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Discovery of a potent benzoxaborole-based anti-pneumococcal agent targeting leucyl-tRNA synthetase

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TARGET VALIDATIONQing-Hua Hu^{1*}, Ru-Juan Liu^{1*}, Zhi-Peng Fang¹, Jiong Zhang², Ying-Ying Ding³, Min Tan¹, Meng Wang¹, Wei Pan³, Hu-Chen Zhou² & En-Duo Wang¹Received
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requests for materials
should be addressed to
H.-C.Z. (hczhou@sjtu.
edu.cn) or E.-D.W.
(edwang@sibcb.ac.
cn)* These authors
contributed equally to
this work.

¹State Key Laboratory of Molecular Biology, Center for RNA research, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, The Chinese Academy of Sciences, Shanghai 200031, China, ²School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, China, ³Department of Microbiology, Shanghai Key Laboratory of Medical Biodefense, Second Military Medical University, Shanghai 200433, China.

Streptococcus pneumoniae causes bacterial pneumonia with high mortality and morbidity. The emergency of multidrug-resistant bacteria threatens the treatment of the disease. Leucyl-tRNA synthetase (LeuRS) plays an essential role in cellular translation and is an attractive drug target for antimicrobial development. Here we report the compound ZCL039, a benzoxaborole-based derivative of AN2690, as a potent anti-pneumococcal agent that inhibits *S. pneumoniae* LeuRS (*SpLeuRS*) activity. We show using kinetic, biochemical analyses combined with the crystal structure of ZCL039-AMP in complex with the separated *SpLeuRS* editing domain, that ZCL039 binds to the LeuRS editing active site which requires the presence of tRNA^{Leu}, and employs an uncompetitive inhibition mechanism. Further docking models establish that ZCL039 clashes with the eukaryal/archaeal specific insertion I4ae helix within editing domains. These findings demonstrate the potential of benzoxaboroles as effective LeuRS inhibitors for pneumococcus infection therapy, and provide future structure-guided drug design and optimization.

Pneumonia is a severe acute respiratory infectious disease that causes lung dysfunction, and it is considered the leading cause of morbidity and mortality among children worldwide¹. The WHO estimated that pneumonia kills over 2 million children each year, accounting for 19 per cent of under-five deaths¹. *Streptococcus pneumoniae* is the primary pathogen of bacterial pneumonia, which resides in the human nasopharynx and can be transmitted through respiratory secretions in all age groups. Currently, pneumonia is treated using several classes of antibiotics such as penicillins, macrolides and vancomycins^{2,3}. The effectiveness of widely available antibiotics is essential for reducing pneumonia mortality. However, pneumococci has developed severe resistance to a variety of drugs and the numbers of clinically isolated resistant strains are in steadily increasing in recent years³. One major resistance of concern is the prevalence of penicillin-resistant *S. pneumoniae* which has been recognized as a community-acquired pathogen. More recently, the development of pneumococci resistance to over 3 kinds of antibiotics have been reported around the world³. The global emergence of multidrug-resistant *S. pneumoniae* and the high cost of vancomycin have restricted the effectiveness of clinically available drugs¹⁻³, presenting a greater threat to public health. Therefore, there is an urgent need for the development of new anti-pneumococcal agents that show no cross-resistance to current drugs.

Bacterial gene expression is a valuable process in the discovery of antibacterial targets⁴⁻⁶. Aminoacyl-tRNA synthetases (aaRSs) play an important role in the first step of protein synthesis. These enzymes have been shown to be promising targets in the development of antimicrobial therapeutic agents⁷. AaRSs constitute an ancient housekeeping family that catalyzes the esterification of amino acids and cognate transfer RNAs (tRNAs) to yield aminoacyl-tRNAs, which then conduct genetic code transfer from messenger RNAs to proteins⁸. The aminoacylation reaction usually starts with the activation of amino acids to generate aminoacyl-adenosine monophosphate (AMP), followed by the charging of tRNA⁸. The presence of multiple natural amino acids and their analogs in cells challenges the accuracy of this process. However, the overall error rate for aaRSs in translation is about 10⁻⁴⁹. This high fidelity can be attributed to the evolved proofreading (editing) function of some aaRSs^{10,11}. To prevent the formation of mischarged tRNA, several aaRSs possess hydrolytic activities toward either misactivated aminoacyl-AMP (pre-transfer editing) or noncognate aminoacyl-tRNA (post-transfer editing), ensuring that the quality of translation and cellular functions are maintained¹⁰. The failure of the generation of aminoacyl-tRNA or



the clearance of mischarged tRNA can disrupt the translation and fidelity, which can severely affect the viability of the organisms¹². Genetic code ambiguity has been reported previously in *Escherichia coli* with an artificial editing-defective isoleucyl-tRNA synthetase (IleRS), which has been shown to retard cell growth and cause global changes in protein function¹³.

Mupirocin, a natural inhibitor of bacterial IleRS¹⁴, which has been widely used in the clinical treatment of *Staphylococcus aureus* infection, has been found to kill bacteria by interrupting the aminoacylation reaction. Mupirocin represents most types of aARS inhibitors that have been developed to date. These inhibitors mimic the natural aminoacyl-AMP intermediates and competitively bind the synthetic site of the enzyme with its natural substrates, including amino acids and ATP^{15,16}. Although these substrate analogs showed excellent inhibitory effects against aARS activities and microorganism growth in the nanomolar range, only few analogs have proceeded into the clinical stage due to their poor absorption and lack of specificity. Benzoxaboroles are a new class of aARS inhibitors that have been recently developed. They displayed broad-spectrum activity to dermatophytes¹⁷. Of these, Tavaborole (AN2690) is currently in a phase III clinical trial for the treatment of onychomycosis. Biochemical and structural studies have revealed that AN2690 inhibits yeast cytosolic leucyl-tRNA synthetase (LeuRS) with an oxaborole tRNA trapping (OBORT) mechanism that depends on the unique boron atom¹⁸. Boron forms covalent bonds with the 2' and 3'-oxygen of the ribose ring of the tRNA terminal A₇₆ to yield a stable tRNA-AN2690 adduct in the LeuRS editing domain, which blocks tRNA translocation and prevents enzyme turnover, consequently arresting protein synthesis¹⁸.

