazole analogues using click chemistry yielded our most potent structure ($K_i$ 90 nM) with >1100-fold selectivity for the *S. aureus* BPL over the human homologue. X-ray crystallography confirmed the mechanism of inhibitor binding. Importantly, the inhibitor showed cytotoxicity against *S. aureus*, but not cultured mammalian cells. The biotin 1,2,3 triazole provides a novel pharmacophore for future medicinal chemistry programmes to develop this new antibiotic class.

Since the discovery and development of penicillin, over 70 years ago, society has grown accustomed to rapid and effective treatment of bacterial infections. While a range of antibiotics has since been developed to target a wide diversity of infectious agents, resistance to these compounds is an inevitable and relentless process. The combination of over-prescription and the waning interest of the pharmaceutical industry in this area over the last 30 years, has contributed to the emergence of widespread life-threatening infections with strains that are resistant to most, if not all, antibiotics in current clinical use (1,2). For
example, *S. aureus* bacteraemia in the USA has almost trebled in the past 20 years, with 50-60% of hospital acquired infections now being due to methicillin resistant strains (MRSA) (3,4). More disturbingly, while the public profile of serious *S. aureus* infections is that they are hospital-acquired, it is important to recognize that 60% of such infections are now thought to begin in the community (3,4).

Screening for anti-microbial activity in natural product extracts identified the majority of currently prescribed classes of antibiotics. The molecular targets for these agents, where known, are generally an essential protein or enzyme that is unique to the prokaryotic pathogen. These obvious drug targets have now been extensively investigated. Since only three new classes of antibiotic have been developed for the clinic in the last 35 years, it is clear that alternative avenues to antibiotic discovery must be considered. One such approach is to target essential proteins and enzymes even if they have homologues in humans. This greatly increases the opportunity to identify new classes of antibiotics with novel modes of action. Importantly, this absolutely necessitates that very high selectivity for the bacterial enzyme is achieved over the human equivalent. A number of antivirals have been identified using this approach, with multiple neuraminidase inhibitors available that have a therapeutic window of >5 orders of magnitude (5,6).

Biotin protein ligase (BPL) presents one such antibacterial drug target. Whilst it is ubiquitously found throughout the living world, protein sequence comparisons show that this enzyme family is segregated into three structural classes. Importantly, the Gram-negative and Gram-positive pathogenic bacteria fall into class I or II, whilst the enzymes from mammals are in a separate class (III) with a large N-terminal extension required for catalysis that is not present in the bacterial enzymes (7,8). BPL catalyses the ATP-dependent addition of biotin onto specific carboxylases that require the cofactor for activity. The BPL in *S. aureus* (*Sa*BPL) has two such substrates, acetyl-CoA carboxylase (ACC) and pyruvate carboxylase (PC), that without biotinylation are totally inactive. We, and others, have proposed BPL as a potential antibacterial target, since ACC is required for membrane lipid biosynthesis (9-11). This metabolic pathway is important in *S. aureus*, as the bacteria can only derive 50% of their membrane phospholipids from exogenous fatty acids (12). Inhibitors of bacterial ACC have demonstrated *in vivo* efficacy in an *S. aureus* infection model of mice (13). PC plays an anaplerotic role in central carbon metabolism, where it replenishes the tricarboxylic acid cycle with oxaloacetate (14). The inhibition of BPL therefore targets both fatty acid biosynthesis and the tricarboxylic acid cycle pathways. Genetic studies support the observation that *Sa*BPL is an essential gene product in *S. aureus* (15-17).

