An azido-oxazolidinone antibiotic for live bacterial cell imaging and generation of antibiotic variants

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ABSTRACT

An azide-functionalised analogue of the oxazolidinone antibiotic linezolid was synthesised and shown to retain antimicrobial activity. Using facile ‘click’ chemistry, this versatile intermediate can be further functionalised to explore antimicrobial structure–activity relationships or conjugated to fluorophores to generate fluorescent probes. Such probes can report bacteria and their location in a sample in real time. Modelling of the structures bound to the cognate 50S ribosome target demonstrates binding to the same site as linezolid is possible. The fluorescent probes were successfully used to image Gram-positive bacteria using confocal microscopy.

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1. Introduction

Bacterial infection is a leading cause of death worldwide, and antimicrobial resistance has become one of the most serious public health concerns.1-2 It has proven very difficult to identify new antibiotics, especially those with activity against drug-resistant bacteria, with very few new classes identified in the past 40 years.3 The growing incidences of these drug-resistant ‘superbugs’ is fuelling a resurgence in microbiology research focused on the identification of new antibiotics, bacterial resistance mechanisms, new biological targets, and mode of action (MoA) studies.

Linezolid 1 (Zyvox®, Zyvoxid®, Zyvoxam®, Pfizer) was the first clinically used example of a novel class of chemically synthesized antimicrobial agents known as oxazolidinones (Fig. 1).4,5 Developed in the 1990s and FDA approved in 2000, linezolid 1 is administered orally or intravenously for the treatment of Gram-positive infections. It is effective against methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin resistant Enterococcus spp. (VRE). Resistance was noted within a year after its clinical introduction4,7 and is still a concern.8 Linezolid 1 acts by inhibiting bacterial protein synthesis by targeting the ribosome, one of the most common antibiotic targets.9 Bacterial ribosomes are known as 70S ribosomes (composed of 30S and 50S subunits), whereas mammalian ribosomes are 80S structures (composed of 40S and 60S subunits).10 Antibiotics bind to bacterial ribosomes selectively because they differ from mammalian ribosomes. Oxazolidinone antibiotics bind to the 50S subunit and block formation of the 70S initiation complex by preventing assembly of the N-formyl-methionyl-tRNA–ribosome–mRNA ternary complex.11,12 In addition, they interfere with translocation of peptidyl-tRNA from the A site to the P site.12 Resistance to linezolid 1 is associated with modifications of its binding site on the ribosome.14 Resistance to oxazolidinones also involves mutation of 23S rRNA, resulting in decreased binding affinity.15

A number of new oxazolidinone derivatives are in clinical development (Fig. 1).16 Tedizolid phosphate 2a (Sivextro, torezolid phosphate, TR-701, DA-7218; Cubist/Trius Therapeutics) is a prodrug that has completed phase-III trials and a New Drug Application (NDA) has been filed in the US and Marketing Authorization Application (MAA) for Europe, with FDA approval granted in June 2014.16-18 Diphosphorylation in vivo unmasks tedizolid 2b, which is active against linezolid resistant strains.19 Cadazodol 3 (ACT-179811; Actelion Pharmaceuticals) is a quinolonyl-oxazolidinone chimeric antibiotic that recently started a phase-III trial for the treatment of patients with Clostridium difficile infection (CD).20,21 Four oxazolidinones have completed or are in phase-II trials. Sutezolid 4 (PNU-100480, PF-02341272; Sequella/Pfizer) is a close analogue of linezolid 1 that was optimized for activity against tuberculosis.22-26 Radezolid 5 (RX-1741; Melinta Therapeutics, formally

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vancomycin linked with a near-infrared (NIR) probe binds to Gram-positive bacteria, allowing invasive infection to be detected by NIR optical imaging. Boron-dipyrromethene (BODIPY)-labeled daptomycin (BDP-DAP) was used to determine a novel resistance mechanism of vancomycin-resistant enterococci (VRE) to cationic antimicrobial peptides. From this study, VRE was shown to resist DAP-elicted cell membrane damage by diverting the antibiotic away from its principal target to other distinct cell membrane regions.