LeuRS consists of a characteristic Rossmann-fold catalytic domain, an appended anticodon-binding domain, a connective peptide 1 (CP1, editing domain), and a C-terminal extension. The overall architecture of LeuRS is conserved across different species as suggested by the crystal structures of bacterial and archaeal LeuRSs^{19–21}. Although only insignificant structural deviations were observed in the ancestral catalytic domain of LeuRS, distinctive structural variations could be found in the CP1 domain. The catalytic core of the CP1 domain is highly conserved among eukaryal and bacterial LeuRSs; however, the active site pocket of the human cytosolic LeuRS CP1 domain is more compact than that in the corresponding bacterial domain²². This can be attributed to the presence of four additional eukaryal-specific insertions around the highly conserved core, among which I4ae covers the opening of the editing pocket²², making it difficult to bind to compounds with larger molecular mass. These structural differences will serve as valuable evidence for the development of novel selective antibacterial agents against LeuRS.

In this work, we established an enzymatic assay system of *S. pneumoniae* LeuRS (*SpLeuRS*) with both the *SpLeuRS* and *SptRNA*^{Leu} obtained from *E. coli* expression system, and performed inhibitors screening. The benzoxaborole compound ZCL039 was identified to be a potent *SpLeuRS* inhibitor that displayed good inhibition of *S. pneumoniae* growth with a minimum inhibitory concentration (MIC) of 5 µg/mL. Human cytosolic and mitochondrial LeuRSs were less susceptible to ZCL039, which is consistent with the lower toxicity of this compound to human cells. The target specificity in bacteria was confirmed by a shift of MIC upon LeuRS overexpression. Enzyme kinetic and mutagenesis analyses, combined with the 2.0 Å crystal structure of ZCL039-AMP in complex with the separated *SpLeuRS* editing domain were used to elucidate the uncompetitive action and binding mode of ZCL039, which requires the presence of tRNA^{Leu}. Moreover, docking models were made to find that ZCL039 clashes with the eukaryal/archaeal specific insertion I4ae helix within editing domains, which establishes the selectivity of ZCL039. The results demonstrated the potential of LeuRS as a target for *S. pneumoniae* infection therapy and benzoxaboroles were found to be a class of useful compounds in the development of novel anti-pneumococcal agents. Furthermore, the findings of this

study may provide structural evidence for rational drug design and optimization.

Results

Initial screening identified an effective *SpLeuRS* inhibitor. To identify active *SpLeuRS* inhibitors, we first established an effective study system of *SpLeuRS*. We used the *E. coli* gene expression system to produce *SpLeuRS* and its cognate tRNA^{Leu}, and a mass of protein and tRNA were obtained. From 5 g of wet cells, approximately 60 mg of *SpLeuRS* (with a molecular mass of 95.6 kDa) and 20 mg of *SptRNA*^{Leu}(CAA) were purified to more than 90% homogeneity, which was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or denatured PAGE (**Supplementary Fig. 1**). The leucine-accepting activity of the crude extracted tRNA was 820 pmol/A₂₆₀, which increased to 1500 pmol/A₂₆₀ after DEAE-Sephacrose anion-exchange chromatography purification, and this increase was accompanied by an improvement in product purity.

Primary sequence alignment revealed that *SpLeuRS* shared 45.4% similarity to *T. thermophilus* LeuRS (*TtLeuRS*), and their CP1 domains shared 52.1% identity. Therefore, we used the structure of *TtLeuRS* (PDB code 2V0G) as a basis of structure-guided inhibitor design. A focused library of benzoxaboroles consisting of a variety of AN2690 derivatives with different linkage groups at C(6) and C(7) was constructed. After an initial examination of their activities in aminoacylation, a compound designated ZCL039 was identified to distinctly inhibit *SpLeuRS*. ZCL039 was derived from substitutions at C(6) of the AN2690 with a phenyl group linked to the benzoxaborole core by a carbinol linkage (**Fig. 1a** and **Supplementary Fig. 2**). This compound showed a dose-dependent inhibition of aminoacylation (**Fig. 1b**) with an IC₅₀ value of 1.73 µM (**Table 1**). However, the PPI exchange activity of *SpLeuRS* was not affected when 200 µM ZCL039, i.e., over 100-fold of its IC₅₀, was used (**Fig. 1c**), indicating that ZCL039 is not involved in leucine activation. We further studied the effect of ZCL039 on *SpLeuRS* editing (**Fig. 1d**). ZCL039 was found to severely block the hydrolysis of mischarged Met-*SptRNA*^{Leu}. These results suggested that ZCL039 inhibits *SpLeuRS* aminoacylation and editing but not amino acid activation.

To elucidate the action mode of ZCL039, we performed kinetic assays of aminoacylation inhibition. The *K_m* values of *SpLeuRS* remained unaffected in the case of ATP and Leu, but the maximum rates decreased (**Table 2**), which is a characteristic feature of noncompetitive inhibition (**Supplementary Fig. 3**). This was consistent with ZCL039 inactivity observed in the ATP-PPI exchange reaction. In the case of tRNA^{Leu}, both the apparent *K_m* and the rate of *SpLeuRS* were significantly reduced (**Table 2**), implying that ZCL039 is uncompetitive with regard to tRNA^{Leu} (**Fig. 1e**). As an uncompetitive inhibitor binds to the enzyme-substrate complex but not to the free enzyme, we inferred that ZCL039 function depends on the presence of tRNA. The inhibitory constant (*K_i*) for ATP and Leu was determined to be 2.63 µM and 2.65 µM, respectively, while for *SptRNA*^{Leu}, ZCL039 exhibited an alpha *K_i* value of 3.53 µM (**Table 2**). Together, these results indicate that the benzoxaborole derivative ZCL039 is a potent inhibitor of pneumococcus LeuRS.

Antibacterial activity. We tested the antibacterial activity of ZCL039 to the growth of *S. pneumoniae*, which had a MIC value of 5 µg/mL (**Table 1**). Furthermore, ZCL039 moderately reduced the survival of *E. coli* (MIC = 60 µg/mL; **Table 1**), which was in accordance with its higher IC₅₀ (8.25 µM; **Table 1**) against *EcLeuRS*. The diverse MICs suggested that ZCL039 displays preferable potential antibacterial activity against pneumococci.