BPL performs protein biotinylation via the synthesis of a reaction intermediate, biotinyl-5’-AMP (1, Fig. 1), where a labile phosphoanhydride linker joins biotin with AMP. An enzymatic mechanism involving an adenylated intermediate is employed by other organic acid ligases, such as amino acyl tRNA synthetases, *o*-succinylbenzoyl-CoA synthetase and phosphopantothenoylcysteine synthetase, amongst others. Using an adenylated intermediate as a basis for developing ligase inhibitors is problematic because of the hydrolytic and enzymatic instability of the component phosphoroanhydride linkage and difficulties of synthesis (18). Nonetheless, several of these enzymes have been the subjects of antibacterial drug discovery studies using inhibitors designed to replace the labile phosphoanhydride with more stable functionalities (19-24). Non-hydrolysable phosphate bioisosteres, such as sulphonyl, phosphodiesters, hydroxylamine and di-keto-ester, have been reported. However, there has been limited success in developing inhibitors that show the required selectivity over the mammalian homologues. This was highlighted in a recent study targeting the BPL from *Mycobacterium tuberculosis* (11) using a simple analogue of the reaction intermediate 1 where the phosphate linker was replaced with a sulfamate group. Whilst the acylsulfamate analogue was a potent inhibitor of *M. tuberculosis* BPL (*K*<sub>D</sub> 0.5 nM), it was not tested for its selectivity *in vitro* with human BPL. However, observed toxicity in a cell culture model suggested poor selectivity. Thus the development of antibiotics based on the inhibition of BPL requires compounds with improved stability, species selectivity, and versatility of
It is important to note that BPL was one of 70 molecular targets investigated by GSK in an antibacterial discovery program using high throughput screening (9). The reported lack of success using this approach highlights the need for a more sophisticated approach to inhibitor discovery, and the importance of combining structural biology, enzymology and biophysical characterization to direct medicinal chemistry. As an important first step we determined the X-ray crystal structures of SaBPL alone and in complex with a chemical analogue of 1, biotinol-5^'-AMP (2, Fig. 1), that contains a non-hydrolysable phosphodiester linker (25). The challenge is to now incorporate structural biology into the design of potent and selective inhibitors of SaBPL. With this in mind we report a rational structure-guided design to obtain and characterize potent and selective BPL inhibitors containing a triazole bioisostere of the phosphate group of 1 that possess narrow-spectrum antimicrobial activity.

**EXPERIMENTAL PROCEDURES**

**Protein methods**

The expression and purification of recombinant SaBPL (10), EcBPL (26) and HsBPL (8) have been previously described. SaBPL and HsBPL were obtained with a C-terminal hexahistidine tag. Quantitation of BPL activity was performed as previously described (27,28) and Supplementary Material. Methodologies for surface Plasmon resonance and circular dichroism are also described in Supplementary material.

**X-ray crystallography**

Apo-SaBPL was buffer exchanged into 50 mM Tris HCl pH 7.5, 50 mM NaCl, 1 mM DTT and 5% (v/v) glycerol, and concentrated to 5 mg/mL. Each compound was then added to BPL in a 10:1 molar ratio. The complex was crystallized using the hanging drop method at 4° C in 8 – 12% (w/v) PEG 8000 in 0.1 M Tris pH 7.5 or 8.0, and 10% (v/v) glycerol as the reservoir. A single crystal was picked using a Hampton silicon loop and streaked through cryoprotectant containing 25% (v/v) glycerol in the reservoir buffer prior to data collection. X-ray diffraction data was collected at the macromolecular crystallography beamline at the Australian Synchrotron using an ADSC Quantum 210r Detector. 90 images were collected for 1 second each at an oscillation angle of 1° for each frame. Data was integrated using HKL, and refined using the CCP4 suite of programs (29). PDB and cif files for the compounds were obtained using the PRODRG web interface. The models were built using cycles of manual modeling using COOT (30) and refinement with REFMAC (29). The quality of the final models was evaluated using MOLPROBITY. Composite omit maps were inspected for each crystal structure and statistics for the data and refinement reported (Table S1). The crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, [www.pdb.org](http://www.pdb.org) [PDB ID codes: SaBPL with 2 4DQ2, SaBPL with 7 3V7C; SaBPL with 14 3V7S].

**Antibacterial Activity Evaluation**

Antimicrobial activity of the compounds was determined by a microdilution broth method as recommended by the CLSI (Clinical and Laboratory Standards Institute, Document M07-A8, 2009, Wayne, Pa.) with cation-adjusted Mueller-Hinton broth (Trek Diagnostics Systems, U.K.). Compounds were dissolved using DMSO. Serial two-fold dilutions of each compound were made using DMSO as the diluent. Trays were inoculated with 5 x 10^4 CFU of each strain in a volume of 100 µL (final concentration of DMSO was 3.2 % (v/v)), and incubated at 35° C for 16-20 hours. Growth of the bacterium was quantitated by measuring the absorbance at 620 nm.