The Cu-catalysed azide–alkyne cycloaddition (an azide–alkyne version of the Huisgen 1,3-dipolar cycloaddition) is a useful reaction for derivatising azide-alkyne substitutes under mild conditions. The ‘click’ generated triazole is very useful in biological studies and medicinal chemistry due to the favourable physiochemical properties of the triazole ring. Triazoles are stable to reductive and oxidative reactions and acidic and basic hydrolysis reactions, under conditions where amides can be hydrolytically cleaved. Furthermore, the aromatic structure of triazoles resists enzymatic degradation and also participates in hydrogen bond formations and π-stacking interactions.

In this study, we prepare an azide-functionalised oxazolidinone antibiotic, and use it as a convenient intermediate in azide–alkyne click reactions to rapidly generate modified analogues, including fluorescent probes.

2. Materials and methods

2.1. Synthesis of azide-derivatised linezolid

The intermediate linezolid derivative 19 (Scheme 1) was synthesised as described in the literature except that a tosylate was used instead of a mesylate for the conversion of 15 to 16. In short, 3,4-difluoronitrobenzene 10 was treated with piperazine, selectively resulting in p-substituted nitrobenzene 11. Catalytic reduction of 11 and subsequent acylation of amine 12 with benzylchloroformate gave protected carbamate 13. Reaction of 13 with (R)-glycidyl butyrate in THF at −78 °C in the presence of n-Buli provided 5-(R)-hydroxymethyl-2-oxazolidinone 14. To form the desired oxazolidinone ring it was essential to use a lithium counter ion in the base for regiochemical control. Sodium and potassium bases generate 5-hydroxymethyl-2-oxazolidinone as a major by-product. Treatment of compound 14 with p-toluensulfonyl chloride at 0 °C in the present of triethylamine gave the tosylated product 15, which was displaced by phthalimide to produce compound 16. Deprotection of phthalimide with methylamine generated the free amine 17 which was acetylated with acetic anhydride to provide 18. Catalytic deprotection of theCbz group with palladium on activated carbon gave the intermediate linezolid derivative 19.

A linker 22 containing the azido group was synthesised in 2 steps from 3-bromo-1-propanol 20, which was treated with sodium azide in THF/H2O. The reaction mixture was refluxed with stirring for 16 h to yield 3-azido-1-propanol 21. Treatment of p-toluensulfonyl chloride at 0 °C in the present of triethylamine gave the tosylated product 22.

Finally, the secondary amine contained in 19 was alkylated with azide linker 22 to yield azide-derivatised linezolid analogue 9 in good yield.

2.2. Synthesis of alkyne-derivatised fluorophores

Two fluorophores, 7-(dimethylamino)-coumarin-4-acetic acid (DMACA, prepared according to literature procedures) and 7-nitrobenzofurazan (NBD, Sigma–Aldrich), were functionalized with an alkyne substituent (Scheme 2) so that they could be coupled to the linezolid–azide analogue 9 by click chemistry. DMACA 23 was

![Figure 1. Oxazolidinone antibiotics in the clinic and the azide-functionalised linezolid analogue 9.](image-url)
reacted with propargylamine in the presence of HATU as coupling agent to give DMACA linked alkyne 24. NBD linked alkyne 26 was prepared by a substitution reaction from NBD-Cl (4-chloro-7-nitrobenzofurazan) 25 by an improved method based on that previously reported in the literature. The use of Cs2CO3 in THF for the substitution gave improved yields compared to aqueous NaHCO3 in MeOH.