To confirm the target specificity of ZCL039, we examined whether the suppression of bacterial growth could be reversed by overexpressing *EcLeuRS* in *E. coli* strain. Indeed, strains containing pET30a-*eclrs* showed an increase of over 4-fold in the MIC (>240 µg/mL;

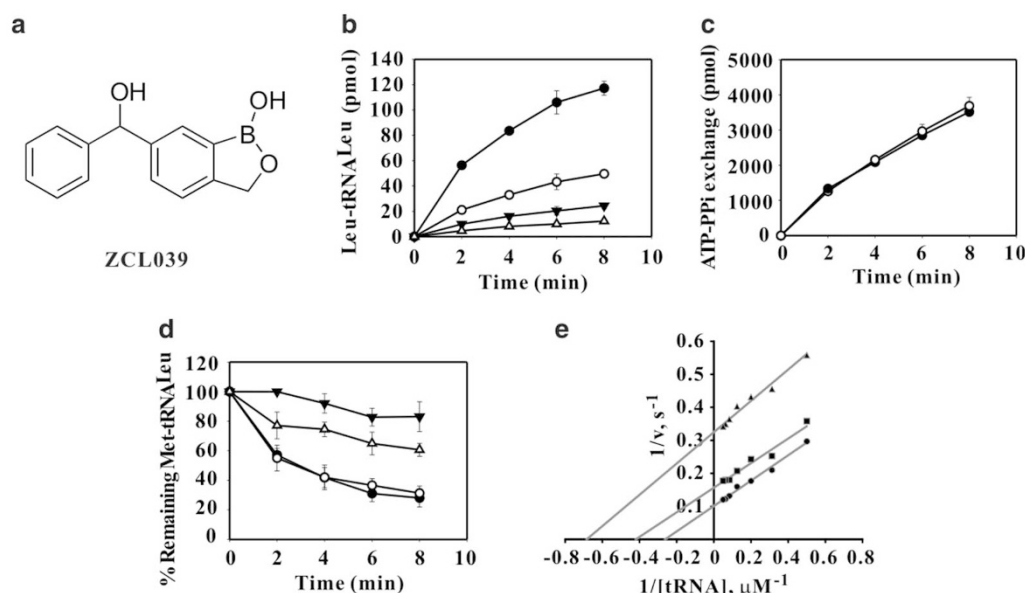


Figure 1 | Effect of ZCL039 on *SpLeuRS* activities. (a) Chemical structure of ZCL039. (b) A dose-dependent inhibition of *SpLeuRS* aminoacylation in the presence of 5 (○), 20 (▼), and 50 μM (Δ) of ZCL039. DMSO (●) was used as the control. (c) ATP-PPI exchange reaction catalyzed by *SpLeuRS* in the presence (●) or absence (○) of 200 μM ZCL039. (d) Hydrolysis of 1 μM [³H]Met-*SptRNA*^{Leu} by *SpLeuRS* in the presence of 5 (○) or 50 μM (Δ) of ZCL039 compared to that with DMSO (●). Spontaneous hydrolysis (▼) as the control was performed in the absence of enzyme. (e) Lineweaver-Burke plot of the substrate-velocity curve of *SptRNA*^{Leu} in the presence of ZCL-039: (●) 0, (■) 2, and (▲) 8 μM. Data in (b–d) are means from three independent experiments; errors bars show s.d.

Table 1) when induced by IPTG as compared to strains containing pET30a (MIC = 60 μg/mL; Table 1). A shift of MIC (>240 μg/mL; Table 1) was also observed in the absence of IPTG; this could be attributed to the leaky expression of *EcLeuRS* (Supplementary Fig. 4). However, there was no difference in susceptibility for ampicillin (MIC = 4 μg/mL; Table 1) when *EcLeuRS* was overexpressed, implying that ZCL039 may target *LeuRS* to play its role in cells.

Selective potency. Considering ZCL039 is structurally analogous to the antifungal agent AN2690, we examined the activity of ZCL039 against yeast cytosolic *LeuRS* which showed little effect (Supplementary Fig. 5). To further investigate the possible toxicity of ZCL039 to human cells, we first determined the effect of ZCL039 on human cytosolic and mitochondrial *LeuRS*s. Both *LeuRS*s were obtained in an *E. coli* expression system^{23,24}. ZCL039 showed IC₅₀ values above 250 μM against these enzymes (Table 1), which were approximately 150-fold less sensitive than *SpLeuRS*. We then examined the susceptibility of ZCL039 to mammalian cells by cell proliferation assays. The growth of A549 and 293T human cells was slightly affected by ZCL039 at detectable concentrations with IC₅₀ values above 200 μM (Table 1). Together, these results suggest that ZCL039 possess selectivity for the bacterial target versus the human orthologs.

ZCL039 targets *SpLeuRS* CP1 domain. According to the resolved structures of benzoxaboroles with *LeuRS*^{18,22}, we suspected that ZCL039 may target the CP1 domain of *LeuRS*. To confirm our hypothesis, two mutations, T252R and Y332D were made within the CP1 domain of *SpLeuRS*, intending to interrupt the binding of ZCL039 or tRNA^{Leu} to confer resistance of *SpLeuRS* to ZCL039. Residue Thr252 is a structural component of the benzoxaborole binding pocket as suggested by known structures^{18,22}, and residue Tyr332 is proposed to contribute to tRNA 3'-terminus binding²⁵, which is required for the action of ZCL039. Both mutants could not hydrolyze mischarged tRNA as shown in *EcLeuRS*²⁵ (data not shown). Aminoacylation inhibition assays showed that ZCL039 did not affect the synthetic activities of the two mutants (Fig. 2a), confirming their insensitivity to ZCL039. We suggested that the T252R mutation may obstruct the binding of ZCL039 with the CP1 domain of *SpLeuRS*, while the Y332D mutation could block the entry of tRNA into the editing site. The inability of ZCL039 to the charging of tRNA of the two mutants supported the assumption that it targets the CP1 domain of *SpLeuRS*.

To further confirm it, we removed *SpLeuRS* CP1 domain (Lys226-Thr411) and replaced it with a nonapeptide (*MmLinker*) to modulate enzyme aminoacylation activity as shown in other *LeuRS*s^{26,27}. The obtained chimeric mutant (*SpLeuRS-MmLinker*) retained approximately half of the tRNA charging activity compared to the wild-type

Table 1 | *In vitro* activity of ZCL039

	IC ₅₀ (μM)				MIC (μg/mL)			
	LeuRSs				Human cell lines		Bacteria	
	<i>S. pneumoniae</i>	<i>E. coli</i>	Human cytosolic	Human mitochondrial	HEK 293	A549	<i>S. pneumoniae</i>	<i>E. coli</i>
ZCL039 ^a	1.72 ± 0.05	8.25 ± 1.2	>250	>250	>200	>200	5	60
Ampicillin							0.0625	4

^aFor ZCL039 (Mr = 240.1), 1 μM equals to 0.24 μg/mL.