**Assay of cell culture cytotoxicity**

HepG2 cells were suspended in Dulbecco-modified Eagle’s medium containing 10% fetal bovine serum, and then seeded in 96-well tissue culture plates at either 5 000, 10 000 or 20 000 cells per well. After 24 hours, cells were treated with varying concentrations of compound, such that the DMSO concentration was consistent at 4% (v/v) in all wells. After treatment for 24 or 48 hours, WST-1 cell proliferation reagent (Roche) was added to each well and incubated for 0.5 hours at 37° C. The WST-1 assay quantitatively monitors the metabolic activity of cells by measuring the hydrolysis of the WST-1 reagent, the products of which are detectable at absorbance 450 nm.
**Synthetic chemistry methods**

All reagents were from standard commercial sources and of reagent grade or as specified. Solvents were from standard commercial sources. Reactions were monitored by ascending TLC using precoated plates (silica gel 60 F254, 250 µm, Merck, Darmstadt, Germany), spots were visualised under ultraviolet light at 254 nm and with either sulphuric acid-vanillin spray, potassium permanganate dip or Hanessian’s stain. Flash chromatography was performed with silica gel (40-63 µm 60 Å, Davisil, Grace, Germany). Melting points were recorded uncorrected on a Reichert Thermovar Kofler microscope. 1H and 13C NMR spectra were recorded on a Varian Gemini (200 MHz) Varian Gemini 2000 (300 MHz) or a Varian Inova 600 MHz. Chemical shifts are given in ppm (δ) relative to the residue signals, which in the case of DMSO-d6 were 2.50 ppm for 1H and 39.55 ppm for 13C and CDCl3 were 7.26 ppm for 1H and 77.23 ppm for 13C. High-resolution mass spectra (HRMS) were recorded on a Thermo Fisher Scientific LTQ orbitrap FT MS equipment (Δ < 2 ppm) at Adelaide Proteomics, University of Adelaide and Brucker micrO TOF-Q at The Australia Wine Research Institute. Purity for assayed compounds was determined by 1H NMR (>95%). Compound 2 (25), 3 (31) and 4 (32) were prepared according to literature procedures.

**RESULTS**

**Molecular basis for inhibitor binding**

To assist with inhibitor design, the structure of SaBPL in complex with inhibitor biotinol-5’-AMP 2 was determined by X–ray crystallography to 2.5 Å resolution. This revealed that the enzyme contains three domains (Fig. 2A). A helix-turn-helix motif at the N-terminus facilitates DNA binding activity. The central and C-terminal domains contain amino acids required for ligand binding and catalysis and adopt SH2 and SH3-like folds, respectively. The overall structures are consistent with the class II enzyme from *E. coli* (33) (RMSD of Ca 1.89 Å over 249 amino acids), and with the catalytic region of class I enzymes from *Pyrococcus hirokoshii* (RMSD 1.89 Å), *Aquifex aeolicus* (RMSD 1.8 Å) and *Mycobacterium tuberculosis* (RMSD 1.89 Å) (11,34-36). The high-degree of structural conservation observed in all available BPL structures highlights the challenge in designing selective inhibitors.

The Ca backbone could be traced from residues Ser 2 to Phe 323 indicating all amino acids were observed in the X–ray diffraction data, with the exception of Met 1. Noteworthy were residues Thr 117 – Lys 131 and Phe 220 – Ala 228 (Figs. 2A and B). In other BPLs these features, known as the biotin-binding loop (BBL) and ATP binding loop (ABL), respectively, are not visible in the unliganded form of the enzyme but are observed when ligand is bound. The disordered to ordered transition that accompanies ligand binding has been previously reported (33-36). In the complex of SaBPL with 2, the BBL folds against the central β-sheet in the central domain to cover the active site and maintain the reaction intermediate in situ (Figs. 2A and B). The side chain of Trp 127 in the BBL becomes buried and forms a barrier over which the biotin and adenosine halves of 2 must bend (Fig. S1), thereby forming distinct biotin and ATP binding pockets. The ribose moiety in 2 assists the inhibitor to bridge the two binding pockets, and forms a hydrogen bond through its 2’ hydroxyl group with the side chain of Arg 227 (Fig. S1). As for other BPLs, the phosphodiester in 2 permits the inhibitor to adopt the same V-shaped geometry observed for 1 binding. The complex with 2 is further stabilized through hydrogen bonding interactions between the phosphate in the linker with residues in the BBL, namely Arg 122 and Arg 125 (Fig. S1). The ATP pocket is dominated by the side chain of Trp 127, which is required for a π – π stacking interaction with adenine. The side chains of hydrophobic residues Phe 220, Ile 224 and Ala 228 in the ABL present at the same plane as the purine ring providing a hydrophobic surface for binding. Hydrogen bonding with the side chain of Arg 227 (Fig. S1). The ATP pocket is dominated by the side chain of Trp 127, which is required for a π – π stacking interaction with adenine. The side chains of hydrophobic residues Phe 220, Ile 224 and Ala 228 in the ABL present at the same plane as the purine ring providing a hydrophobic surface for binding. Hydrogen bonding with the side chain of Arg 227 (Fig. S1). The ATP pocket is dominated by the side chain of Trp 127, which is required for a π – π stacking interaction with adenine. The side chains of hydrophobic residues Phe 220, Ile 224 and Ala 228 in the ABL present at the same plane as the purine ring providing a hydrophobic surface for binding. Hydrogen bonding with the side chain of Arg 227 (Fig. S1).
show the biotin pocket to be relatively small and hydrophobic as required to accommodate the ureido and thiophane rings of biotin. This is consistent with reported literature that showed chemical modifications to these heterocycles produced biotin analogues that were unable to be used as substrates by the BPLs from a wide range of species (38). Hence, targeting the biotin site alone is an unattractive approach for inhibitor design. We propose that targeting the ATP binding pocket would provide significant opportunity for introducing selectivity. A comparison of our SaBPL crystal structure with others available in the PDB and also a model of the human BPL active site (7), revealed the amino acid residues in the ATP binding sites to be far more divergent. For example, five of the residues that define the nucleotide-binding pocket for SaBPL are not conserved with the sequence of the human BPL. These residues are located immediately around the adenine-binding site (Gln 125, Phe 220 and Arg 227) and in the BBL (His 126 and Ser 128). The position of these residues is shown in Fig. 2C (yellow) relative to the adenosine portion of 2 bound in the SaBPL structure.