2.3. Click chemistry of azide-derivatised oxazolidinone

To demonstrate the potential of the azide-functionalised oxazolidinone to generate analogues to explore antimicrobial SAR and produce fluorescent probes, two different alkynes and the two alkyne derivatised fluorophores were reacted with the azide. Lin ezolid azide analogue 9 was reacted with propargyl alcohol, phenylacetylene, and alkyne fluorophores 24 and 26 in the presence of copper sulfate and sodium ascorbate to provide the corresponding triazole derivatives 27, 28, 29, and 30, respectively (Scheme 3). The cycloaddition reactions of the alkyne-fluorophores and azide 9 at room temperature were found to be very slow, hence were conducted at 50 °C with completion after 16 h. All products were purified by reverse-phase HPLC.

2.4. MIC assays

Compounds were tested for antimicrobial activity against twelve Gram-positive bacterial strains: Enterococcus faecalis (VanA clinical isolate), Enterococcus faecium (MDR Van A ATCC 51559), Streptococcus pneumoniae (MDR ATCC 700677), Staphylococcus aureus (MRSA ATCC 43300, MRSA clinical isolate, MRSA daptomycin resistant clinical isolate, GISA NRS 1, GISA NRS 17, VRSA NARSA VRS1, VRSA NARSA VRS4 and VRSA NARSA VRS10). All experiments were performed in duplicate with vancomycin, and linezolid used as positive controls (see Table 1). Positive growth control rows of bacteria and DMSO + bacteria as well as a negative control row of only media were included for every plate.

2.5. Fluorescent properties of probes

The fluorescent spectra of the two oxazolidinone-fluorescent probe conjugates 29 and 30 were measured (Fig. 2). Note that the DMACA emission peak of 29 (λmax = 490 nm) overlaps with the NBD absorption peak of 30 (λmax = 475 nm).
2.6. Fluorescent imaging of bacteria

The ability of the fluorescent probes to label bacteria was examined using log phase cultures of two S. aureus strains (an MRSA clinical isolate and a VISA NARSA VRS3b) and an Enterococcus faecium strain (MDR Van A ATCC 51559). Probes 29 and 30 were incubated with the bacteria for one hour at a concentration of 64 \( \mu \)g/mL, after which the bacteria were smeared onto glass slides. After fixation, washing and mounting steps, fluorescent images of the slides were obtained using a confocal microscope (Zeiss LSM 510 META).

2.7. Docking studies of oxazolidinone derivatives

The derivatives were modelled into the crystal structure of native Deinococcus radiodurans large ribosomal subunit (D50S) bound with linezolid (Pdb: 3DLL) using Schrödinger software package and its Induced Fit Docking module (Induced Fit Docking protocol 2013-2, Glide version 5.9, Prime version 3.2, Schrödinger, LLC, New York, NY, 2013), to account for the reported structural flexibility of the peptidyl transferase centre.

3. Results and discussion

3.1. Design of azide-derivatised linezolid analogue

The oxazolidinone antibiotic linezolid possesses 4 key structural elements; the A-, B-, and C-rings, and the C-5 position of the A-ring (Fig. 3). Modification of linezolid's A-ring, B-ring, and C-5 position influence its antimicrobial activity. Most modified oxazolidinone antibiotics keep the B-ring to maintain potent activity.

| Compound | MRSA ATCC 43300 | MRSA | DaptRs | GISA NRS1 | GISA NRS17 | VRSA NARS1 | VRSA NARS4 | VRSA NARSA | VRSA NARSA | VRSA NARSA | VRSA NARSA MDR ATCC 700677 | Enterococcus faecalis Van A Clin. Isol. | Enterococcus faecium Van A ATCC 51559 |
|----------|-----------------|------|--------|-----------|------------|-----------|-----------|-----------|-----------|-----------|------------------------|-----------------------------|-----------------------------|
| Vancomycin | 1 | 1 | 2 | 8 | 4 | >64 | 32 | 1 | 1 | >64 | >64 | 0.5 |
| Linezolid 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 2 | 2 |
| 19 | 8 | 2 | 2 | 4 | 4 | 4 | 32 | 8 | 4 | 8 | 2 |
| 27 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 4 | 4 | 4 | 4 |
| 29 | 64 | 32 | >64 | 16 | 16 | 16 | 64 | 32 | 64 | 16 | 16 |
| 30 | 16 | 8 | 8 | 8 | 16 | 16 | 32 | 32 | 4 | 32 | 16 |