The IC₅₀ values are means from at least two independent experiments; errors show s.d.



Table 2 | Inhibitory kinetic constants of SpLeuRS in aminoacylation with respect to ZCL039

Substrate	ZCL039			
	-	+2 μM	+8 μM	
ATP	K_m (μM)	2335 \pm 452	2138 \pm 395	2231 \pm 429
	k_{cat} (s^{-1})	11.45 \pm 1.15	6.35 \pm 0.65	2.80 \pm 0.32
	k_{cat}/K_m ($\text{s}^{-1} \text{mM}^{-1}$)	4.89	2.97	1.26
	K_i (μM)		2.63 \pm 0.26	
Leu	K_m (μM)	23.37 \pm 2.88	23.74 \pm 3.07	22.38 \pm 2.81
	k_{cat} (s^{-1})	10.86 \pm 0.79	6.44 \pm 0.55	2.61 \pm 0.16
	k_{cat}/K_m ($\text{s}^{-1} \text{mM}^{-1}$)	464.70	271.27	116.62
	K_i (μM)		2.65 \pm 0.22	
SptRNA ^{Leu}	K_m (μM)	3.80 \pm 0.42	2.84 \pm 0.31	1.45 \pm 0.16
	k_{cat} (s^{-1})	10.09 \pm 1.12	6.62 \pm 0.48	2.89 \pm 0.24
	k_{cat}/K_m ($\text{s}^{-1} \text{mM}^{-1}$)	2655.26	2330.99	1993.10
	alpha K_i (μM)		3.53 \pm 0.16	

Kinetics was determined under 2 nM SpLeuRS.
All the values in the table are means from three independent experiments; errors show s.d.

enzyme, and this was not affected by ZCL039 (Fig. 2b). This result may have occurred due to an abolishment of the binding of ZCL039 to the CP1 domain of SpLeuRS. Together, these results suggested that the editing active site of SpLeuRS is the binding pocket of ZCL039.

Insight into the structure of ZCL039 bound with SpLeuRS CP1 domain. To determine the mechanism of action of ZCL039 and improve its efficacy, we obtained a high resolution of the crystal structure of the SpLeuRS-CP1 (Thr228-Val410) complex with ZCL039 and AMP. The final models of the apo form (PDB code: 4K48) and the complex form (PDB code: 4K47) of SpLeuRS-CP1 were refined to 2.5 Å and 2.0 Å, respectively. Data collection parameters and refinement statistics are summarized in Table 3.

Overall, the structure of SpLeuRS-CP1 is very similar to that of other bacterial LeuRSs. It is a globular β -barrel composed of 7 β -strands surrounded by 6 α -helices (Fig. 3a). The highly conserved catalytic core (colored deep blue) is present in the order β 1- β 2- α 1- β 3- β 4- β 5- β 6- α 2- α 3- β 7 in the secondary structure. Two additional bacterial insertions, I2b and I4b, are found beside the catalytic core (Fig. 3a). The I2b insertion is composed of three helices inserted between the β 3 and β 4 of the catalytic core. The I4b insertion is a long loop located between α 2 and α 3 helices. The active site of the CP1 domain features in the “T-rich region”, the “GTG loop” and the strictly conserved Asp residue (Asp347 in SpLeuRS; Supplementary Fig. 6). The T-rich region (T²⁴⁷TRPDT²⁵²) is located between β 2 and β 3 strands, containing two extremely conserved Thr residues that are important for the hydrolysis function: Thr247 at the end of the β 2 strand and Thr252 in the small helix between β 2 and β 3 strands

(Fig. 3b). The “GTG loop”, which is involved in the binding of tRNA Ade76, is located between β 5 and β 6 strands (Fig. 3b). The universally conserved Asp347 residue (Asp347 in TtLeuRS and Asp345 in EcLeuRS), which is supposed to be critical for amino acid editing, is located at the center of the α 2 helix (Fig. 3b).

In the complex structure, ZCL039 formed a covalent adduct with AMP (ZCL039-AMP) through the boron atom with the 2' and 3'-oxygen atoms of adenosine (Fig. 3c) at the active site of SpLeuRS-CP1. As shown in Fig. 3d, the adduct, especially the AMP part, has a broad interaction network with the catalytic core. Notably, the side chain of Tyr332 forms a hydrogen bond with the phosphate group of the AMP, confirming its role in binding for tRNA²⁵. This interaction explained the inactivity of ZCL039 to the Y332D mutant, revealing a cooperation of ZCL039 with tRNA. The benzoxaborole core of ZCL039, which is common to other benzoxaboroles, interacts mainly with the conserved residues within the T-rich region (Fig. 3d). The derived phenyl group of ZCL039 exhibited wide and moderate interactions with residues from the catalytic core, such as the T-rich region (Arg249 and Thr252) and the I4b insertion (Thr373; Fig. 3d). Specifically, the hydroxyl group from the carbinol bridge of ZCL039 and the side chain of Asp344 were located close to each other, within 2.8 Å, implying a strong hydrogen bond. To examine the role of this interaction for the action of ZCL039, we mutated Asp344 to alanine (Ala). This mutation reduced the sensitivity of SpLeuRS to ZCL039 (Fig. 3e), implying the importance of Asp344 in the binding of ZCL039-AMP. The complex structure of SpLeuRS-CP1 with the ZCL039-AMP adduct illuminated the binding and inhibitory mechanisms of ZCL039, suggesting that the SpLeuRS

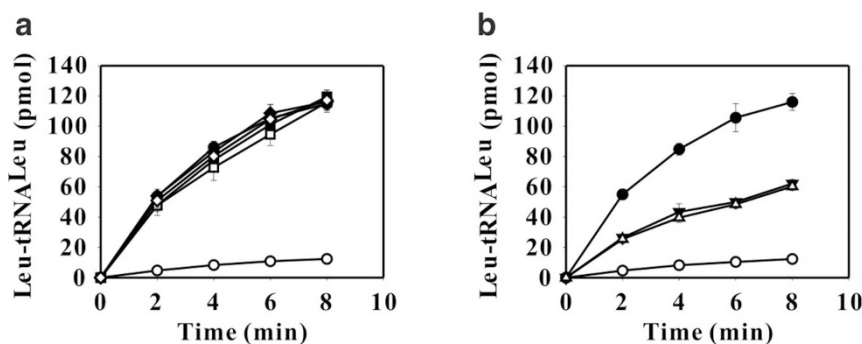


Figure 2 | Sensitivity of ZCL039 to SpLeuRS mutants. Leucylation carried out by 5 nM SpLeuRS or its mutants including T252R, Y332D (a), and SpLeuRS-MmLinker (b). Symbols are ●, ■, ◆, and ▼, respectively, in the absence of ZCL039; ○, □, ◇ and △, respectively, in the presence of 50 μM ZCL039. Data are means from three independent experiments; errors bars show s.d.