**Biotin binding induces the nucleotide-binding pocket**

An understanding of the ligand-binding mechanism provided important information for inhibitor design. Biophysical analysis of the Class II BPL from *E. coli* has shown that the enzyme possesses an ordered binding mechanism during catalysis with biotin binding first, triggering the disorder to order transition that forms the nucleotide-binding pocket (33,39). This ordered binding mechanism was confirmed for SaBPL using surface plasmon resonance (SPR). The addition of MgATP to SaBPL that was covalently attached to the matrix did not result in binding (Figs 3A & B). In contrast, biotin binds in a concentration dependent manner with fast association and dissociation kinetics (Fig. 3A). This is consistent with the immobilized enzyme retaining biotinyl-5'-AMP synthetase activity, and the formation of a stable holoenzyme complex. Addition of protein substrate in the running buffer induced a decrease in response of the surface plasmon, as would be expected when SaBPL discharges the reaction intermediate 1 during protein biotinylation (data not shown). This finding agrees with studies on *E. coli* BPL that demonstrated the holo-enzyme complex is stable with a half-life of 30 minutes (40,41). This result has a significant impact on inhibitor design. To target the ATP pocket, a successful inhibitor must induce the conformational changes required to form this pocket. We thus chose to incorporate a biotinyl moiety into our inhibitor design. In support of this, SPR analysis showed that MgATP could bind to SaBPL but only following the formation of an enzyme complex with biotin analogue 3 (Figs. 3B and C). It is important that an inhibitor competes with biotin binding because once the ligand occupies the enzyme, the saturating concentration of ATP in the bacterial cell (~3 mM, vs $K_M$ of SaBPL for ATP 180 µM, Table S2) will drive the formation of intermediate 1 and subsequent biotinylation of the protein substrates.

**Triazole linker as a phosphate isostere**

Currently, there are no reports of selective BPL inhibitors in the literature. Biotinol-5'-AMP (2) (25) and the sulfamate analogue 5'-Amino-5'-N-(biotinyl)sulfamoyl-5'-deoxyadenosine (11) are close mimics of the reaction intermediate (1) that contain non-hydrolysable linkers. We demonstrated that 2 inhibited SaBPL with a $K_i = 0.03 \pm 0.01$ µM in *in vitro* BPL enzyme assays. However, it was also a potent inhibitor of the human BPL with a $K_i = 0.21 \pm 0.03$ µM. Alternative bioisosteres were considered as a means to link biotin and adenine components identified in our inhibitor design to access selective inhibitors. The 1,2,3-triazole motif is a versatile heterocycle with a number of desirable attributes that made it a good candidate. It is stable to acid/base hydrolysis, reductive and oxidative conditions, rendering it resistant to metabolic degradation. A 1,2,3-triazole ring has three potential hydrogen bond acceptor sites (nitrogens), a polarized proton and can also participate in $\pi - \pi$ interactions. In addition, a 1,2,3-triazole is readily synthesized by an azide alkyne Huisgen cycloaddition reaction under chemically benign conditions (42,43). As a result,
the 1,2,3-triazole has found some applicability as bioisosteric analogues for phosphomonoesters (44), pyrophosphate (45), phosphodiester linkers (46) and phosphoanhydrides (47).