Clin. Isol. = clinical isolate; DaptRs = daptomycin resistant; MRSA = methicillin resistant S. aureus, GISA = glycopeptide insensitive S. aureus, MDR = multidrug resistant, VRSA = vancomycin resistant S. aureus.
However, the 4'-position of the C-ring was tolerant to alteration and does not show significant loss of activity after replacement with different functional groups. 12,56-58 This correlates with structural analysis of different ribosomal subunits in comparison with the binding mode of linezolid 35,59 in which the nucleotide U2585 at the peptidyltransferase centre shows considerable conformational flexibility, and is able to accommodate large substituents at the 4'-position of linezolid. Therefore, an azide-derivatized linezolid 9 was designed to explore the 4'-position of the C-ring.

### 3.2. ‘Click’ chemistry and antimicrobial activity of conjugates

Four different alkynes were successfully employed for proof-of-principle Cu-catalysed azide–alkyne cycloadditions with the azide-derivatised oxazolidinone, generating the triazole-linked conjugates. The reactions proceeded in good yield to give the desired products. As expected, the reaction was tolerant of a range of functionalities on both the azide and alkyne components.

The linezolid/sutezolid analogue 19, replacing the morpholine oxygen with a nitrogen, consistently lost approximately 4-fold potency, with the exception of multidrug resistant *E. faecalis* ATCC 51559, where potency was gained. Substitution with the azidopropyl substituent, in the azide-derivatised linezolid intermediate 9, gave a compound that retained similar activity to linezolid against all strains tested (MIC 2 to 4 µg/mL). Triazole formation with phenylacetylene or propargyl alcohol resulted in significantly different activity, with the phenyl-substituted triazole 27 much less active (MIC 32 to >64 µg/mL) than the hydroxymethyl derivative 28 (MIC 2 to 32 µg/mL). The linezolid-DMACA probe 29 lost most antimicrobial activity (MIC 32 to >64 µg/mL) whereas the linezolid-NBD probe 30 retained some activity (4 to 32 µg/mL) against most strains tested. From the in silico docking studies the azide-derivatised oxazolidinone 9 was able to fit into a similar position observed for linezolid in the crystal structure of ribosomal subunit 50S of *D. radiodurans* (Fig. 4). 55 Due to the extended size of the molecule an induced fit workflow had to be applied in order to accommodate the molecule. As reported in other crystal structures, the nucleotide U2585 was able to adopt a variation of conformations, while the piperazine azide moiety oriented itself along the P-site. The fact that some activity was retained for the fluorescent probes was promising, as it indicated that some compound was penetrating the bacterial membrane, and for staining of live bacteria sub-MIC concentrations could be used.

### 3.3. Fluorophore-derivatised oxazolidinone antibiotics

Two different fluorophores, 7-(dimethylamino)-coumarin-4-acetic acid (DMACA) and 7-nitrobenzofurazan (NBD) were selected as initial fluorophores due to their low molecular size compared to most other fluorophores (such as rhodamines and fluoresceins), increasing both the potential for cellular penetration of the antibiotic-fluorophore probes, and the likelihood that they could be accommodated within the linezolid binding site. They also have differing fluorescence colours (blue and green, respectively), with an emission/excitation overlap that may be useful for FRET studies. The azide-derivatised fluorophores were readily coupled with the azide-oxazolidinone 9 to generate fluorescent probes 29 and 30. Incubation of these probes with Gram-positive bacteria resulted in selective staining of the bacteria cells (Fig. 5), with the staining pattern consistent with internalization when compared to other fluorophore probes known to stain the cell surface (e.g., similar to internal staining pattern of DAPI, 4,6-diamidino-2-phenylindole, which binds internal nucleic acids, but not like the membrane binding dye FM4-64, N-(3-triethylammoniumpropyl)-4-(6-(4-diethylamino)phenyl)-hexa-triencylpyridinium dibromide). The probes labelled both *S. aureus* and *E. faecium* despite relatively poor MIC values.