Table 3 | Data collection and refinement statistics

SpLeuRS-CP1	Apo	+AMP and ZCL039
Data collection		
Wavelength (Å)	0.9794	0.9792
Resolution range (Å)	50.0–2.50 (2.59–2.50)	50.0–2.00 (2.07–2.00)
Number of total reflections	1,424,646	1,559,012
Number of unique reflections	11,374	21,307
I/σ	32.7 (11.7)	36.3 (6.6)
Completeness (%)	99.7 (100)	99.6 (99.9)
R _{merge}	0.12 (0.45)	0.07 (0.50)
Redundancy	17.4 (18.5)	10.4 (10.7)
Space group	P6 ₁ 22	P6 ₁ 22
Unit cell dimensions		
α, β, γ (°)	88.02, 88.02, 135.40	88.35, 88.35, 137.16
α, β, γ (°)	90.00, 90.00, 120.00	90.00, 90.00, 120.00
Refinement		
Resolution (Å)	50.0–2.49	50–2.02
R _{work} /R _{free}	0.204/0.241	0.224/0.256
Number of reflections	10,726	20,052
Number of atoms		
Protein atoms	1,401	1,401
Water/other	28	135/4 sulfate
ZCL039-AMP	0	1
RMSD		
Bond lengths (Å)	0.009	0.010
Bond angles (°)	1.171	1.214
Average B factor (Å ²)	59.5	41.2
Ramachandran statistics (%)		
Most favored	96.7	98.3
Allowed	3.3	1.7

Values in parentheses are for highest resolution shell.

CP1 domain is a potential target site for benzoxaborole-based anti-bacterial inhibitors.

Discussion

The emerging benzoxaboroles have been explored in the development of novel antifungal and antiprotozoal agents as they possess excellent bioactivity and favorable pharmacokinetic properties^{17,18,28}. However, few studies have reported the antibacterial properties of these compounds²⁹. This study revealed a potent benzoxaborole-based anti-pneumococcal agent with a unique oxaborole tRNA trapping (OBORT) mechanism, which were highlighted by the crystal structure of ZCL039 bound to SpLeuRS-CP1. ZCL039 inhibited the aminoacylation of SpLeuRS with over 150-fold more sensitivity than to human cytosolic LeuRS. We docked the ZCL039-AMP adduct to the CP1 domain of human cytosolic LeuRS to help determine its specificity. From the docking model, the phenyl group of the ZCL039 was found to sterically clash with the long α-helix of the eukaryal-specific insertion I4ae, and the location of the phenyl group is held by the presence of Tyr468 at the I4ae insertion (Fig. 3f). Furthermore, the primary sequence alignment showed that the SpLeuRS Asp344, which forms a strong interaction with ZCL039-AMP, is replaced by Ser419 in human cytosolic LeuRS (Supplementary Fig. 6). This suggests ZCL039 may exhibit a decreased binding affinity towards human LeuRS. These rendered the large boron-containing compound incompatible with the smaller hydrophobic editing pocket of the eukaryal LeuRS. Similar results can also be obtained with archaeal (*P. horikoshii*) and other eukaryal cytosolic (*C. albicans* and *G. lamblia*) LeuRSs (Fig. 3f), which possess the conserved I4ae insertion and Tyr468 (in human LeuRS).

On the contrary, ZCL039 could be docked well into the editing pockets of structure-known bacterial LeuRSs, including *E. coli*, *T. thermophilus*, and *A. aeolicus*, without any steric conflicts with the surrounding residues (data not shown). Structurally, the bacterial LeuRS CP1 domain contains two other specific insertions, namely

I2b and I4b, that do not interfere with the binding of the phenyl group of ZCL039, and the strictly conserved T-rich region and the Asp344 residue to provide strong interactions for the binding of ZCL039 (Supplementary Fig. 6). Considering that ZCL039 and archaeal/eukaryal cytosolic LeuRSs are incompatible, the structural bases suggest that ZCL039 could be developed into a broad-spectrum antibacterial agent. ZCL039 showed different degrees of inhibition towards bacterial LeuRSs of different species, for instance, the IC₅₀ of ZCL039 towards SpLeuRS was 5-fold higher than towards EcLeuRS. A moderate dose-dependent inhibition of aminoacylation was also observed in *Mycobacterium tuberculosis* LeuRS (Supplementary Fig. 7). These results indicate that ZCL039 could be designed to obtain species-specific inhibitors targeting LeuRS, such as antitubercular drugs.

The interactions between ZCL039 and SpLeuRS-CP1 show similarity to that observed in other benzoxaboroles. The benzoxaborole moiety of ZCL039 interacts with the conserved catalytic editing core in the same way as all benzoxaboroles^{18,22}. Specifically for the derived part of ZCL039, a strong interaction was observed between the hydroxyl group from the carbinol bridge of ZCL039 and the Asp344 residue (Fig. 3d). The absence of any other strong interactions probably accounts for the moderate affinity of ZCL039, providing some clues for further optimization to enhance the antibacterial activity of this compound. The derived phenyl group was found to be located near to the residues from the conserved T-rich region (Arg249 and Thr252) and also from the bacterial insertion I4b (Thr373) (Fig. 3d). However, the distances between ZCL039 and these residues are approximately 4 Å or larger, suggesting that the interaction between this phenyl group and SpLeuRS-CP1 is considerably weak. It is noteworthy that the phenyl group points to the I4b direction; therefore, introducing small groups into the phenyl ring can aid in setting up stronger interactions between them. The resulting compounds could more efficiently bind to SpLeuRS and lead to increased potency. Furthermore, it should be noted that the

