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Design, synthesis and biological evaluation of Leu_{10}-teixobactin analogues

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A B S T R A C T

Two thioamino acids and four fluorinated amino acids were employed to substitute either partially or entirely the Ile_2, Ser_3, Ile_6, and Ser_7 residues of Leu_{10}-teixobactin to prepare ten analogues and the bioactivity of them was investigated. The SAR studies revealed that Ile_6 was tolerable for both thioamidation and fluoridation, while Ser_7 was identified as the most tolerable site for thioamidation. Analogue **1a** demonstrated comparable or slightly improved antibacterial activity, superior protease stability compared to Leu_{10}-teixobactin, while not exhibiting obvious cytotoxicity against mammalian cells.
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Teixobactin, a novel antibiotic isolated from soil bacterlum *Eleftheria terrae* by Lewis and co-workers in 2015, is the hope to break the antimicrobial resistance standoff [1]. It exerts an unparalleled activity against Gram-positive bacteria, while without detectable resistance. Unlike other antibiotics, teixobactin binds to the conserved peptidoglycan-pyrophosphate motifs of lipid II, thus inhibiting peptidoglycan synthesis and disrupting the membrane integrity [2]. Teixobactin is a cyclodepsipeptide that contains a tedious synthesis amino acid, L-*allo*-enduracididine (L-*allo*-End_{10}) (Fig. 1) [3,4]. However, the structure-activity relationship (SAR) studies reveal that L-*allo*-End can be replaced by other amino acids without significantly altering the antibacterial activity [5,6]. For example, Singh group found that Leu_{10}-teixobactin (Fig. 1) and Ile_{10}-teixobactin displayed highly potent antibacterial activity that comparable to native teixobactin [7,8]. The ease of synthesis and the superior potency of Leu_{10}- and Ile_{10}-teixobactin offered solid structural foundations for the development of teixobactin analogues possessing desirable drug like properties. Based on these results, Nowick group designed and evaluated the *O*-acyl isopeptide prodrugs of Leu_{10}-teixobactin, resulting in enhanced solubility [9]. Aside from low solubility, another drawback that may hamper the clinical application of these peptides is that they are prone to be hydrolyzed by proteases. However, a comprehensive approach to modifying teixobactin analogues in order to mitigate their susceptibility to protease cleavage, while preserving or enhancing their antibacterial activity, has yet to be established.

A number of modification methods have been developed to improve the enzymatic stability of peptides [10-12]. Among them, *N*-methylation has been applied in modifying Leu_{10}-teixobactin, leading to backbone *N*-methylated analogues with significantly decreased or even abolished antibacterial activity, possibly because the conformation of Leu_{10}-teixobactin was altered by the lack of amide protons engaging in intramolecular H-bonds formation [13]. Unlike backbone *N*-methylation which often influences the H-bond pattern, backbone thioamidation only slightly alters the electron distribution of the peptide owing to a simple oxygen-sulfur exchange in peptide [14]. Thioamide serves as a *quasi*-isosteric substitute for amide due to the slightly larger size of sulfur compared to oxygen, and the slightly longer C=S bond in comparison to C=O bond [15]. The weak binding of thioamide to sol-

