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Application of *tert*-Butyl Disulfide-Protected Amino Acids for the Fmoc Solid-Phase Synthesis of Lactam Cyclic Peptides under Mild Metal-Free Conditions

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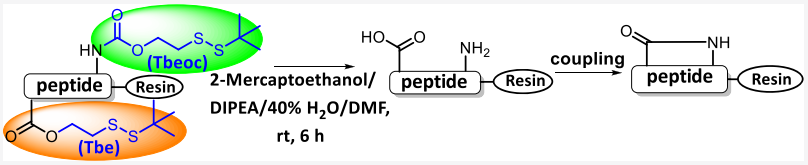

ABSTRACT: Lactam cyclic peptides are a class of interesting and pharmaceutically active molecules, but their previous syntheses have required the use of heavy metals and/or forcing conditions. Here, we describe the efficient application of the previously reported *tert*-butyl disulfide-protected amino acids and their use in the efficient, solid-phase synthesis of a series of lactam cyclic peptides under mild, metal-free conditions.

INTRODUCTION

Lactam cyclic peptides incorporating an amide bond between the α or side-chain carboxyl group of Asp/Glu and the side-chain amino group of Lys are a class of interesting and pharmaceutically active molecules.[1] For example pasireotide,[2] which incorporates cyclized backbone chain amide, and bremelanotide,[3] incorporating side chain amide cyclization, have been developed to treat acromegaly and sexual dysfunction (Scheme 1a). Such cyclic peptides can be prepared relatively easily using Fmoc solid-phase synthesis (SPPS), although their synthesis in the solution phase is accompanied by the formation of oligomers.[4]

The successful execution of an Fmoc-SPPS strategy for the synthesis of lactam cyclic peptides depends on the orthogonal protection of Lys, Glu, or Asp, such as with allyloxycarbonyl/allyl or *p*-nitrobenzyl-oxycarbonyl/*p*-nitrobenzyl groups.[5] However, such protecting groups compel the use of heavy metal reagents (Pd and Sn) for their deprotection, which may result in contamination of the product cyclic peptide with heavy metals.[6] To obviate this problem, metal-free synthetic strategies such as those using vinyl ether benzyloxycarbonyl, 4-methyltrityl, and azidomethyl carbamate protected amino acids have recently been reported.[7] Further development of more metal-free orthogonal types of protected amino acids can expand the flexibility of the toolbox, providing the possibility to prepare cyclic peptides incorporating multiple lactam bridges.

Here, we report a new strategy for Fmoc-SPPS of lactam cyclic peptides under mild, metal-free conditions using *tert*-butyl disulfide-protected amino acids. This strategy stems from our previous discovery (in protein ligation studies) that the 2-(*tert*-butyldisulfanyl)ethoxycarbonyl (Tbeoc) group can be used to protect secondary amine of L-thiazolidine-4-carboxylic acid and then can be easily removed in aqueous solution of reducing agent tris(2-carboxyethyl)phosphine (TCEP).[8] Thus, we envisioned the use of *tert*-butyl disulfide to protect the carboxyl groups of Asp and Glu or the amino groups of Lys, Orn, and Dap as part of a new strategy for the synthesis of lactam cyclic peptides under mild and metal-free conditions. However, the TCEP/H_2O conditions we used to remove the Tbeoc group are not compatible with Fmoc-SPPS, precluding its use for the synthesis of amide cyclic peptides. Therefore, we screened a series of deprotecting conditions and found that a mixed solvent containing 2-mercaptoethanol/DIPEA [40% H_2O/DMF (v/v)] can remove the *tert*-butyl disulfide group mildly and quickly. Using this new type of amino acid, we have efficiently prepared a series of lactam cyclic peptides, indicating the high efficiency and easy operation of the new strategy.