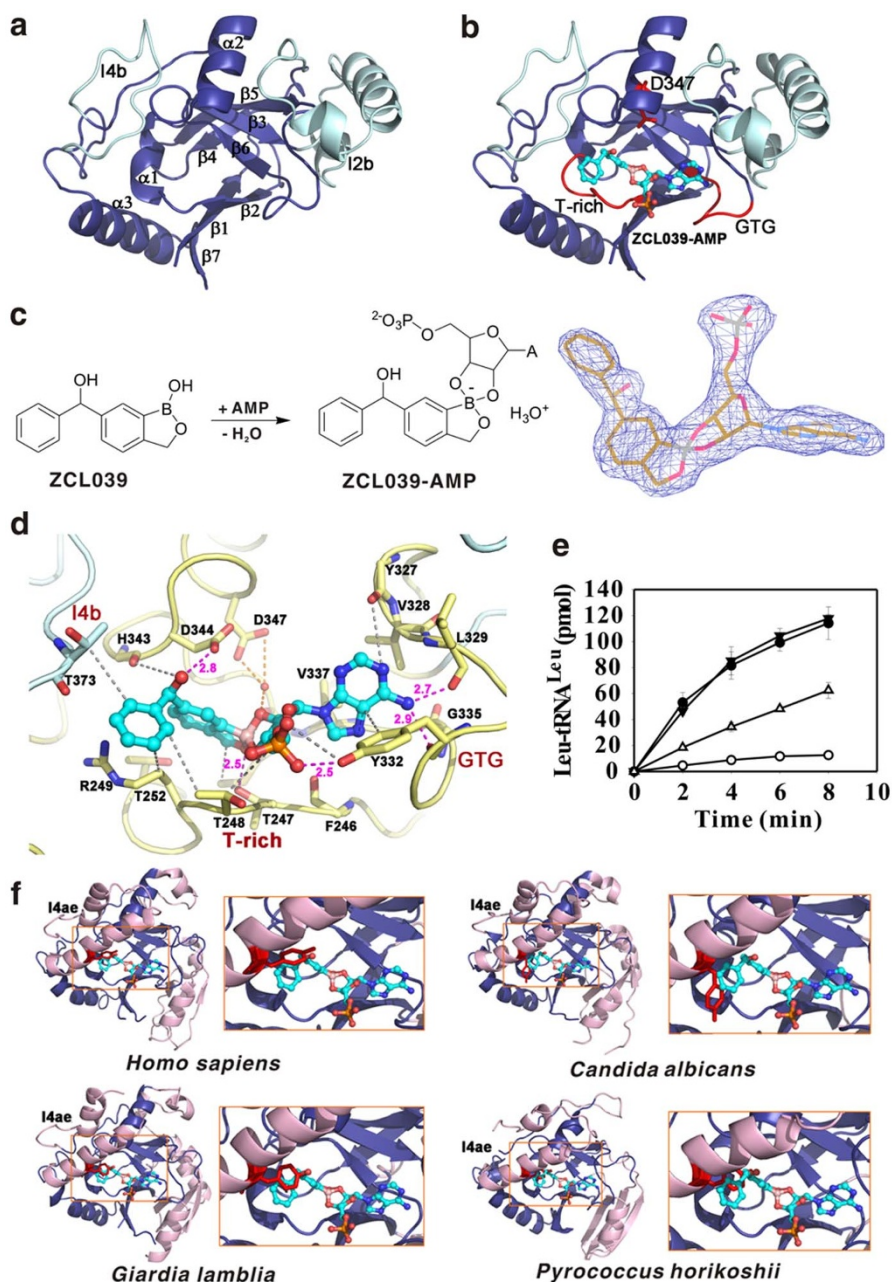


Figure 3 | Insight into the structure of ZCL039 bound with *SpLeuRS*-CP1 and the selectivity of ZCL039. (a) Ribbon diagram of the apo form of *SpLeuRS*-CP1: the catalytic core with secondary structure elements labeled (deep blue) and the I2b and I4b insertions (light cyan). (b) *SpLeuRS*-CP1 in complex with ZCL039-AMP (ball and stick): T-rich region, GTG loop, and the catalytic Asp347 residue are shown in red. (c) ZCL039 and AMP form the ZCL039-AMP adduct at the active site of *SpLeuRS*-CP1. The electron density (contoured at 1.5σ) is shown beside its schematic diagram (“A” represents the adenosine moiety). (d) Details of the ZCL039-AMP bound at the active site: the backbone of *SpLeuRS*-CP1 is shown in yellow, and all the residues within 4 Å from ZCL039-AMP are shown as sticks. The red ball located near Asp347 represents a water molecule. (e) Aminoacylation of the D344A mutant compared to the wild-type *SpLeuRS* in the presence (Δ and \circ , respectively) or absence (\blacktriangledown and \bullet , respectively) of 50 μM ZCL039. (f) Docking models of ZCL039-AMP with archaeal/eukaryal LeuRS-CP1s. ZCL039-AMP is docked with LeuRS-CP1s from *H. sapiens*, *C. albicans*, *G. lamblia*, and *P. horikoshii*. An enlarged view corresponding to each docking model is shown in the right box. The catalytic cores are shown in deep blue, the archaeal/eukaryal specific insertions are shown in pink, and the archaeal/eukaryal conserved Lys (Lys 468 in human cytosolic LeuRS) is shown in red stick. Data in (e) are means from three independent experiments; errors bars show s.d.

I4b insertion is not conserved in bacterial LeuRSs, conferring specificity of each LeuRS. Thus, it would be possible to design benzoxaborole compounds specific to LeuRSs from different species.

Previous studies have investigated several active *S. pneumoniae* aaRSs inhibitors. Phenyl-thiazolylurea-sulfonamides are described as a novel class of phenylalanyl-tRNA synthetase (PheRS) inhibitors that showed broad-spectrum antimicrobial activity towards both gram-positive and gram-negative bacteria and high levels of species

specificity³⁰. REP8839 is a potent selective methionyl-tRNA synthetase (MetRS) inhibitor³¹, which was found to suppress a variety of gram-positive microorganisms and is currently undergoing phase II clinical trials. These compounds act similarly by competitively binding to the synthetic active site of the enzyme with respect to amino acids. Therefore, their antibacterial activity can be reversed by nutrient supplementation. Furthermore, REP8839 is exclusively effective on MetRS1 and possessed no activity towards MetRS2. As



S. pneumoniae expresses both these forms, it will be less susceptible to REP8839³¹. The benzoxaboroles studied here represent another potent new class of *S. pneumoniae* LeuRS inhibitors that target the CP1 domain. The novel oxaborole tRNA trapping (OBORT) mechanism of benzoxaboroles would not arouse cross-resistance with the drugs that are currently used to treat *S. pneumoniae* infections. Furthermore, benzoxaboroles are characterized as slow-tight-binding inhibitors, and very slow recovery of the enzyme activity was detected¹⁸. Further analysis of the genomic sequence of several major pathogenic bacteria have suggested that LeuRS is exclusively encoded by an open reading frame (ORF) reported to date, supporting its potency as a potential drug target for treating bacterial infection.