Our first example of this new class of BPL inhibitors was prepared by Huisgen cycloaddition of biotin acetylene (3) with adenosine azide (4) in the presence of copper nanopowder, followed by removal of the isopropylidene diol protecting group gave the biotin 1,4-disubstituted triazole-adenosine (5), as shown in Scheme 1. The related triazoles (6, 8, and 9, see Fig. 1) were similarly prepared by reaction of the appropriate azide and acetylene as described in the Supplementary information. In vitro enzyme inhibition studies demonstrated that the triazole (5) was a competitive inhibitor of SaBPL with a $K_i$ of 1.17 ± 0.3 µM. The ribose 2',3'-d'iol was deemed not important for activity as the isopropylidene protected analogue of 5 (see 7, Fig. 1) was as equally potent as the unprotected analogue ($K_i$ 1.83 ± 0.33 µM, $P = 0.2$, 5 vs 7). Significantly, it was also observed that shortening or lengthening the valeric acid chain on the biotinyl moiety of 7 by one carbon (see 6 and 8 respectively) abolished inhibitory activity. Similarly, the 1,5-disubstituted triazole-adenosine isomer of 7 (see 9, Fig. 1) was also inactive against SaBPL, presumably since this moiety does not provide the appropriate V-shaped geometry required for active site binding as discussed earlier. This was supported by a crystal structure of 7 bound to SaBPL that revealed the expected mode of binding (discussed later). These data highlight the need for the precise positioning of the isostere in the inhibitor.

The biotin triazoles 5 and 7 were next tested for inhibitory activity against recombinant E. coli and human BPLs, with all being inactive at the highest concentration achievable without precipitation in the assay medium (typically 200 µM). These are the first compounds that show significant selectivity for SaBPL. The biotin-triazole pharmacophore thus provides a scaffold for further development of selective inhibitors of SaBPL.

Improving inhibitor potency and selectivity

In addition to providing a phosphodiester bioisostere, the triazole linkage provides an ideal opportunity to rationally advance the inhibitor design. A selection of readily available azides, the side chains of which might occupy the ATP pocket, were linked to the acetylene 3 under standard Huisgen cycloaddition conditions to give a second series of triazole-based inhibitors (see Supplementary Experimental Procedures). Key features of the earlier inhibitors were considered in this stage of the design: (i) the 1,4-disubstituted triazole was retained to allow the inhibitor to adopt the desired V-shape for binding with BPL, (ii) the ribose sugar of 1 was removed as the kinetic data with inhibitors 5 (with unprotected diol) and 7 (protected diol) demonstrated it was not essential for binding and (iii) the optimum 5-carbon linker length between the triazole and biotin groups of 5 and 7 was retained. Two linker lengths between the triazole and potential adenine replacements were investigated, with the choice of analogue guided by the hydrobobicity of the ATP binding pocket, defined by the side chains of Phe 220, Ile 224 and Ala 228, and the potential for π interactions with Trp 127. Thus we chose to target triazoles containing aliphatic and aromatic groups (see R group in Table 1) that might be predicted to substitute for adenine and bind in the hydrophobic ATP pocket. A privileged 2-benzoxazolone scaffold (48,49) was also included in this series (see structures 14 and 16 in Table 1).

The triazole with the appended aliphatic tertiary butyl ester (10) was inactive against SaBPL, as were the phenyl and 1-naphthyl analogues 11 and 12, respectively. Interestingly, the analogous 2-naphthyl derivative 13 showed encouraging activity with a $K_i$ of 1.17 ± 0.17 µM. The triazole with the appended 2-benzoxazolone (14) proved to be particularly potent with a $K_i$ of 0.09 ± 0.02 µM (Fig. 4A). Of particular significance was the observation that both compounds 15 and 16 were inactive, despite containing the favored aryl groups but a linker reduced by one carbon. This suggests that the correct nature and positioning of the aryl group in the ATP pocket is critical for optimal π–π stacking and hydrophobic-hydrophobic interactions (see discussion below on the X-ray crystal structures of complexes with 7 and 14).

Biotin triazole 14 was confirmed as a competitive inhibitor versus biotin using Lineweaver-Burke analysis. Critically for this study, the biotin triazoles 13 and 14 were inactive
against both human and E. coli BPLs in vitro at concentrations limited by solubility. For biotin triazoles 14 the selectivity for SaBPL was >1100-fold over the human isozyme (Fig. 4A), making this compound by far the most significant example of a selective BPL inhibitor reported to date.