RESULTS AND DISCUSSION

Our synthesis of the *tert*-butyl disulfide group-protected Fmoc-amino acid building blocks is depicted in Table 1, and it is based on the reaction of commercially available Fmoc-protected amino acids with (*tert*-butyl disulfide)chloroethyl

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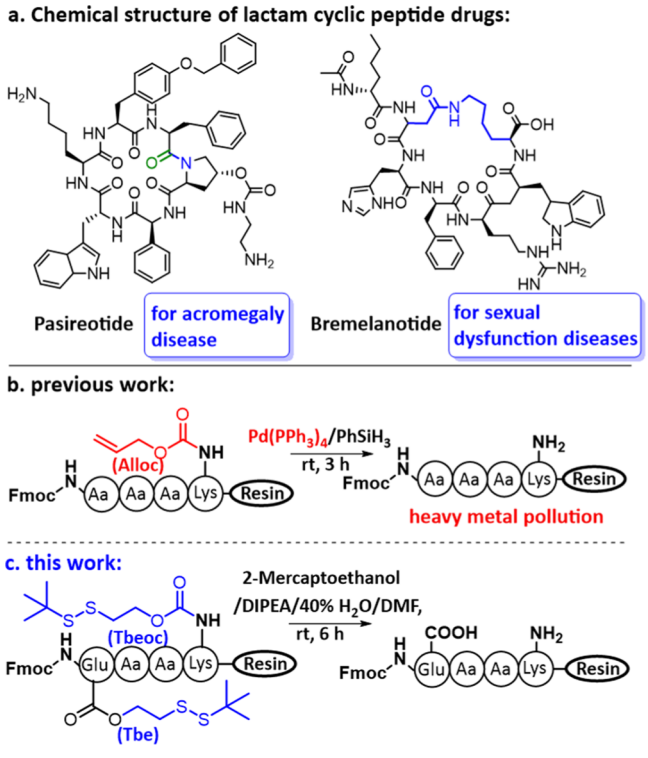
Scheme 1. Selective Removal of Side Chain Protecting Groups on the Resin

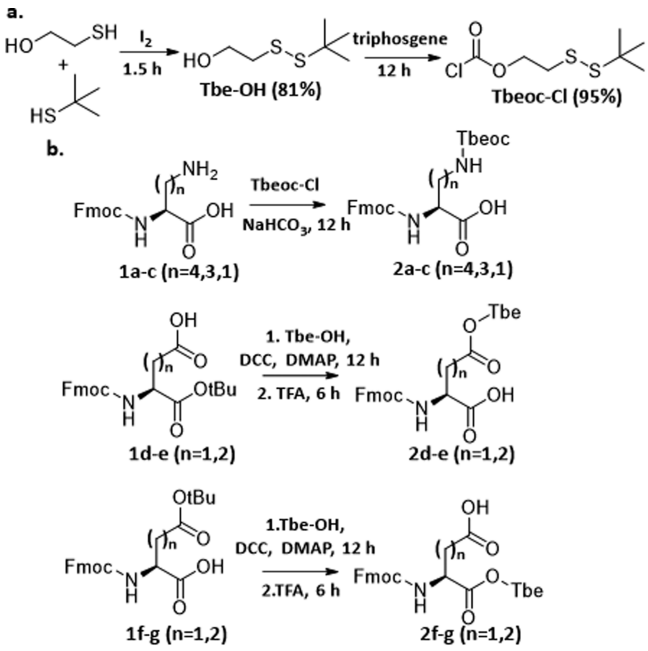


carbonate (Tbeoc-Cl). This was prepared by reacting phosgene with Tbe-OH, obtained by mixing 2-mercaptoethanol (1 equiv) with 2-methyl-2-propanethiol (10 equiv) in the presence of iodine. Coupling of Tbeoc-Cl with Fmoc-Lys-OH in aqueous solution of 1,4 dioxane gave **2a** in a high yield of 81%. Using the same strategy, we obtained the Tbeoc-protected unnatural amino acids Fmoc-Orn(Tbeoc)-OH **2b** and Fmoc-Dap(Tbeoc)-OH **2c** (yield 62 and 63%), which could be used to synthesize enkephalin analogs[9] and urotensin-II.[10]

In the case of Fmoc-Aa(Otbu)-OH and Fmoc-Aa-O*t*Bu, 2-(*tert*-butyldisulfanyl)ethanol (Tbe) was chosen to protect the α or side-chain carboxyl group of the Fmoc amino acid. After Tbe-OH was coupled to the side-chain carboxyl group or α-carboxy group of Fmoc-amino acids, the protecting group can be removed using 95% trifluoroacetic acid (TFA), to efficiently give requisite Fmoc-Aa(OTbe)-OH or Fmoc-Aa-OTbe. Similarly, Tbe-OH can also be coupled with the side-chain carboxyl group of unnatural amino acid Fmoc-3-carboxy-Phe-OAllyl, and then, $Pd(PPh_3)_4$ was used to remove the allyl protecting group to obtain Fmoc-3-carboxy-Phe(OTbe)-OH (yield 46%) for the modification of special amino acids (Table 1b).