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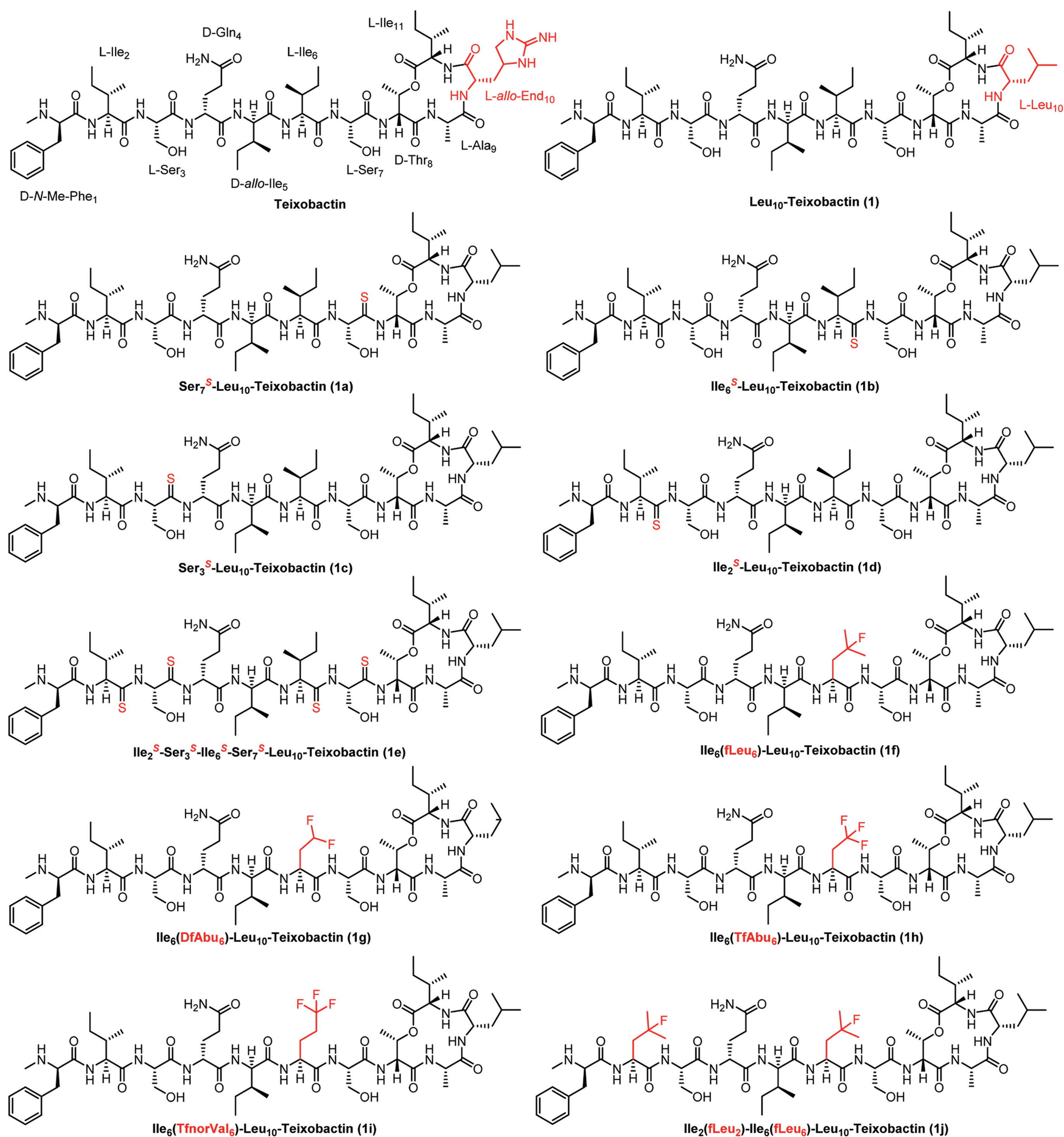
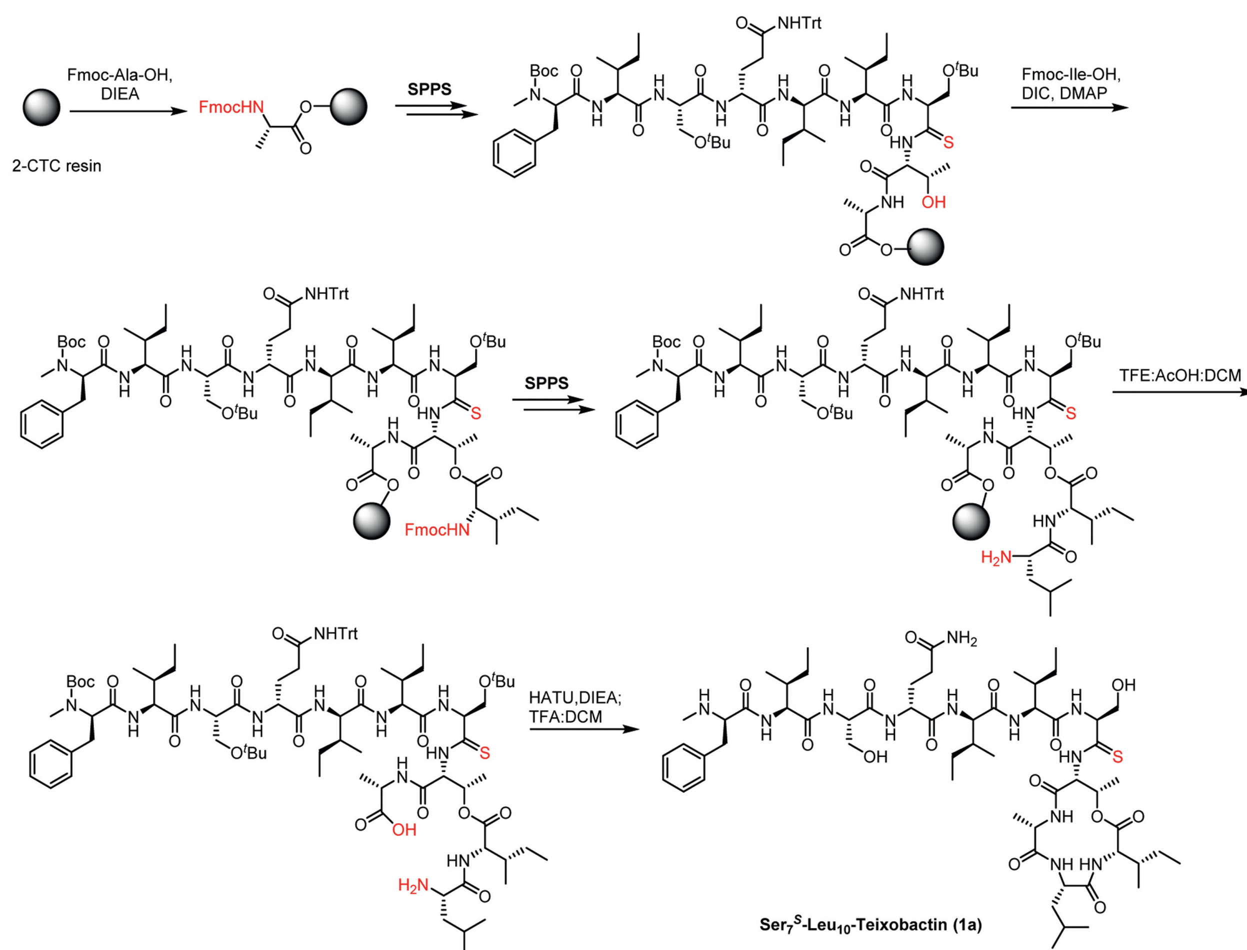