**Anti-microbial activity of biotin-triazoles**

The anti-microbial activity of selected SaBPL inhibitors was measured against S. aureus ATCC strain 49775 using a microbroth dilution assay. Bacteriostatic activity was observed with the pan inhibitor 2, demonstrating for the first time that the BPL target is indeed drug-gable in S. aureus in an in vitro setting (MIC 8 – 32 µg/ml). The most potent of the triazole inhibitors (14) also reduced cell growth by 80% when 8 µg/ml was included in the growth medium (Fig. 4B). Interestingly triazole 13 did not show anti-microbial activity even though it is only 13-fold less potent than 14 in an in vitro enzyme inhibition assay. This suggests that additional factors, such as uptake across the bacterial membrane, impact on the utility of this class of compounds. Biotin triazoles 13 and 14 were both inactive against E. coli ATCC strain 25922 in the micro-broth dilution assay. This is consistent with the fact that both were inactive against E. coli BPL. Importantly, 5, 7, 13 and 14 did not show any toxicity in a cell culture model using HepG2 cells (Fig. 4C). Cells seeded at three different densities were treated with 64 µg/ml in the growth media for 48 hours with no inhibition of cell growth, consistent with the lack of inhibitory activity against human BPL in vitro.

**X-ray structures of biotin-triazoles bound to SaBPL**

The X-ray crystal structures of SaBPL in complex with 7 reveals the adenine ring adopts an analogous binding mechanism to that observed with the holo enzyme complex with inhibitor 2 (discussed earlier). Hence, the requisite properties necessary for binding in the ATP pocket are maintained, namely π – π stacking interaction with Trp 127, hydrophobic interactions with the ABL and hydrogen-bonding interactions with Asn 212 and Ser 128 located at the bottom of the ATP pocket (Fig. 5B). Crystal structures of 7 and 14 in a co-complex with SaBPL all revealed that the inhibitors occupied both biotin and ATP pockets as per our inhibitor design and again with the expected V-shaped geometry essential for binding (Fig. 5C).

Additionally, many of the hydrogen-bonding interactions were retained in all structures. The isopropylidene protecting group on inhibitor 7 caused the displacement of the Arg 227 side chain away from its position noted in our other X-ray structures, thereby disrupting a potential hydrogen bond (Fig. S3). However, the fact that the protecting group showed no effect on inhibitor potency (see 5 vs 7 above) suggests that the ribose ring in 1 is dispensable, consistent with inhibitor design principles described previously. Importantly, the triazole group assists to stabilize the enzyme : inhibitor complex through hydrogen bonding interactions with residues in the BBL, namely N2 with the guanidinium side chain of Arg 125, and N3 and the backbone amide at Arg 227 (Fig. 5B). The proton at C5 of the triazole ring also formed a hydrogen bond with the carboxylate side chain of Asp 180. In addition, the triazole ring participates in an edge-tilted-T shape interaction with Trp 127 (Fig. 5C). Crystal structures of 7 and 14 in a co-complex with SaBPL all revealed that the inhibitors occupied both biotin and ATP pockets as per our inhibitor design and again with the expected V-shaped geometry essential for binding (Fig. 5C).
the first evidence of a so-called privileged 2-benzoxazolone scaffold binding in an ATP pocket.

Arg 125 contributes to selectivity of biotin-triazoles

To further probe the mechanism of selectivity, we addressed the role of Arg 125 in the binding mechanism by mutagenesis. As the equivalent amino acid residue in human BPL is a non-conservative asparagine, we proposed that Arg 125 in SaBPL was likely to be a key determinant in the selective binding mechanism employed by the biotin triazoles. To test this hypothesis, we generated a mutant SaBPL with an arginine to asparagine substitution (SaBPL Arg 125→Asn), as well as an alanine substitution (SaBPL Arg 125→Ala). CD analysis demonstrated the amino acid substitutions did not destabilise the SaBPL secondary structure (Fig. S5). Similarly, kinetic analysis demonstrated that the affinity for biotin was unaltered by the mutation and the $K_i$ for MgATP had a modest increase of only two-fold (Table S2). Significantly, the largest difference observed between the enzymes was the turnover rate with the $k_{cat}$ reduced by >500-fold by both amino acid substitutions. This implied a key role for this residue, and the BBL, in catalysis. As expected, the pan inhibitor 2 also functioned as an inhibitor against Arg 125→Asn ($K_i = 0.199 \pm 0.028 \mu M$, $P < 0.05$ vs Wt SaBPL, $P < 0.001$ vs human BPL). In contrast the inhibitory activity of biotin triazole 5 was abolished. Whilst 14 retained inhibitory activity against both muteins (Arg 125→Ala $K_i = 4.38 \pm 0.39 \mu M$, Arg 125→Asn $K_i = 4.47 \pm 0.47 \mu M$), it remained significantly more potent against wildtype SaBPL (Wt $K_i = 0.09 \pm 0.01 \mu M$, $P < 0.0001$ Wt vs mutein) (Fig. 4A). Together these data support the hypothesis that Arg 125 plays a key role in the selective inhibition by the biotin triazoles.