In summary, we identified ZCL039 as a potent anti-pneumococcal agent targeting LeuRS. The determination of the structure of ZCL039 in complex with SpLeuRS-CP1 domain can aid in improving its pharmacological activity and species specificity for the treatment of pneumococcal infection. The LeuRS CP1 domain and benzoxaboroles can be further explored for efficient antibacterial therapeutics against LeuRS.

Methods

Chemicals, strains, and plasmids. Benzoxaboroles, including compound ZCL039 ([6-(1-phenyl-1-hydroxymethyl)-1,3-dihydro-1-hydroxy-2,1-benzoxaborole) were synthesized as previously described²⁸. Genomic DNA of *S. pneumoniae* was a gift from Professor Guo (Shanghai Jiao Tong University, School of Medicine). The *S. pneumoniae* strain was clinically isolated from children with community-acquired pneumonia hospitalized in Changhai Hospital (Shanghai, China) in 2002 and stored at the Department of Microbiology, Second Military Medical University. *Escherichia coli* strain BL21(DE3) was obtained from Invitrogen. The plasmids pET30a and pET30a-*ecLrs* were from Novagen and our laboratory, respectively.

Gene cloning, expression, and purification of SpLeuRS and SptRNA^{Leu}. The gene encoding SpLeuRS (NC_011900.1) was amplified from *S. pneumoniae* genomic DNA by polymerase chain reaction (PCR) using the following primers: forward primer, 5'-ATCGCCATGGCCATGAGTGTATTTATAATC-3' (*NcoI* site in italics), and reverse primer, 5'-ATCGCTCGAGTGGTATTTACAC-3' (*XhoI* site in italics). The PCR products were cleaved and ligated into the corresponding sites of the pET30a vector to yield the recombinant plasmid pET30a-*splrs* with six histidine-encoding sequences at the N-terminal. The genes encoding SpLeuRS mutants were constructed on the basis of the pET30a-*splrs* template using KOD-plus-Mutagenesis Kit (Toyobo). The identities of all the genes were confirmed by DNA sequencing (BioSune Bioscience, Shanghai, China). All enzymes were purified from *E. coli* strain BL21(DE3). The cells transformed with the expression plasmids were cultured in the presence of 200 μ M isopropyl β -D-1-thiogalactopyranoside (IPTG) at 22°C. The enzymes were purified to 95% homogeneity by one-step affinity chromatography using Ni²⁺-NTA superflow resin (Qiagen).

The gene encoding SptRNA^{Leu} (CAA isoacceptor in this study) was chemically synthesized using the sequence from the genomic tRNA database (<http://gtrnad-b.ucsc.edu>) and inserted between the *EcoRI* and *PstI* sites of the pTrc99B vector. The SptRNA^{Leu} was purified from *E. coli* strain MT102 that was transformed with the recombinant expression plasmids. The cells were cultured in the presence of 300 μ M IPTG at 37°C. Overexpressed tRNAs were confirmed and purified as previously described³². The tRNA concentrations were quantified by UV absorbance at 260 nm. The pTrc99B and MT102 were gifts from Dr. Gangloff of the Institut de Biologie Moléculaire et Cellulaire du CNRS, Strasbourg, France.

Preparation of mischarged [³H]Met-SptRNA^{Leu}. Previous studies showed that Ile, Met, and several non-standard amino acids could be misactivated by LeuRS *in vitro*^{33,34}, and further charged to tRNA^{Leu} by editing defective mutants such as *E. coli* LeuRS (*EcLeuRS*) D345A³⁵, yielding mischarged tRNA^{Leu}. Therefore, SptRNA^{Leu} was methionylated at 37°C in a 300- μ L volume containing 100 mM Tris-HCl (pH 7.8), 30 mM KCl, 12 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 4 mM ATP, 40 μ M [³H]Met, 20 μ M purified SptRNA^{Leu}, and 0.5 μ M *EcLeuRS*-D345A mutant for 30 min. The mixtures were isolated by acid phenol/chloroform extraction (pH 4.5) and ethanol precipitation followed by resuspension in 50 mM potassium phosphate buffer (pH 5.2).

ATP-PPI exchange, aminoacylation, and post-transfer editing assays. Leucine activation was performed at 37°C in a 50- μ L reaction mixture containing 100 mM HEPES (pH 7.5), 10 mM KF, 10 mM MgCl₂, 4 mM ATP, 5 mM leucine, 2 mM tetrasodium [³²P]PPI (15 cpm/pmol), and 10 nM SpLeuRS in the presence or absence of 200 μ M of ZCL039 dissolved in DMSO. The synthesized ³²P-labeled ATP was separated and counted as described previously²⁶. Aminoacylation reaction was initiated by 4 mM ATP at 37°C in a 50- μ L mixture containing 100 mM Tris-HCl (pH 7.5), 12 mM MgCl₂, 120 mM KCl, 0.5 mM DTT, 40 μ M [³H]leucine, 20 μ M purified SptRNA^{Leu}, various concentrations of ZCL039, and 5 nM SpLeuRS or its variants. To determine the inhibitory kinetic constants, reactions were performed with various

concentrations of ATP, Leu, or tRNA^{Leu} in the presence or absence of ZCL039 (2 or 8 μ M). Lineweaver-Burke plots were generated by linearizing substrate-velocity curve by fitting the data to a reciprocal equation using GraphPad Prism. Deacylation assay was carried out at 37°C in a 50- μ L reaction mixture containing 100 mM Tris-HCl (pH 7.5), 12 mM MgCl₂, 120 mM KCl, 0.5 mM DTT, 1 μ M [³H] Met-tRNA^{Leu}, 5 nM SpLeuRS or its mutants, and various concentrations of ZCL039. Before the start of the reaction, the mixtures containing enzyme and compound were pre-incubated for 20 min.

To determine the half-maximal inhibitory concentration (IC₅₀), at least six concentrations of ZCL039 were tested in aminoacylation with equal activity of LeuRSs from different species that corresponded to the formation of 10 pmol of Leu-tRNA^{Leu} in 5 min. For human cytosolic and mitochondrial LeuRSs, assays were performed at 30°C under the same reaction conditions used in our previous studies^{23,24}. Data were fitted to a dose-response curve using GraphPad Prism.