Fig. 1. The structure of teixobactin, Leu_{10}-teixobactin and analogues (**1a**-**1j**).

vent water molecules can be attributed to the fact that sulfur acts as a weak hydrogen bond acceptor. Consequently, the introduction of thioamide in the peptide backbone greatly enhances its lipophilicity [11]. In addition, backbone thioamidation can increase the metabolic stability with minimal perturbation of the peptide [16]. Besides backbone thioamidation, the incorporation of fluorine serves as an alternative approach to substantially modifying the biological functionality of peptides. Fluorine has comparable atomic size to hydrogen, allowing for the substitution of hydrogen with fluorine without causing significant structural alterations [17,18]. Additionally, the C-F bond, as the strongest single bond in organic chemistry, exhibits high polarization and reactivity resistance, thereby impeding enzymatic cleavage [19].

To explore whether thioamidation and fluorination would alter the bioactivity of teixobactin analogues, we sought to introduce the thioamide and fluorine-containing groups into Leu_{10}-teixobactin. Considering that the macrocyclic ring and D-amino acids are more tolerable towards enzymatic hydrolysis [20], we hypothesized that either partially or entirely substituting the four L-amino acids (Ile_2, Ser_3, Ile_6, and Ser_7) with thioamide- or fluorine-containing amino



Scheme 1. The synthetic route for **1a**.

acids could enhance enzymatic stability while preserving or augmenting the bioactivity of Leu$_{10}$-teixobactin. Therefore, we designed and synthesized ten analogues and evaluated their bioactivities in this work (Fig. 1).