We next tried to remove the *tert*-butyl disulfide protecting group simultaneously in SPPS under user-friendly conditions. We first tested the previously reported solution of TCEP in PBS buffer to remove the Tbeoc group of lysine on the model peptide but found that the efficiency of the reaction was low, even after a 12 h reaction time (Table 2a, entry 1). Next, we tried to use 2-mercaptoethanol/DIPEA solution to remove the Tbeoc group. However, intermediate 2-mercaptoethyl carbamate produced during the removal process could not be cleaved. Knowing that water can promote the decomposition of carbamate bonds by adjacent nucleophilic sulfhydryl groups,

Table 1. Synthesis of the *tert*-Butyl Disulfide-Protected Amino Acid Library[a]



no	abbreviation	total isolated yield[%]
2a	Fmoc-Lys(Tbeoc)-OH	81
2b	Fmoc-Orn(Tbeoc)-OH	62
2c	Fmoc-Dap(Tbeoc)-OH	63
2d	Fmoc-Asp(OTbe)-OH	77
2e	Fmoc-Glu(OTbe)-OH	72
2f	Fmoc-Asp-OTbe	69
2g	Fmoc-Glu-OTbe	77
2h	Fmoc-3-carboxy Phe(OTbe)-OH	46

In particular, the total isolated yield of 2d-2g is a two-step yield.

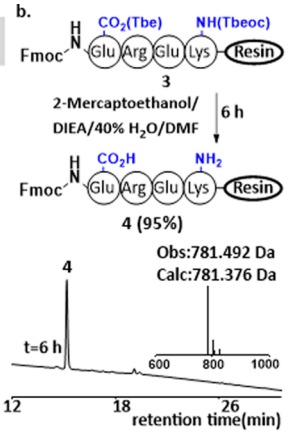
[a]In particular, the total isolated yield of **2d**−**2g** is a two-step yield.

Table 2. Optimization of Deprotection Conditions of the *tert*-Butyl Disulfide Group in SPPS

a.

Entry	solvent	Time(h)	Conv %
1	PBS Buffer[b]	12	45
2	DMF	2	50
3	DMF	6	55
4	10% H_2O/DMF	2	56
5	10% H_2O/DMF	6	64
6	20% H_2O/DMF	2	67
7	20% H_2O/DMF	6	86
8	30% H_2O/DMF	2	84
9	30% H_2O/DMF	6	94
10	40% H_2O/DMF	2	85
11	40% H_2O/DMF	6	98

b.



[a]Reaction condition: 2-mercaptoethanol (6 mmol), DIPEA (9 mmol). [b]PBS buffer [TCEP (20 mmol), pH 7.0].

we screened a mixed-solvent system containing different ratios of water and DMF.[11] As shown in Table 2, the efficiency of the removal of the Tbeoc group improved with the increasing concentration of water. Finally, using an optimized solvent [2-mercaptoethanol/DIPEA dissolved in 40% H_2O/DMF (v/v)], we were able to simultaneously remove the Tbeoc and Tbe