DISCUSSION

In this study we present the first selective inhibitors of *S. aureus* biotin protein ligase using an analogue of the reaction intermediate, biotinol-5’AMP (2), as the starting point for inhibitor design. This new class of inhibitor contains a 1,2,3-triazole-based bioisostere of the chemically unstable phosphate-based linkages found in the native reaction intermediate and inhibitor 2. We also considered the hypothesis that a close mimic of the reaction intermediate 1 that targets the BPL active site would have a high barrier for developing drug resistance due to spontaneous mutation (6). However, the conserved reaction mechanism employed by all BPLs that utilises adenylated biotin, coupled with the high degree of homology in the primary and tertiary structures suggested that this would be challenging (50). Here we utilized structural biology to assist in the rational design of inhibitors that overcome this problem. An important consideration was the disordered-to-ordered transition that accompanies catalysis. The BBL that is observed in the crystal structures of holo BPLs, but not apo structures, plays a key role to stabilize the BPL:ligand complex. The reaction intermediate is maintained *in situ* in the active site through interactions with amino acids in this loop, especially with the phosphate containing linkers in 1 and 2. We argued that an effective mimic of 1 should also interact with amino acids in the BBL. Indeed, replacement of the phosphate linker with a 1,2,3-triazole bioisostere yielded a series of potent and selective inhibitors of SaBPL. The triazole linker provided selective binding via a key amino acid (Arg 125) present in the BBL of SaBPL. This amino acid is not conserved between BPLs, thereby contributing to the selective inhibition. Mutation studies targeting Arg 125 confirmed the mode of binding and demonstrated that this amino acid is important for BPL activity and selectivity. The mutein displayed a reduced enzyme catalytic turnover rate of >500-fold. This reduction of specific enzyme activity should be a significant barrier for the development of drug resistance to the biotin-triazoles by spontaneous mutation, as we initially proposed.

The triazole linkage provides an opportunity to develop BPL inhibitors using well-documented reaction conditions that give rise to both the 1,4- and 1,5-disubstituted triazole isomers. BPL provides an attractive template for a fragment-based approach with the biotin and ATP pockets juxtaposed in the crystal structures. Compounds that reside in the two pockets can be joined via a 1,2,3-triazole that constrains the partners in the same V-shaped geometry naturally observed for enzyme bound 1. The biotin-dependent ATP binding mechanism employed by
BPLs prevents targeting the ATP site alone. However, by incorporating a biotin moiety into the inhibitor, the ATP pocket can be formed and explored as an avenue towards an optimized inhibitor. There have been reports of large pharma repositioning their nucleotide-analogue libraries, assembled for screening eukaryotic protein kinase targets, for antibiotic discovery. Successful examples in the literature include acetyl CoA carboxylase (51), histidine kinase (52) and D-alanine-D-alanine ligase (53). The availability of high-resolution crystal structures of SaBPL is necessary to direct future chemical optimisation. The mechanism of selective inhibition reported in this study provides the first approach to antibiotics based on the selective inhibition of BPL. To date, target-based antibiotic discovery has focused upon metabolic enzymes and pathways that are found exclusively in bacterial pathogens (54,55). As a result, targets with close equivalents in mammalian hosts have typically been excluded from consideration. We demonstrate an important new approach to antibiotic discovery based on the selective inhibition of a bacterial target (BPL) that has a mammalian homologue. Examples of antibiotics in clinical use that selectively target a bacterial protein include ribosome inhibitors, such as macrolides, aminoglycosides, tetracyclines and linezolid (56). In this example however, the differences between the bacterial and eukaryotic ribosomal proteins are large and provide adequate opportunities for selective binding of drugs. More challenging for antibiotic drug discovery are the drug targets that more closely resemble their mammalian counterparts, as addressed in this paper. The implication from work presented in this study refocuses discussion on what constitutes a druggable target for antibiotic discovery. Given the clinical demand for new agents to combat drug resistance across the globe, these studies are timely.