Unlike commercially available Fmoc-protected fluorine-containing amino acids, to replace the amide bond of Leu$_{10}$-teixobactin by thioamide bond at Ile$_2$, Ser$_3$, Ile$_6$ or Ser$_7$ position, two thioamide precursors, Fmoc-Ile^S-NBt (NBt: benzotriazolide) and Fmoc-Ser^S(O^{t}Bu)-NBt, were prepared according to the previous report (Scheme S1 in supporting information) [21]. With the Fmoc-protected thiobenzotriazolides and fluorine-containing amino acids in hand, the total synthesis of these peptides was carried out. Cyclization is one of the key steps in the synthesis of cyclopeptides. Given that the steric hindrance of Ala$_9$ is smaller than that of Ile$_{11}$ bearing a branched side chain, the cyclization site was selected at Ala$_9$ and Leu$_{10}$ junction, resulting in a linear precursor with *C*-terminus Ala$_9$ and *N*-terminus Leu$_{10}$ residues [22]. In addition, the esterification between D-Thr$_8$ and Ile$_{11}$ is another key step for the synthesis of this kind of cyclodepsipeptides. To circumvent O→N acyl migration during removal of Fmoc protecting groups from D-Thr$_8$ [23], the esterification step should be carried out after the assembly of the main peptide chain. Three generic strategies for the synthesis of teixobactin and its analogues have been developed, including a convergent strategy [24], a stepwise Fmoc-SPPS and solution-phase cyclization [25,26], and a stepwise Fmoc-SPPS synthesis and "on-resin" cyclization [27]. In this research, a modified Fmoc-SPPS and solution-phase cyclization strategy was applied to synthesize the Leu$_{10}$-teixobactin analogues efficiently. The synthetic route is displayed as Scheme 1. The synthesis was commenced with the loading of Fmoc-Ala-OH onto 2-chlorotritylchloride (2-CTC) resin (initial substitution is 1.0 mmol/g). The residual active sites on the resin were capped by using MeOH/DIEA/DCM. Then, Fmoc deprotection was performed by using 20% piperidine in DMF and the free amine was detected by using the Kaiser Test. According to the standard Fmoc-SPPS procedure (SI), the peptide chain was elongated from Fmoc-D-Thr-OH to Boc-*N*-Me-D-Phe-OH. To be noted, Fmoc-D-Thr-OH without any protection at the side chain was directly used in the coupling reaction to avoid the additional deprotection procedure needed to release the free hydroxyl group before the esterification step [28]. Fortunately, *O*-acylation of D-Thr$_8$ was not observed in the subsequent rounds of SPPS. In addition, to reduce the epimerization of the thioamidated peptides during removal of the Fmoc protecting groups, shorter deprotection time was applied (5 min × 2) [21]. Additionally, immediate coupling of the next amino acid to the peptidyl resin following Fmoc group removal is necessary to prevent the instability of the thioamino acids. Next, esterification of the hydroxyl group was successfully accomplished on the solid support by using excess Fmoc-Ile-OH, DIC and DMAP [29]. After Fmoc deprotection, Fmoc-Leu-OH was coupled to the peptidyl resin. Remarkably, DKP (2,5-diketopiperazine) side product was not detected during removal of Fmoc groups from Leu$_{10}$, which formation is a frequently occurring side reaction in SPPS [30]. Then, the side chain protected linear peptide was cleaved off from the resin by using TFE/AcOH/DCM. After purification, the linear peptide was cyclized between *C*-terminus Ala$_9$ and *N*-terminus

Table 1
The MIC (μg/mL) of the Leu_{10}-teixobactin and **1a**-**1j**.

Compound	*S. aureus* ATCC 29213 (MSSA)	*S. aureus* ATCC 43300 (MRSA)	*E. faecalis* ATCC 700802 (VRE)	*B. subtilis* ATCC 6633
Leu_{10}-teixobactin	2^a	2^a	4^a	1^a
	1^b	0.5^b	2^b	0.25^b
1a	2^a	2^a	2^a	1^a
	0.5^b	0.5^b	1^b	0.25^b
1b	4^a	4^a	8^a	2^a
1c	64^a	64^a	$>128^a$	64^a
1d	16^a	16^a	64^a	8^a
1e	$>128^a$	$>128^a$	$>128^a$	8^a
1f	2^b	2^b	8^b	1^b
1g	2^b	1^b	4^b	0.5^b
1h	1^b	1^b	2^b	0.5^b
1i	2^b	2^b	8^b	1^b
1j	32^b	16^b	64^b	8^b
Levofloxacin	0.25^a	0.25^a	0.5^a	0.06^a

[a] Culture media without polysorbate 80.
[b] Culture media containing 0.002% polysorbate 80.

