New Series of BPL Inhibitors To Probe the Ribose-Binding Pocket of *Staphylococcus aureus* Biotin Protein LigaseJiage Feng,^{†,‡} Ashleigh S. Paparella,[§] William Tieu,^{†,||} David Heim,[§] Sarah Clark,[†] Andrew Hayes,[§] Grant W. Booker,[§] Steven W. Polyak,[§] and Andrew D. Abell^{*,†,‡}[†]Department of Chemistry, [§]Department of Molecular and Cellular Biology, and [‡]Centre for Nanoscale BioPhotonics (CNBP), University of Adelaide, Adelaide, South Australia 5005, Australia

Supporting Information

ABSTRACT: Replacing the labile adenosyl-substituted phosphoanhydride of biotinyl-5'-AMP with a N1-benzyl substituted 1,2,3-triazole gave a new truncated series of inhibitors of *Staphylococcus aureus* biotin protein ligase (SaBPL). The benzyl group presents to the ribose-binding pocket of SaBPL based on *in silico* docking. Halogenated benzyl derivatives (**12t**, **12u**, **12w**, and **12x**) proved to be the most potent inhibitors of SaBPL. These derivatives inhibited the growth of *S. aureus* ATCC49775 and displayed low cytotoxicity against HepG2 cells.

	R	K_i SaBPL (μ M)	K_i HsBPL (μ M)
12t	3F	0.28 ± 0.02	>10
12u	4F	0.60 ± 0.10	>10
12w	3Cl	0.39 ± 0.04	>10
12x	4Cl	1.10 ± 0.07	>10

KEYWORDS: Enzyme inhibitors, antibiotics, biotin protein ligase, *Staphylococcus aureus*

Biotin protein ligase (BPL) catalyzes the reaction of biotin **1** and ATP **2** to give biotinyl-5'-AMP **3**, which then biotinylates and activates essential metabolic enzymes required for fatty acid biosynthesis and gluconeogenesis, specifically acetyl CoA carboxylase and pyruvate carboxylase (Figure 1).^{1–5}

Figure 1. General mechanism of BPL catalyzed biotinylation.

A number of analogues of biotinyl-5'-AMP have recently been reported as inhibitors of BPL as shown in Figure 2. Some of these compounds have potential as antibacterial agents by inhibiting BPL from clinically important pathogens such as *Staphylococcus aureus*,⁶ *Escherichia coli*,^{7,8} and *Mycobacterium tuberculosis*.^{9,10} A range of bioisosteres have been investigated as replacements for the labile phosphoanhydride of biotinyl-5'-AMP **3**, including phosphodiester **4**,^{11,12} hydroxyphosphonate **5**,¹³ ketophosphonate **6**,¹³ acylsulfamate **7**,¹¹ and sulphonmyl amide **8**¹⁰ (Figure 2). We have also reported biotin triazoles (e.g., **9–11**) as a novel class of BPL inhibitor that selectively targets BPL from the clinically important bacterial pathogen *Staphylococcus aureus* over the human homologue.^{3,14,15}

Figure 2. Reported BPL inhibitors, with isosteric replacements for the phosphoanhydride of biotinyl-5'-AMP **3** shown in the box.

Without exception, all isostere-based BPL inhibitors reported to date contain a biotin and an adenine group, or analogue thereof, as discussed above and as shown in Figure 2. These two groups occupy well-defined binding pockets in the enzyme as per biotinyl-5'-AMP **3**, as supported by X-ray crystallographic and mutagenesis studies.^{3,16} The ribose group of the triazole series can be removed as in **10**, and the adenine can be modified as in **11**, which has improved stability and >1000-fold specificity for the BPL from *S. aureus* over the human homologue.³ We now report the first examples of truncated 1,2,3-triazole-based BPL inhibitors with a 1-benzyl substituent

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designed to interact with the ribose binding pocket of *S. aureus* BPL (SaBPL), see **12a–y**, Figure 3. These derivatives are the

Figure 3. Benzyl-substituted 1,2,3-triazole analogues.

first examples of isostere-based BPL inhibitors lacking an appended adenine or analogue thereof and the associated tether as discussed above. The ribose-binding pocket is composed of amino acids that provide potential hydrogen bonding sites, specifically through the side chains of K187, R122, R125, and R227 as well as the backbone peptide atoms from H126 and S128 (Figure 4a). This series of inhibitors provides an important starting point for further optimization and antibiotic development.