### 4. Conclusion

In conclusion, we have demonstrated that a morpholine-to-piperidine analogue of the oxazolidinone antibiotic linezolid can be alkylated with azidopropene to produce an azide-modified derivative that retains the antibacterial activity of linezolid. This versatile intermediate can be applied to rapidly assess structure–activity relationships using Cu-catalysed azide–alkyne cycloadditions with substituted alkynes, and to easily conjugate fluorophores with different physicochemical and photochemical properties. The oxazolidinone-fluorophore probes successfully labelled a number of different types of Gram-positive bacteria. This approach allows for a rapid 'mix and match', with alternate fluorophore colours readily attached to the derivatised antibiotic in a single step. Future studies will examine the utility of these probes in bacterial detection systems, and their ability to detect internal bacterial structures when combined with high resolution imaging, such as 3D structured illumination microscopy (3D-SIM-Microscopy). The azide-modified oxazolidinone will also be combined with other functionalized antibiotics to rapidly generate hybrid antibiotics similar to cadazoloid 3.
5. Experimental

5.1. Chemistry

5.1.1. General
All materials, unless otherwise noted, were obtained from commercial suppliers and used without further purification. Non-aqueous reactions were conducted under an inert atmosphere of nitrogen. Reactions were monitored by thin layer chromatography (TLC). Analytical TLC was performed on Merck TLC alumina sheets pre-coated with Silica Gel 60 F254, and compounds were visualized using UV lamp and appropriate TLC stains. Column chromatography was performed using silica gel 60 (0.063–0.200 mm), 70–230 mesh ASTM. Biotage Isolera and Grace Reveleris chromatography systems were used for compound purification.

$^1$H (600 MHz) and $^{13}$C (125 MHz) NMR spectra were obtained using a Bruker Avance-600 spectrometer equipped with a TXI cryoprobe. Chemical shifts are reported relative to the residual solvent signals in parts per million ($\delta$) (CDCl$_3$: $^1$H: $\delta$ 7.27, $^{13}$C: $\delta$ 77.2; CD$_3$OD: $^1$H: $\delta$ 3.30, $^{13}$C: $\delta$ 49.5; DMSO-$d_6$: $^1$H: $\delta$ 2.50, $^{13}$C: $\delta$ 39.5). High resolution mass spectrometry (HRMS) was performed on a Bruker Micro TOF mass spectrometer using (+)-ESI calibrated to NH$_4$OAc.

5.1.2. Synthesis of linezolid analogue 19

Linezolid analogue 19 was synthesized by a slight modification (mesylate displacement replaced with tosylate) of a literature method as shown in Scheme 1.

5.1.3. 3-Azidopropyl 4-methylbenzenesulfonate 22

The mixture of 3-bromo-1-propanol 20 (1.0 g, 7.19 mmol) and sodium azide (1.4 g, 21.58 mmol) in THF/H$_2$O (20:5 mL) was stirred at 80 °C for 16 h. The reaction mixture was extracted with CH$_2$Cl$_2$, dried over MgSO$_4$, and concentrated under reduced pressure to give compound 21 (0.59 g, 81%) as an oil which was used for the next reaction without further purification.

To the solution of 3-azidopropan-1-ol 21 (1.47 g, 14.55 mmol) in CH$_2$Cl$_2$ was added p-toluenesulfonyl chloride (3.05 g, 16.01 mmol). The reaction mixture was stirred at rt overnight. The reaction was checked by TLC for completion. The resulting residue was diluted with water and extracted with CH$_2$Cl$_2$, dried over MgSO$_4$, and concentrated under reduced pressure. Purification by column chromatography (silica gel, 50% EtOAc/Hexane) gave compound 22 (2.58 g, 69%) as an oil.