Leu_{10} with HATU/DIEA under high diluted condition (1 mg/mL, in DCM) [31]. Finally, global deprotection of the cyclodepsipeptide was performed by using TFA/DCM. The crude product was purified by semi-preparative RP-HPLC. According to this procedure, we synthesized Leu_{10}-teixobactin and ten analogues, the structures of which were confirmed by HRMS and ^1H NMR (supporting information).

After successful synthesis these peptides, the minimum inhibitory concentration (MIC) assay was conducted to assess the antibacterial activity of them. Levofloxacin and Leu_{10}-teixobactin were chosen as a positive control and a benchmark for activity, respectively. The MIC values are listed in Table 1. As observed, among the thioamidated analogues, **1a** exhibited the most potent antibacterial activity, which was comparable or slightly superior to that of Leu_{10}-teixobactin. In contrast, **1b** displayed slightly reduced antibacterial activity, while **1c**, **1d** and **1e** showed greatly decreased or even completely lost antibacterial activity. These data indicated that thioamide substitution at Ser_7 position was the most tolerable, followed by Ile_6, and then Ile_2 and Ser_3, suggesting that the impact of thioamidation on the antibacterial activity of Leu_{10}-teixobactin depends on the specific amino acid and the site being substituted. According to the previous SAR studies, Ser_7 of teixobactin mainly binds to the pyrophosphate group of lipid II through the backbone amide and side chain hydroxyl group, Ile_6 primarily serves as a membrane anchor with its hydrophobic side chain [32]. Furthermore, hydrogen bonds can be established between specific amino acid residues within the β-sheet interfaces of teixobactin analogues, such as Ser_3 and Ser_7, Ile_2 and Ile_5. In an aligned fibril structure, additional hydrogen bonds may form between Ser_7 and *N*-Me-D-Phe^{I}_1, as well as between Ser_3 and D-*allo*-Ile_5 [25]. Additionally, the presence of $C\alpha$-$C\alpha$ contacts has been found between residues Ile_2-Ser_7, Ile_2-Ile_6, and Ser_3-Ser_7 in the antiparallel β-strands of [R4L10]-teixobactins [33]. Therefore, we propose that the backbone thioamidation in **1a** may not only decrease the H-bonds formation between Ser_7 and water molecules, but also Ser_7 and Ser_3, Ser_7 and *N*-Me-D-Phe_1 to increase lipophilicity, which facilitates Ser_7 to bind with the pyrophosphate group of lipid II, resulting in good antibacterial activity. On the other hand, the small conformational change induced by thioamidation in **1b** interferes the binding of Ile_6 to membranes, leading to the slightly reduced antibacterial activity. As reported, the *N*-terminal residues are important for the formation of antiparallel β-sheet of teixobactin analogues that bound to the target [25]. In this regard, thioamidation of *N*-terminal residues, Ser_3 or Ile_2, possibly may not only result in the disruption of the dimer, but also in the impairment of the supramolecular assembly of **1c** and **1d**, thus causing the greatly decrease in their antibacterial activity. These findings indicated that O to S substitution in amide bond of *N*-terminal residues impacts the antibacterial activity of Leu_{10}-teixobactin analogues, while thioamide substitution at Ser_7 is found to be tolerable, providing **1a** with good antibacterial activity. In addition, similarly enhanced antibacterial activity of Leu_{10}-teixobactin and **1a** was observed in the presence of polysorbate 80, which could prevent peptide binding to plastic surfaces [1,34].