Figure 4. (a) X-ray structure of the SaBPL active site with biotinyl-5'-AMP 3 (yellow) bound PDB ID 3RIR.¹⁰ Amino acids that encompass the ribose-binding pocket are shown. Dashed lines represent hydrogen bonds. (b) *In silico* docking poses for biotin triazole analogues **12a**, **12b**, and **12c** containing a hydroxyl group at C2- (pink), C3- (blue), and C4- (green), respectively.

In silico docking experiments were carried out in order to explore possible binding modes by which this series of benzyl analogues might occupy the active site of SaBPL. Flexible ligand docking was carried out using AutoDockTools (version 1.5.6). The docking protocol was first validated by removing compound **11** from its cocrystal structure with *S. aureus* BPL (PDB 3V7S) and then redocking. This occurred with a high degree of commonality as revealed by superimposition of the docked and crystallized ligands in the active site. We next docked benzylated triazoles **12a–c** (Figure 3) into SaBPL. Each of these structures contains a single hydroxyl substituent on the benzyl ring capable of forming a hydrogen bond as per the diol in the ribose of biotinyl-5'-AMP 3. Gratifyingly, the top ranking poses of all three analogues placed the hydroxyl group in the site occupied by the ribose diol of **3** (Figure 4a). Rotation around the alkyl linker connecting the triazole and benzyl moieties produced subtly different poses with regards to the ribose-binding site (Figure 4b). These binding modes minimized steric clashes with the protein and facilitated hydrogen bonding between the alcohol group and R122 for **12a** and R227 for **12b** and **12c**. This data reflects an apparent openness of the solvent exposed pocket and suggests that this

site can accommodate a variety of functional groups as exemplified in the extended series depicted in Figure 3. Synthesis and biological testing of this series provides an opportunity to probe potential interaction with the ribose pocket.

The synthesis of the 1,2,3-triazoles **12a–y** was carried out as summarized in Scheme 1. The key benzyl azides **14a–y** were

Scheme 1^a

^aConditions and reagents: (a) (i) PPh₃, CCl₄, DMF; (ii) NaN₃, DMF, rt; (b) NaN₃, DMF, rt; (c) Cu₂SO₄ ascorbate, DMSO/H₂O, rt, 12 h (give **12a–y** (18%–55%)).

prepared from commercially available benzyl alcohols (**13a–i** and **13n**) and bromides (**15j–m** and **15o–y**). Specifically, commercially available benzyl alcohols **13a–i** and **13n** were converted directly¹⁷ into the corresponding azides **14a–i** and **14n** on reaction with triphenylphosphine, in the presence of carbon tetrachloride and sodium azide at ambient temperature. The second series of benzyl azides (**14j–m** and **14o–y**) was prepared from commercially available benzyl bromides **15j–m** and **15o–y** on reaction with sodium azide in DMF as shown. Huisgen cycloaddition of biotin alkyne **16**¹⁸ with each of the benzyl azides **14a–y**, in the presence of copper sulfate and sodium ascorbate,³ then gave the desired 1,2,3-triazole **12a–y** as shown.

The activity profiles of 1,2,3-triazoles **12a–y** were determined using established biochemical and microbiological assay protocols.^{3,19} Compounds displaying inhibitory activity against SaBPL and cytotoxic activity against bacteria, but not mammalian cells, are considered important candidates for further antibiotic development. The *in vitro* potency and selectivity profiles of the 1,2,3-triazoles **12a–y** were measured using recombinant BPLs from *S. aureus* and *Homo sapiens*. Here the enzymatic incorporation of radiolabeled biotin onto an acceptor protein was measured in the presence of varying concentrations of each compound with the results shown in Tables 1 and 2. Previous enzymology and X-ray crystallography studies have demonstrated that the biotin triazoles are competitive inhibitors against biotin,^{3,15,16} and as such, inhibitory constants (*K_i*) were calculated from IC₅₀ values using the known *K_M* for biotin as previously described.²⁰ The antibacterial activity of the compounds was also determined using *S. aureus* strain ATCC 49775.¹⁸ Growth of the bacteria 20 h post-treatment was measured spectrophotometrically at 600

Table 1. *In Vitro* Biotinylation and Antibacterial Assay Results for Benzyl Triazole Series 1

ID	R	K_i SaBPL (μ M)	K_i human BPL (μ M)	anti- <i>S. aureus</i> activity ^a
12a	2-OH	>10	>16	–
12b	3-OH	>10	>16	–
12c	4-OH	1.59 $\pm$ 0.08	>16	–
12d	2-OMe	0.53 $\pm$ 0.05	>16	–
12e	3-OMe	1.17 $\pm$ 0.1	>16	–
12f	4-OMe	>10	>16	–
12g	2-NH ₂	1.48 $\pm$ 0.14	>16	+
12h	3-NH ₂	>10	>16	–
12i	4-NH ₂	>10	>16	–
12j	3-Me	0.71 $\pm$ 0.04	>16	+
12k	4-Me	>10	>16	–
12l	3-CF ₃	>10	>16	–
12m	4-CF ₃	>10	>16	–
12n	4- <i>t</i> Bu	1.22 $\pm$ 0.07	>16	+
12o	4-COOH	0.67 $\pm$ 0.06	>16	–
12p	2-Br	0.96 $\pm$ 0.13	>16	+
12q	3-I	>10	>16	–
12r	4-I	0.56 $\pm$ 0.06	>16	+