Antimicrobial susceptibility testing. Broth microdilution was performed according to the procedures standardized in the CLSI document M7-A7³⁶. Approximately 2 \times 10⁸ CFU/mL of *S. pneumoniae* and 5 \times 10⁷ CFU/mL of *E. coli* were added to the wells of a 96-well microtiter plate containing Mueller Hinton broth. For *S. pneumoniae*, the medium was supplemented with 2.5% lysed sheep blood. Serial dilutions of ZCL-039 were added to the wells and incubated at 37°C for 24 h. The MIC was determined as the lowest concentration that suppressed the visible growth of bacteria. To measure the MIC upon overexpression of *EcLeuRS*, IPTG (10 μ M) was inoculated with transformants containing pET30a-*ecLrs* plasmid, and pET30a vector was used as the control. Ampicillin was used to monitor bacterial susceptibility.

Cell proliferation assay. The toxicity of ZCL039 on mammalian cell proliferation was determined using a CellTiter 96[®] AQ_{ueous} Assay (Promega). HEK293T and A549 human lung carcinoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂. Then, 1 \times 10⁵ cells/mL was diluted and 50- μ L aliquots were dispensed into the wells of a 96-well plate. After allowing the cells to adhere overnight, ZCL039 was added (2.5–100 μ g/mL) to reach a final concentration of 0.5% DMSO. Fresh medium was added to make up a total volume of 200 μ L. Each concentration was assayed in five parallel wells. The plates were incubated at 37°C for 0, 24, 48, and 72 h, supplemented with a 40 μ L of mixture of MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]) and PMS (phenazine methosulfate) with a ratio of 20 : 1, and incubated for another 4 h. Absorbance was measured at 492 nm using a Thermo MK3 reader. DMSO was used as the negative control, and culture medium was used as the background. The IC₅₀ value was determined by nonlinear regression of the dose-response curve of the absorbance plotted against the logarithm of the ZCL039 concentration using GraphPad Prism software.

Cloning, expression, and purification of SpLeuRS CP1 domain. The DNA fragment encoding the CP1 domain of SpLeuRS (Thr228-Val410) was amplified from pET30a-*splrs* using primers: 5'-ACTGCATATGACTGGTGCCAAATGTAAC-3' and 5'-ACTGCTCG AGAACCTTCTCTGACCACAG-3'. The PCR products were cleaved and inserted between the *NdeI* and *XhoI* sites of pET22b (Novagen), yielding the recombinant construct including a C-terminal six histidine-encoding sequence. The *E. coli* BL21(DE3) cells transformed with the expression plasmids were induced with 200 μ M IPTG at 22°C. High amounts of soluble proteins were purified by affinity chromatography using the Ni²⁺-NTA superflow resin followed by gel filtration chromatography with Superdex 200 (GE Healthcare). The protein was designated SpLeuRS-CP1.

Crystallization, diffraction data collection, and processing. SpLeuRS-CP1 was concentrated to 20 mg/mL for crystallization. More than 300 crystallization conditions from Hampton Research were used for the initial screening; however, no crystals were obtained until a protein surface residue (Cys399) was substituted by serine (Ser). The C399S mutation did not affect the full-length enzyme activities and sensitivity to ZCL039 comparable to the wild-type SpLeuRS (Supplementary Fig. 8); therefore, it was used in the structure analysis of SpLeuRS-CP1. Crystals were obtained at 20°C in sitting drops containing 1 μ L of proteins and 1 μ L of reservoir solution (0.1 M Bis-Tris (pH5.5), 2 M ammonium sulfate) by vapor diffusion. Complexes of SpLeuRS-CP1 with ZCL039 and AMP were grown under the same conditions with a molecular ratio of 1 : 4 : 4. Crystals were directly mounted on a nylon loop (Hampton Research) and flash-cooled in liquid nitrogen. For the complex crystals of SpLeuRS-CP1 with ZCL039, higher concentrations of ammonium sulfate were used as a cryoprotectant. Diffraction data were collected at the Shanghai Synchrotron Radiation Facility 100 K beamline BL-17U1. Data were processed using the HKL2000 program package³⁷.

Structure determination. The structure of the apo form of SpLeuRS-CP1 was solved by molecular replacement implemented in the program MOLREP of the CCP4 suite^{38,39} using the structure of *Aquifex aeolicus* LeuRS CP1 (PDB entry: 3PZ5)⁴⁰ as the starting search model. Initial structure refinement was carried out using the program REFIN in PHENIX⁴¹ according to the standard protocol. Final structure refinement was performed using the program REFMAC⁴². Model building was manually carried out using the program COOT⁴³. The structure of SpLeuRS-CP1 bound to ZCL039 was then determined by molecular replacement (MOLREP) using the structure of the apo form of SpLeuRS-CP1 as the starting model. Model building and refinement were



carried out as described above. Structure refinement was performed using the program REFMAC5 and PHENIX^{41,42}. Throughout the refinement, 5% of randomly chosen reflections were set aside for free R-factor monitor. The quality of the final models was evaluated by PROCHECK⁴⁴. All the molecular graphics were generated using PyMOL (DeLano Scientific; <http://www.pymol.org>).

Docking model of ZCL039 to various LeuRSs. The docking models of ZCL039 bound to the active sites of the LeuRS CP1 domains from various organisms were all constructed based on superposition of the catalytic core of the CP1 domains. The LeuRS crystal structures of *Thermus thermophilus* (1H3N), *E. coli* (2AJG), *A. aeolicus* (3PZ0), *Candida albicans* (2WFE), *Homo sapiens* (2WFD), *Giardia lamblia* (3PZ6), and *Pyrococcus horikoshii* (1WKB) were used.

Accession codes. Protein Data Bank: the structures of the apo form and the complex form of SpLeuRS-CP1 have been deposited under accession code 4K48 and 4K47, respectively.

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Author contributions

Q.H.H., H.C.Z. and E.D.W. designed the experiments. Q.H.H., R.J.L., H.C.Z. and E.D.W. analyzed the data and wrote the manuscript. R.J.L. resolved the crystal structures. Z.P.F. assisted in the gene cloning, expression and purification of tRNA^{leu}. J.Z. and H.C.Z. designed, synthesized and purified the compounds. Y.Y.D. and W.P. performed the MIC determination. M.T. and M.W. assisted in *EcLeuRS-D345A* mutant and human mitochondrial LeuRS preparation, respectively. Q.H.H. performed all the other experiments.

Additional information

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