Since the synthesis of fluorine-containing serine amino acid is difficult, we focused on selective modification of Ile_6 and Ile_2, and synthesized five fluorine-containing analogues (**1f**-**1j**). The MIC assays of these analogues were carried out in the presence of polysorbate 80. It was found that **1f**-**1i** exhibited good activity against the tested pathogens. Among them, **1h** showed the best antibacterial activity, which was comparable or slightly inferior to that of Leu_{10}-teixobactin. Compared to **1h**, **1g** displayed retained or slightly decreased antibacterial activity, while **1f** and **1i** manifested 2–4 times decrease in activity. These data demonstrated that the subtle structural changes of Ile_6 with the replacement of fluorinated amino acid would not completely damage the activity. The difference of antibacteria activity between fluorinated analogues **1f**-**1i** could be closely related to the structural similarity of the fluorine-containing amino acids with Ile. These fluorine-containing residues are analogues of Leu-with hydrogen and/or methyl group at the side chain are replaced by fluorine. The size of the trifluoromethyl group approximates twice than that of a methyl group so the steric effects of the trifluoromethyl group is close to isopropyl group, or even larger *sec*–butyl and cyclohexyl groups [35]. The better antibacteria activity found for **1h** indicated that TfAbu is more structurally similar to Ile, compared to fLeu, DfAbu and TfnorVal incorporated in **1f**, **1g** and **1i**, respectively. **1j** with fluorine substitution at both Ile_2 and Ile_6 caused 8–16 times loss of the activities in comparison with **1f** with the same fluorine substitution at Ile_6. This observation may be attributed to the structural difference between the side chain of fLeu and Ile_2, which possibly influences the interaction with the membrane lipid [36]. These findings indicated that replacement of the Ile by fluorine-containing amino acids is tolerable at position 6, while less tolerable at position 2, which is consistent with the result of backbone thioamidation.

1a was then selected for further cytotoxicity and plasma stability investigation (supporting information) since it exhibited the most potent antibacterial activity among all the analogues. No obvious cytotoxicity (≥80% viability) was detected in HUVEC and HeLa cells treated with analogue **1a** for 48 h at concentrations up to 100 μmol/L (121.77 μg/mL), which was well above the MIC values (1–2 μg/mL, 61–122 times) (Fig. S1 in supporting information). As expected, **1a** had an extended half-life ($t_{1/2} > 72$ h) compared to Leu_{10}-teixobactin, indicating that the protease stability of **1a** was improved owing to the presence of the thioamide group at Ser_7 (Fig. 2). Since proteases exhibit a notable degree of accuracy and specificity in the hydrolysis of peptide bonds [37,38], the decreased degradation rate of **1a** could be attributed to the three-dimensional structure changes caused by oxygen-sulfur exchange at Ser_7 which may alter the recognition of **1a** by proteases within the plasma. These results demonstrated that the incorporation of a thioamide at Ser_7 into Leu_{10}-teixobactin is not only absence of cytotoxicity but sufficient to prevent enzymatic hydrolysis.

In conclusion, ten thioamidated and fluorinated Leu_{10}-teixobactin analogues were designed and synthesized based on the bioisosterism principles. The conducted SAR investigations revealed the significance of the *N*-terminal Ser_3 and Ile_2 residues in Leu_{10}-teixobactin for its antibacterial efficacy, as substitutions at these positions resulted in a substantial decrease in bioactivity. Furthermore, the Ile_6 residue exhibited tolerance towards both backbone thioamidation and fluoridation. Notably, the Ser_7 residue

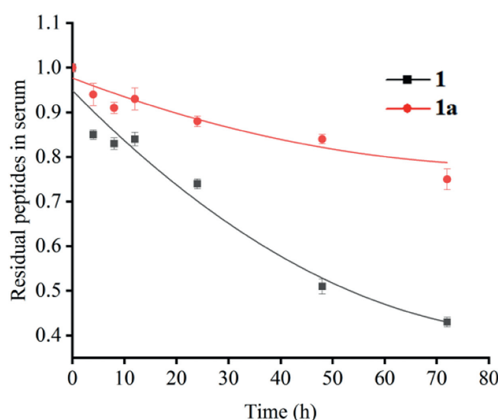


Fig. 2. The stability of Leu_{10}-teixobactin (**1**) and **1a** in plasma.

displayed the highest tolerance for thioamidation. In contrast to prior knowledge, our findings suggest that Ser_7 and Ile_6 of Leu_{10}-teixobactin can undergo additional modifications using structurally similar groups to furnish more potent analogues. The newly developed compound (**1a**) exhibited favorable antibacterial activity, enhanced enzymatic stability, and absence of cytotoxicity, thus has great potential for further exploration in antibacterial drug discovery.

Ethical statement

The experiment of plasma stability was performed under the guideline of Institutional Ethical Committee of Animal Experimentation of Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Yanye Fan: Writing – original draft, Methodology, Investigation, Data curation. **Jingjing Chen:** Investigation. **Bichun Chen:** Investigation. **Jinyu Bai:** Investigation. **Bowen Yang:** Investigation. **Feng Liang:** Project administration, Conceptualization. **Lijing Fang:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2024.110075.

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