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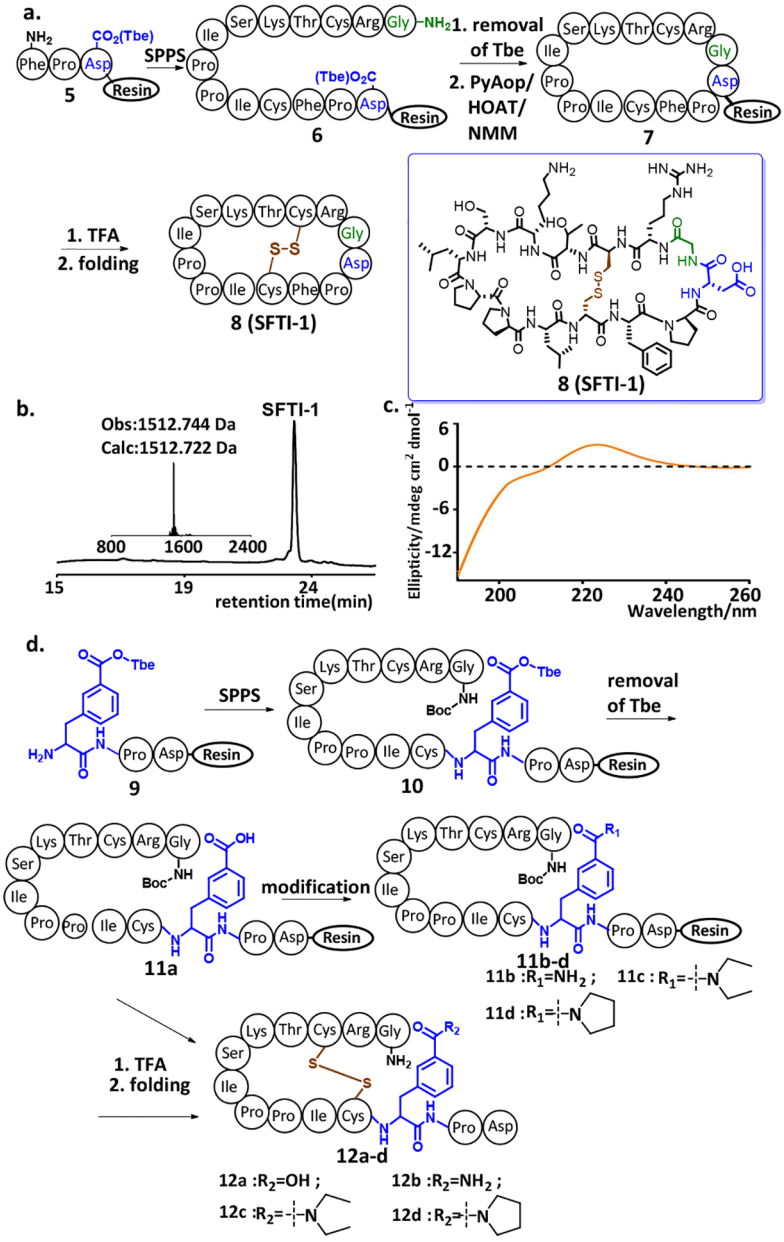

Figure 1. (a) Solid-phase synthetic route for the preparation of cyclization of linear peptides using **2f**. (b) HPLC traces and ESI-MS analysis of purified STFI-1. (c) CD spectrum of SFTI-1 in water. (d) Solid-phase synthetic route for the preparation of site-specific modified SFTI derivatives using **2h**.

groups of peptide segment **3** at room temperature with 95% efficiency (Table 2b). It is worth mentioning that the use of a pair of protective groups (Tbeoc/Tbe) that can be removed simultaneously to prepare lactam cyclic peptides can simplify the deprotection steps and improve the synthesis efficiency. In addition, we also found that this mixed solvent can efficiently remove the Tbeoc groups on the side chains of ornithine and diaminopropionic acid on the model peptide (Scheme S8).

Having successfully prepared a library of *tert*-butyl disulfide-protected amino acids, we next sought to exemplify their utility by the synthesis of a natural product. Sunflower trypsin inhibitor (SFTI), one of the smallest disulfide-bridged cyclic peptides to have been discovered and the most potent known Bowman−Birk protease inhibitor, was selected as the first target, along with its derivatives.[12] SFTI-1 has been previously synthesized twice—once by cyclization of linear SFTI-1 in solution and once through intramolecular native chemical ligation.[12a] However, both methods also resulted in oligomer formation, diminishing the yield of the product. Our synthesis of SFTI-1 is depicted in Figure 1a. Fmoc-Asp-OTbe, Pro, and Phe were sequentially coupled to the 2-chlorotrityl chloride (2-CTC) resin to give **5**. The remaining 11 amino acids were assembled to **5** by using standard Fmoc-SPPS to obtain linear peptide **6**. After removal of the Tbe group by 2-mercaptoethanol/DIPEA/40% H_2O/DMF, the side-chain carboxyl group of Asp was efficiently cyclized with the N-terminal amino group of Gly using (3-hydroxy-3*H*-1,2,3-triazolo[4,5-*b*]pyridinato-O)tri-1-pyrrolidinylphosphonium hexafluorophosphate (PyAOP)/3-hydroxytriazolo[4,5-*b*]-pyridine(HOAT)/*N*-methylmorpholine (NMM) to give **7**.