^a+, Optical density of the culture reduced by >40% of nontreated controls. –, compound did not inhibit bacterial growth.

Table 2. *In Vitro* Biotinylation and Antibacterial Assay Results for Benzyl Triazole Series 2

ID	R	K_i SaBPL (μ M)	K_i Human BPL (μ M)	anti- <i>S. aureus</i> activity ^a	cytotox HepG2 ^b
12s	2-F	>10	>16	–	N/D
12t	3-F	0.28 $\pm$ 0.02	>16	+	>40
12u	4-F	0.6 $\pm$ 0.1	>16	+	>40
12v	2-Cl	>10	>16	–	N/D
12w	3-Cl	0.39 $\pm$ 0.04	>16	+	>40
12x	4-Cl	1.1 $\pm$ 0.07	>16	+	>40
12y	3,4-diF	>10	>16	–	N/D

^a+, optical density of the culture reduced by >40% of nontreated controls. –, compound did not inhibit bacterial growth. ^bCompounds were assayed at 40 μ g/mL.

nm. Finally, selected compounds were assessed for potential toxicity using a cytotoxicity assay with cultured mammalian HepG2 cells (ATCC HB-8065).³

The initial series of alcohol analogues **12a–c** docked against SaBPL were first assayed against the enzyme (Table 1) with the compound containing a C4 hydroxyl group (**12c**) showing modest activity (K_i = 1.59 μ M). Interestingly, the C2 and C3 hydroxylated derivatives (**12a** and **12b**, respectively) were devoid of activity. It thus appears that the ribose pocket is sensitive to the position of the hydroxyl group and more so than predicted by the modeling. This observation is supported on analysis of the results for compounds containing other substituents, although there is little consistency regarding which position is most favored. Specifically, derivatives with a methoxyl group at C2 and C3 were both active (**12d**, K_i = 0.53 μ M; **12e**, K_i = 1.1 μ M), while the C4 analogue (**12f**) was inactive. For an amino substituent, C2 is active (**12g**, K_i = 1.49 μ M), while both C3 (**12h**) and C4 (**12i**) were inactive. Of the other derivatives initially tested, C3 methyl (**12j**, K_i = 0.71 μ M), C4 carboxyl and tertiary butyl (**12o**, K_i = 0.67 μ M; **12n**, K_i = 1.1 μ M) were active. The 2-bromo and 4-iodo derivatives **12p** and **12r** showed good activity against *S. aureus* BPL (K_i = 0.96

and 0.56 μ M, respectively), and these compounds provided impetus for the expanded series of halogenated compounds shown in Table 2 and as discussed in detail below. A strongly electron withdrawing trifluoromethyl group at C3 and C4, resulted in compounds (**12l** and **12m**) that were devoid of activity against SaBPL. All of the biotin triazoles tested were inactive against human BPL when 100 μ M of compound was included in the assay medium. This is an important finding as it demonstrates that benzyl truncated triazoles retain the selectivity profile (i.e., active against *S. aureus* but not human BPL) of the earlier and more complex triazoles.^{3,15}

The compounds shown in Table 1 were also assayed for antibacterial activity against *S. aureus* ATCC 49775. Compounds were designated as antibacterial if they reduced the optical density of the culture by >40% relative to the nontreated controls (Figure 5). Of the 18 compounds assessed,

Figure 5. Inhibition of *S. aureus* growth *in vitro*. (a) Compounds **12g** ($\square$), **12j** ($\blacklozenge$), **12p** ($\circ$), **12r** ($\triangle$), **12t** ($\blacksquare$), **12u** ($\bullet$), **12w** ($\blacktriangledown$), and **12x** ($\blacktriangle$) were tested against *S. aureus* strain ATCC 49775. (b,c) Mechanism of action studies for **12g** (b) and **12t** (c). Growth curves for *S. aureus* RN4220 harboring the plasmid pCNS1 (open boxes, negative control) or pCNS1-BPL (solid boxes, for recombinant BPL overexpression) are shown. Growth media contained 16 μ g/mL of compound, except, for no treatment controls (dashed line).