$^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 7.76 (d, $J = 8.2$ Hz, 2H), 7.33 (d, $J = 8.4$ Hz, 2H), 4.07 (t, $J = 5.8$ Hz, 2H), 3.34 (t, $J = 6.5$ Hz, 2H), 2.42 (s, 3H), 1.85 (quin, $J = 6.2$ Hz, 2H); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 145.2, 132.8, 130.1, 128.0, 67.2, 47.4, 28.6, 21.8; (+)-ESI-HRMS calc for C$_{10}$H$_{13}$N$_3$NaO$_3$S [M+Na$^+$]: 278.0575, found 278.0580.

5.1.4. (S)-N-((3-(4-(3-Azidopropyl)piperazin-1-yl)-3-fluorophenyl)-2-oxooxazolidin-5-yl)methyl)-acetamide 9

To the mixture of 19 (100 mg, 0.30 mmol), 22, and Nal (10 mol %, 5 mg, 0.003 mmol) in EtOH (40 mL), NEt$_3$ was added. The mixture was stirred under reflux for 6 h and then concentrated under reduced pressure. Purification by column chromatography (Grace MPLC; C18 reverse phase with eluents 0.1% TFA in MeOH/
0.1% TFA in H2O) gave compound 9 (46 mg, 37%) as white solid. 1H NMR (600 MHz, CD3OD): δ 7.48 (dd, J = 14.7, 2.5 Hz, 1H), 7.16 (dd, J = 8.8, 2.6 Hz, 1H), 7.05 (t, J = 9.4 Hz, 1H), 4.80 (m, 1H), 4.11 (t, J = 9.0 Hz, 1H), 3.80–3.77 (m, 1H), 3.55 (d, J = 5.0 Hz, 2H), 3.40 (t, J = 6.6 Hz, 2H), 3.10 (br s, 4H), 2.69 (s, 4H), 2.55 (t, J = 7.5 Hz, 2H), 1.96 (s, 3H), 1.85–1.80 (m, 2H); 13C NMR (125 MHz, CD3OD): δ 174.2, 156.9 (d, J = 9.0 Hz, 1H), 130.7, 128.9, 127.9, 125.1, 121.6, 119.9 (d, J = 3.4 Hz), 114.1 (d, J = 10.6 Hz), 71.6, 53.0, 51.2, 47.4, 47.3, 46.9, 41.2, 24.3, 22.4; (+)-ESI-HRMS calc for C27H35F3O7 [M+H]^+; 522.6279, found 522.6277.

5.1.8. (S)-N-((3-Fluoro-4-4(3-(4-hydroxymethyl)-1H-1,2,3-triazol-1-yl)propyl)piperazin-1-yl)phenyl)-2-oxoazolidin-5-yl)methylacetamide 28

To a solution of 9 (24 mg, 0.057 mmol) and propargyl alcohol (3.2 μl, 0.057 mmol) in MeOH (4 ml) was added a solution of CuSO4 (5 mol %, 0.01 mg, 0.0029 mmol) and treated with aqueous sodium ascorbate (10 mol %, 1.13 mg, 0.0057 mmol). The reaction was stirred vigorously for 16 h. The reaction was concentrated under reduced pressure. Purification by column chromatography (Grace MPLC, C18 reverse phase with eluents 0.1% TFA in MeOH/0.1% TFA in H2O) gave compound 28 (13.3 mg, 49%) as a white solid. 1H NMR (600 MHz, DMSO-d6): δ 8.24 (t, J = 5.8 Hz, 1H), 8.02 (s, 1H), 7.51 (dd, J = 14.7, 2.5 Hz, 1H), 7.20 (dd, J = 8.9, 2.2 Hz, 1H), 7.13 (t, J = 9.2 Hz, 1H), 4.73–4.69 (m, 1H), 4.53 (s, 2H), 4.45 (t, J = 6.8 Hz, 2H), 4.08 (t, J = 9.0 Hz, 1H), 3.71–3.69 (m, 1H), 3.59 (br s, 2H), 3.41–3.36 (m, 4H), 3.19 (br s, 4H), 3.01 (br s, 2H), 2.26, (br s, 2H), 1.83 (s, 3H); 13C NMR (125 MHz, CD3OD): δ 174.3, 157.1 (d, J = 245.4 Hz), 156.7, 136.4 (d, J = 10.6 Hz), 135.7 (d, J = 9.5 Hz), 121.3 (d, J = 3.2 Hz), 115.6 (d, J = 2.9 Hz), 108.6 (d, J = 263.7 Hz), 73.7, 56.5, 55.4, 53.6, 49.2, 48.4, 43.3, 25.9, 22.6; 13C NMR (125 MHz, DMF-d6): δ 172.8, 170.7, 160.7, 155.4, 152.9, 151.0, 126.0, 109.4, 109.1, 108.1, 97.5, 80.9, 73.3, 39.7, 38.4, 28.2; (+)-ESI-HRMS calc for C32H28N5NaO6 [M+Na]^+: 591.2220, found 591.2219.