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The head-to-tail cyclization peptide was cleaved from the resin, purified by HPLC, and refolded under neutral conditions (20:1 GSSG/GSH, pH 7.5) to form final bicyclic peptide **8** (Figure 1a). HPLC and electrospray ionization mass spectrometry (ESI-MS) analysis were used to assess the purity of synthetic SFTI-1 (Figure 1b). The circular dichroism (CD) spectra of SFTI-1 were consistent with those previously reported and suggesting that SFTI-1 adopts a well-defined β-sheet structure (Figure 1c).[12b] Subsequently, Fmoc-Asp(OTbe)-OH was used to synthesize SFTI-2, a head-to-side chain cyclization derivative of SFTI-1, through a similar procedure (Scheme S10).

In addition to wild-type SFTI-1, SFTI derivatives bearing different modifications are also of interest—for example, for the screening of protease inhibitors with better activity.[12c] Accordingly, we further used our Tbe-protected amino acids to obtain various chemically modified SFTI derivatives. Specifically, Asp, Pro, and **2h** were sequentially coupled to the 2-CTC resin to obtain **9**. The remaining 11 amino acids were assembled by SPPS to obtain linear peptide **10**. After removing the Tbe-protecting group as described above, peptide **11a** was obtained. We then used ammonium hydroxide, diethylamine, and pyrrolidine to modify the exposed carboxyl group of **11a** to obtain derivatives **11b−c**. Finally, the resulting peptide was cleaved from the resin and purified by HPLC, which was refolded under neutral conditions (20/1 GSSG/GSH, pH 7.5) to give product **12a−d** (Figure 1d). HPLC and ESI-MS analysis demonstrated the purity and correct molecular weight of peptide **12a−d** (total yield 8, 6.4, 6.2, 7.8%, respectively, Scheme S11).

Tyrocidine A, a homodetic head-to-tail cyclic decapeptide antibiotic effective against a variety of bacterial species such as Gram-positive bacteria and a broad range of filamentous fungi, was also selected for total synthesis.[13] Thus, we used Fmoc-Glu-OTbe to gradually couple on the Rink amide AM resin to give linear tyrocidine A. After removing the Tbe group, cyclization was performed to obtain tyrocidine A **13** (yield 16%) (Figure 2a). After determining the purity and correct molecular weight of the product (Figure 2b), we tested its antibacterial activity. *Bacillus subtilis* and *Staphylococcus aureus* were cultured to 10^8 CFU at 37 °C using Luria-Bertani (LB) medium. The minimum inhibitory concentration (MIC) of tyrocidine A necessary to inhibit *B. subtilis* and *S. aureus* was determined to be 4 and 8 μg/mL respectively, using the two-fold dilution method—results consistent with those of a previous study (Figure 2d).[13] These results indicated that cyclic peptides synthesized using Tbe-protected amino acids have a similar biological activity to natural cyclic peptides. Subsequently, we characterize the structure of our synthetic tyrocidine A by CD spectroscopy and found it to incorporate a well-defined β-sheet structure—again, consistent with results previously reported (Figure 2c).[13b]

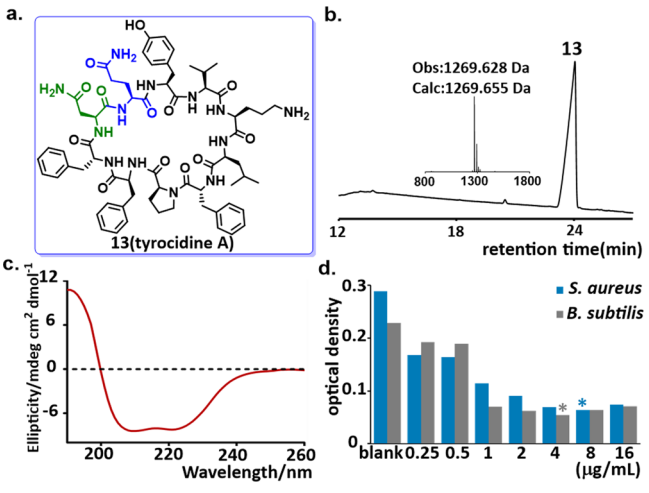

Figure 2. (a) Structure of tyrocidine A. (b) HPLC traces and ESI-MS analysis of purified tyrocidine A. (c) CD spectrum of tyrocidine A in water. (d) MIC of tyrocidine A against *B. subtilis* and *S. aureus*.