only five (those bearing amine, methyl, tertiary butyl, bromo, and iodo substituents, see **12g**, **12j**, **12n**, **12p**, and **12r**, respectively) were active in the whole cell assays. The fact that these compounds also inhibited SaBPL is consistent with a mechanism of antibacterial action being through the BPL target. It is possible that those BPL inhibitors devoid of whole cell activity, namely, **12c–e**, **12g**, **12j**, and **12p**, are unable to penetrate the bacterial membrane, a problem often encountered in antibacterial discovery.²¹

Of the extended halogenated series (**12s–x** in Table 2) four compounds displayed good activity against SaBPL, see **12t**, **12u**, **12w**, and **12x** that had K_i values of 0.28, 0.6, 0.39, and 1.1 μ M, respectively. A 3-halo substituent was most favored for

activity as in **12t** and **12w**. In these cases, the inhibition constants were approximately 2- and 3-fold lower for the 3- vs 4- halogenated analogues, cf. **12t/12u** and **12w/12x**. A 3-fluoro substituent (**12t**) provided the most potent compound in this series with a $K_i = 0.28 \mu\text{M}$. The incorporation of a halogen at C2 removed all activity (see **12s** and **12v**) as did the introduction of a second fluoro substituent as in **12y**. Again all active compounds in this series showed excellent selectivity for SaBPL over the human homologue. In addition, **12t**, **12u**, **12w**, and **12x** did not show cytotoxicity toward mammalian HepG2 cells at a single concentration of $40 \mu\text{g/mL}$.

Finally, to demonstrate that compounds inhibited protein biotinylation *in vivo*, antibacterial susceptibility assays were performed using a *S. aureus* strain engineered to overexpress the BPL target. Similar approaches to establish the mechanism of action have been employed on *M. tuberculosis*,¹⁰ but not previously for *S. aureus*. Bacteria were grown in media containing $16 \mu\text{g/mL}$ of either **12g** (series 1) or **12t** (series 2) for 16 h, with the optical density of the culture measured every 30 min. Overexpression of the BPL target abolished the antibacterial activity of both compounds, as *S. aureus* grew at the same rate as nontreated controls (Figure 5b,c). Bacteria harboring the parent cloning vector pCNS1²² that did not express additional BPL, remained highly sensitive to both inhibitors and failed to grow in their presence. Together these data show that the mechanism of action of **12g** (from series 1) and **12t** (series 2) is clearly via the inhibition of BPL.

The 1-benzyl substituted 1,2,3-triazoles reported here represent a new class of BPL inhibitors that lack the adenine group, or analogue thereof, found in all other isostere-based BPL inhibitors. These compounds have much reduced molecular weight and are relatively easy to prepare. Importantly, the biochemical and microbiological data provide a clear relationship between *in vitro* inhibition of BPL and anti-*S. aureus* activity, with our most potent enzyme inhibitors generally providing our most promising antibacterials (see **12j**, **12p**, **12r**, **12t**, **12u**, and **12w**). In antibiotic drug discovery, this is not always the case as a number of external factors contribute to bioactivity, such as cell permeability and susceptibility to efflux mechanisms and metabolic degradation.

The best lead compound in this new series (**12t**) with a 3-fluoro substituted benzyl group, has a K_i of 280 nM against SaBPL and demonstrated mechanism of antibacterial activity consistent with the inhibition of protein biotinylation. It is essentially nontoxic to mammalian HepG2 cells and is devoid of activity against human BPL. This compares to the extended 1,2,3-triazole **11** that has K_i of 90 nM against SaBPL. This compound provides clear interactions with both the biotin and adenine pockets of SaBPL and, like the new benzyl series, is essentially inactive against human BPL.³ Our initial SAR data on the new benzyl series provides confidence that further optimization of *in vitro* inhibition will lead to improved antibacterial activity. *In silico* docking supports a binding mechanism in which the benzyl group interacts with the ribose pocket of SaBPL. The compounds reported here provide important new scaffolds for further chemical modification and activity optimization, specifically to interact with the adjacent adenylation site in the enzyme. Such studies are currently underway, particularly the inclusion of extended substituents on the benzyl group and also substitution at C5 of the triazole.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.6b00248.

Biological assays, synthetic procedures, and data for selected compounds (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. Medicinal chemistry was performed by J.F., W.T., S.C., and A.D.A., biochemical assays were performed by A.S.P. and S.W.P., antibacterial susceptibility assays were performed by D.H. and A.H. under the guidance of S.W.P. and G.W.B., and cell culture assays were performed by A.H. and S.W.P.

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Notes

The authors declare no competing financial interest.

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