5.1.6. 7-Nitro-N-(prop-2-yn-1-yl)benzo[c][1,2,5]oxadiazol-4-amine 26

To a solution of 3-[3-(4-fluoro-4-(3-(4-(((7-nitrobenzo[4,5]cycloheptadien-4-yl)carboxylic acid (23) (0.3 g, 1.21 mmol) in DMF (5 ml) was added HATU in DMF (5 ml) followed by DIPEA (386 μl) and propargylamine (71 μl, 1.1 mmol). The solution was stirred at rt overnight. The reaction was evaporated under reduced pressure to remove DMF. The residue was diluted with water, evaporated with ethyl acetate, dried over MgSO4, and concentrated under reduced pressure. The crude product was recrystallized in CH2Cl2. The solid was filtered and washed with CH2Cl2 to give pure compound 24 (0.149 g, 48%) as a green solid. 1H NMR (600 MHz, DMSO-d6): δ 8.65 (t, J = 5.4 Hz, 1H), 7.52 (d, J = 9.0 Hz, 1H), 6.72 (dd, J = 9.1, 2.6 Hz, 1H), 6.55 (d, J = 2.6 Hz, 1H), 6.00 (s, 1H), 3.65–3.56 (m, 2H), 2.63 (s, 2H), 2.32 (s, 4H), 1.31 (t, J = 2.5 Hz, 1H), 3.01 (s, 6H); 13C NMR (125 MHz, DMSO-d6): δ 167.7, 160.7, 155.4, 125.9, 141.1, 126.0, 109.4, 109.1, 108.1, 97.5, 80.9, 73.3, 39.7, 38.4, 28.2; (+)-ESI-HRMS calc for C23H25N4NaO6: [M+Na]^+: 591.2220, found 591.2219.

5.1.7. (S)-N-((3-(3-Fluoro-4-4(3-(4-phenyl-1H-1,2,3-triazol-1yl)propyl)piperazin-1-yl)phenyl)-2-oxoazolidin-5-yl)methylacetamide 27