Having successfully prepared head-to-tail and head-to-side chain lactam cyclic peptides, we next used Tbeoc- and Tbe-protected amino acids to prepare side chain-to-side chain cyclization peptides. Such cyclic peptides have been known to adopt a stable active conformation, which improves their affinity with their target protein, metabolic stability, and membrane penetration.[14] For example, the introduction of a lactam bridge to side chain amide-cyclized lactoferricin-like synthetic fragment HLBD1 (amino acids 16−40), part of the antibacterial β-helix region of human lactoferrin, enhances its antifungal effect.[15] The sequential coupling of Arg, Lys, and Ile to the Rink amide AM resin gave **14**. Subsequently, the linear peptide containing orthogonal building blocks **2a** and **2e** was continuously coupled to resin **14** to obtain **15**. After the removal of the Tbeoc- and Tbe-protecting groups, cyclization was performed and the resulting peptide was cleaved from the resin and purified by HPLC to give the lactam bridge peptide, which was refolded under neutral conditions (pH 7.5) to give final product **16** (Figure 3a). HPLC analysis of the purified products gave rise to a single peak, and ESI-MS analysis was consistent with the molecular weight of **16** (Figure 3b). The CD spectrum of **16** is similar to the previous report (Figure 3c).[15]

CONCLUSIONS

In summary, applications of a series of *tert*-butyl disulfide-protected amino acids have been demonstrated as key building blocks for the SPPS of Fmoc/*t*Bu-orthogonal-protected lactam cyclic peptides under mild, metal-free conditions. The utility of these new building blocks was exemplified in the efficient preparation of a series of lactam cyclic peptides, including native and site-specific modified SFTI derivatives, tyrocidine A, and a lactam-bridged human lactoferrin derivative. Accordingly, these new building blocks are proposed as the basis of a general method for the efficient preparation of lactam cyclic peptides under mild and metal-free conditions.

EXPERIMENTAL SECTION

General Information. *Materials and Reagents.* The Rink amide AM resin and 2-CTC resin were bought from Tianjin Nankai HECHENG (TianJin, China). 5-Chloro-1-[bis(dimethylamino)-methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HCTU), HOAT, PyAop, and all of the Fmoc-protected amino acids were bought from CS Bio, GL Biochem (Shanghai, China). DMF, DCM, DIPEA, TFA, and diethyl ether were purchased from Sinopharm Chemical Reagent. Thioanisole, dithioglycol, and TFA (HPLC grade) were purchased from energy-chemical (Shanghai, China). Fmoc-Lys-OH (**1a**), Fmoc-Orn-OH (**1b**), Fmoc-Dap(Boc)-OH (**1c**), Fmoc-Asp-O*t*Bu (**1d**), Fmoc-Glu-O*t*Bu (**1e**) Fmoc-Asp(O*t*Bu)-OH (**1f**), Fmoc-Glu(O*t*Bu)-OH (**1g**), and Fmoc-3-acrboxy-Phe(O*t*Bu)-OH were purchased from Bide Pharmatech Ltd. Analytical TLC was performed on silica gel 60 F254 (250 layer thickness). Flash column chromatography was carried out using

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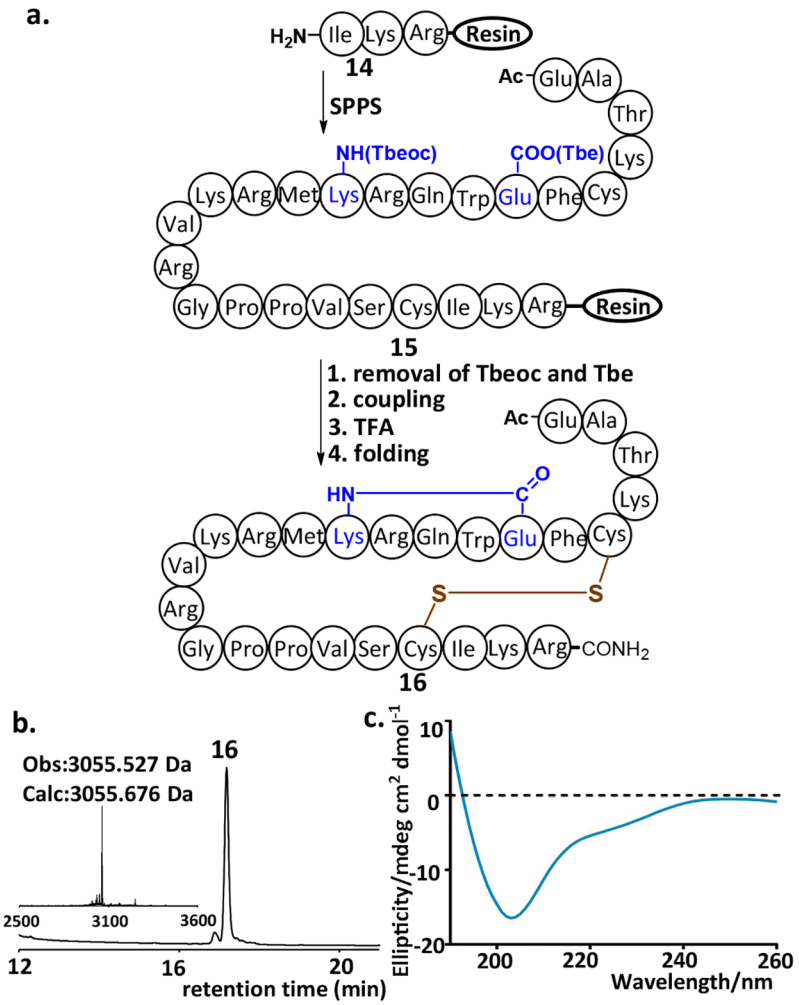