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REFERENCES

**FOOTNOTES**
† = equal contributions to the work  
# = equal contributions as senior author

**Abbreviations**
BPL – biotin protein ligase
FIGURE AND SCHEME LEGENDS:

Figure 1. Chemical structures of key compounds in this study.

Figure 2. *S. aureus* BPL in complex with biotinol-5’-AMP.
A) *Sa*BPL consists of three structured domains, an N-terminal DNA binding domain (red), a central domain (cyan) and C-terminal domain (dark blue). B) Close up of the inhibitor-binding site shows the relative positions of the ATP-binding loop (ABL) and biotin-binding loop (BBL). The final 2F_o − 2F_c map is contoured at the 1σ level is shown on inhibitor 2 in ball and stick representation. C) The ATP pocket of *Sa*BPL in complex with 2 is shown in space filled mode with the adenine portion shown in ball and stick representation. Amino acid residues that line the pocket and that are not conserved between *Sa*BPL and human BPL are highlighted in yellow.

Figure 3. Binding at the ATP-site is biotin dependent
The mechanism of ligand binding was monitored using surface plasmon resonance with immobilized *Sa*BPL. A) Overlaid are the SPR traces that resulted from the inclusion of either biotin (pink) or MgATP (dark blue) in the running buffer, or both ligands co-injected together (cyan). Arrows indicate the start and end of the ligand injection phase. B) and C) ATP binding is dependent upon the formation of a biotin-enzyme complex. The SPR traces are shown when increasing concentrations of MgATP were applied to B) apo *Sa*BPL or C) *Sa*BPL in a pre-formed complex with biotin analogue 3.

Figure 4. Biological assays
A) Differential inhibition. BPL activity was measured *in vitro* with varying concentrations of 14. The assays were performed using recombinant BPL from *S. aureus* (●), *E. coli* ( ), and *H. sapiens* (X). The mutant Arg 125→Asn was also included (○). B) Anti-staphylococcus activity. Inhibition of the growth of *S. aureus* ATCC 49775 was measured using a microbroth dilution assay with varying concentrations of 2 (○), 7 (△) and 14 (●). No inhibitor (n) and erythromycin (X) served as negative and positive controls respectively. C) The cytotoxicity of the biotin triazole series was assessed on HepG2 cells using an assay for metabolic activity. Cells were seeded at either 20 000, 10 000 or 5 000 cells per well and treated for 48 hours with media containing 64 µg/ml of compound and 4% (v/v) DMSO. The treatments in this series were the DMSO vehicle control (white bar), 5 (grey bar), 7 (horizontal stripe), 13 (diagonal stripe), and 14 (black bar).

Figure 5. Mode of inhibitor binding
A) The backbone atoms of *Sa*BPL in complex with inhibitor 2 (dark blue) and inhibitor 7 (cyan) were superimposed to reveal the remarkable overlap in the conformations imparted by the triazole bioisostere. (B) Hydrogen bonding interactions between *Sa*BPL and the trizole ring are shown. (C). The crystal structure of biotin triazole 14 (dark blue) bound in the active site of *Sa*BPL. Hydrogen bonding contacts with the amino acids of *Sa*BPL are shown. The BBL is highlighted in yellow, and the ABL in orange.

**Scheme 1:** Conditions and reagents: a) i) Cu nano-powder, 2:1 MeCN/H_2O, 35 °C; ii) 90% TFA_{aq}, DCM.
### Table 1: SAR series of biotin triazoles

*Selectivity is calculated by $K_i$ human BPL / $K_i$ SaBPL, where the $K_i$ for human BPL is the maximum concentration of compound possible in assay medium due to solubility restraints (90 µM).

<table>
<thead>
<tr>
<th>ID</th>
<th>R</th>
<th>$K_i$ SaBPL (µM)</th>
<th>Selectivity*</th>
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<tbody>
<tr>
<td>10</td>
<td></td>
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<td>N/D</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>&gt; 10</td>
<td>N/D</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>&gt; 10</td>
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<tr>
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</table>
Figure 2
Figure 3
Figure 4

A  

B  

C  

% BHR Activity

% of Control

Absorbance 

Concentration (cells/well)
Scheme 1
Selective inhibition of Biotin Protein Ligase from Staphylococcus aureus

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