To a solution of 9 (18 mg, 0.045 mmol) and phenylacetylene (5 μl, 0.045 mmol) in MeOH (4 ml) was added a solution of CuSO4 (5 mol %, 0.56 mg, 0.0023 mmol) and treated with aqueous sodium ascorbate (10 mol %, 0.89 mg, 0.0045 mmol). The reaction was stirred vigorously for 16 h. The reaction was concentrated under reduced pressure. Purification by column chromatography (Grace MPLC, C18 reverse phase with eluents 0.1% TFA in MeOH (0.1% TFA in H2O) gave compound 27 (5.66 mg, 24%) as a white solid. 1H NMR (600 MHz, DMSO-d6): δ 8.62 (s, 1H), 8.24 (t, J = 5.8 Hz, 1H), 7.86–7.84 (m, 2H), 7.51 (dd, J = 14.7, 2.5 Hz, 1H), 7.48–7.45 (m, 2H), 7.35 (tt, J = 7.4, 1.2 Hz, 1H), 7.21 (dd, J = 8.8, 2.2 Hz, 1H), 7.14 (t, J = 9.4 Hz, 1H), 4.73–4.69 (m, 1H), 4.54 (t, J = 6.7 Hz, 2H), 4.08 (t, J = 9.0 Hz, 1H), 3.71–3.69 (m, 1H), 3.61 (br s, 2H), 3.45–3.39 (m, 4H), 3.24 (br s, 4H), 3.01 (br s, 2H), 2.43 (br s, 2H), 1.83 (s, 3H); 13C NMR (125 MHz, DMSO-d6): δ 170.0, 154.5 (d, J = 243.6 Hz), 154.0, 146.4, 143.4 (d, J = 10.2 Hz), 130.7, 128.9, 127.9, 125.1, 121.6, 119.9 (d, J = 3.4 Hz), 114.1 (d, J = 10.6 Hz), 71.6, 53.0, 51.2, 47.4, 47.3, 46.9, 41.2, 24.3, 22.4; (+)-ESI-HRMS calc for C27H35F3O7 [M+H]^+: 522.6279, found 522.6277.
of CuSO₄ (5 mol %, 1.5 mg, 0.006 mmol) and treated with aqueous sodium ascorbate (10 mol %, 2.4 mg, 0.012 mmol). The reaction was stirred vigorously at 50 °C for 16 h. Purification by column chromatography (Grace MPLC; C18 reverse phase with eluents 0.1% TFA in ACN/0.1% TFA in H₂O) gave compound 54 (51.8 mg, 68%) as a orange solid. ¹H NMR (600 MHz, DMSO-d₆): δ 9.88 (t, J = 6.1 Hz, 1H), 8.53 (d, J = 8.9 Hz, 1H), 8.24 (t, J = 5.8 Hz, 1H), 8.16 (s, 1H), 7.51 (d, J = 14.8, 2.3 Hz, 1H), 7.20 (dd, J = 8.8, 1.9 Hz, 1H), 7.12 (t, J = 8.9 Hz, 1H), 6.51 (d, J = 8.9 Hz, 1H), 4.78 (br s, 2H), 4.73–4.69 (m, 1H), 4.44 (t, J = 6.8 Hz, 2H), 4.08 (t, J = 9.0 Hz, 1H), 3.71–3.69 (m, 1H), 3.56 (br s, 2H), 3.40–3.35 (m, 4H), 3.19 (br s, 4H), 3.0 (br s, 2H), 2.24 (br s, 2H), 1.83 (s, 3H); ¹³C NMR (125 MHz, DMSO-d₆): δ 170.0, 154.5 (d, J = 243.3 Hz), 154.0, 145.7, 144.5, 144.0, 142.7, 137.7, 134.2, 123.6, 119.9, 114.0 (d, J = 2.2 Hz), 106.5 (d, J = 26.0 Hz), 99.8, 71.6, 52.9, 52.0, 51.1, 47.2, 47.7, 41.3, 38.5, 24.2, 22.4; (+)-ESI-HRMS calc for C₂₈H₃₁FN₁O₆ [M+H]⁺: 638.2599, found 638.2602.

5.2. Biology

5.2.1. Determination of MIC (minimum inhibitory concentration)

MICs were determined by a two-fold serial broth microdilution according to the recommendation of CLSI standards with an inoculum of 5 × 10⁵ cfu/mL. The compounds along with standard antibiotics were serially diluted twofold across the wells of 96-well plates at pH 7.0. The analysis of the different ligand orientations were limited to poses similar to the orientation of linezolid in the crystal structure.

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Supplementary data

Supplementary data (NMR spectra and HPLC chromatograms for 9, 22, 24, and 27–30) associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.05.054. These data include MOL files and InChIKeys of the most important compounds described in this article.

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