Figure 3. (a) Solid-phase synthetic route for side chain-to-side chain cyclization peptides using **2a** and **2e**. (b) HPLC traces and ESI-MS analysis of the human lactoferrin derivative containing a lactam bridge. (c) CD spectrum of the human lactoferrin derivative in water.

silica gel (300−400 mesh). Visualization was accomplished by using potassium permanganate and UV light. Analytical and semipreparative HPLC was performed using a Welch column (analytical column Welch "Ultimate XB-C18", 250 × 4.6 mm, 5 μm particle size, semipreparative column Welch "Ultimate XB-C18", 250 × 10 mm, 10 μm particle size).

High-Performance Liquid Chromatography. Analytical and semipreparative HPLC was run on a SHIMADZU (LC-20AT) instrument using acetonitrile (0.08% TFA in acetonitrile) and H_2O (0.1% TFA in H_2O). The analytical injections were monitored at 214 and 254 nm.

Mass Spectrometry and NMR. LC-MS-2020 (SHIMADZU) was obtained using Agilent 6210 ChemStation. ^1H and ^{13}C NMR spectra were recorded on a Bruker (400 MHz or 101 MHz, respectively) spectrometer using deuteriochloroform ($CDCl_3$) as the solvent ($CDCl_3$: 7.26 ppm, as internal reference) unless otherwise stated.

CD Spectroscopy. The targeted peptide was dissolved in H_2O and added to a quartz cell (path length of 1 mm). The CD spectra were measured from 260 to 190 nm on a Pistar π-180 spectrometer at 25 °C.

Tbe-OH.[8] 2-Mercaptoethanol (16 mmol, 1.12 mL) and 2-methyl-2-propanethiol (144.5 mmol, 16.3 mL) were dissolved in 95% ethanol solution. The solution of iodine (30 mmol, 7.62 g) was added in 95% EtOH (40 mL) until the color changed from colorless to brown. After 1.5 h, the solution was concentrated in vacuo. Then, saturated aqueous Na_2CO_3 was added until pH > 7. The residue was extracted with ethyl acetate and the organic layer was washed with 10% Na_2SO_3 and brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo. The product was purified by flash column chromatography (10:1, petroleum ether/EtOAc) to afford the desired product with a yield of 81% (2.14 g, 12.9 mmol). ^1H NMR (400 MHz, $CDCl_3$): δ = 3.88 (q, J = 5.4 Hz, 2H), 2.86 (t, J = 5.9 Hz, 2H), 2.33 (s, 1H), 1.35 (s, 9H).

Tbeoc-Cl.[8] The product of Tbe-OH (0.31 g, 1.87 mmol) was added to dry THF (3.3 mL) containing solid triphosgene (0.554 g, 1.87 mmol), stirred at room temperature for 10 h, and concentrated in vacuo to obtain acid chloride with a yield of 95% (0.41 g, 1.79 mmol).

General Protocol for the Synthesis of Fmoc-Aa(Tbeoc)-OH. Either **1a**, **1b**, or **1c** (4.07 mmol) and $NaHCO_3$ (1.9 g, 22.62 mmol) were dissolved in 204 mL of 1,4-dioxane/H_2O (1:1, v/v) at 0 °C. The solution of 1,4-dioxane containing Tbeoc-Cl (1.55 g, 6.78 mmol) was added to the reaction system and stirred for 1 h, and then, the reaction mixture was gradually heated to room temperature and stirred overnight. The mixture was then evaporated, and the aqueous phase was acidified to pH = 2 with 4 M HCl. The aqueous suspension was extracted by EtOAc, dried over Na_2SO_4, filtrated, and evaporated. The residue was purified by flash column chromatography (petroleum ether/EtOAc 6:1, with 1% acetic acid) to afford desired product **2a**−**c**.

Fmoc-Lys(Tbeoc)-OH (2a). The crude product was purified by flash column chromatography (petroleum ether/EtOAc 6:1, with 1% acetic acid) to give **2a** as a white powder in 81% yield (1.84g). ^1H NMR (400 MHz, chloroform-d): δ 7.72 (d, J = 7.6 Hz, 2H), 7.55 (t, J

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= 7.2 Hz, 2H), 7.35 (t, J = 7.6 Hz, 2H), 7.27−7.22 (m, 2H), 5.88 (d, J = 8.1 Hz, 1H), 5.03 (t, J = 6.1 Hz, 1H), 4.41−4.09 (m, 6H), 3.10 (q, J = 6.9 Hz, 2H), 2.86 (dt, J = 30.4, 6.6 Hz, 2H), 1.77 (dd, J = 58.8, 9.2 Hz, 2H), 1.40 (dt, J = 37.8, 6.4 Hz, 4H), 1.30 (d, J = 4.3 Hz, 9H). $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 156.6 (2C), 143.9, 143.7, 141.3 (2C), 127.7 (2C), 127.1 (2C), 125.1 (2C), 120.0 (2C), 67.1, 63.1, 54.7, 47.9, 47.1, 40.4, 39.1, 31.6, 29.9 (3C), 29.2, 22.3. HRMS (ESI-TOF) m/z: $[M + Na]^+$ calcd for $C_{28}H_{36}N_2O_6S_2Na$, 583.1912; found, 583.1914. IR (cm^{-1}, neat): 3324, 2923, 1700, 1521, 1450, 1363, 1338, 1245, 1049, 757, 740, 620. $[\alpha]D^{20}$ (c = 1.00, $CHCl_3$): +8.56.

Fmoc-Orn(Tbeoc)-OH (2b). The crude product was purified by flash column chromatography (petroleum ether/EtOAc 6:1, with 1% acetic acid) to give **2b** as a white powder in 62% yield (1.36g). ^1H NMR (400 MHz, chloroform-d): δ 7.69 (d, J = 7.4 Hz, 2H), 7.52 (t, J = 7.0 Hz, 2H), 7.33 (t, J = 7.0 Hz, 2H), 7.27−7.20 (m, 2H), 5.98 (s, 1H), 5.21 (s, 1H), 4.36−4.06 (m, 6H), 3.10 (s, 2H), 2.89−2.74 (m, 2H), 1.69−1.42 (m, 4H), 1.29 (s, 9H). $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 156.6 (2C), 143.7 (2C), 141.2 (2C), 127.7 (2C), 127.1 (2C), 125.2 (2C), 120.0 (2C), 67.1, 63.1, 54.3, 47.9, 47.1, 40.3, 39.1, 29.8 (3C), 29.3, 26.0. HRMS (ESI-TOF) m/z: $[M + Na]^+$ calcd for $C_{27}H_{34}N_2O_6S_2Na$, 569.1756; found, 569.1761. IR (cm^{-1}, neat): 3334, 2925, 1697, 1525, 1450, 1413, 1361, 1342, 1251, 1051, 759, 738, 620. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +7.10.

Fmoc-Dap(Tbeoc)-OH (2c). The crude product was purified by flash column chromatography (petroleum ether/EtOAc 6:1, with 1% acetic acid) to give **2c** as a white powder in 63% yield (1.33g). ^1H NMR (400 MHz, chloroform-d): δ 7.71 (d, J = 7.0 Hz, 2H), 7.55 (s, 2H), 7.35 (t, J = 7.0 Hz, 2H), 7.26 (t, J = 4.3 Hz, 2H), 6.30 (s, 1H), 5.62 (s, 1H), 4.53−4.12 (m, 6H), 3.61 (s, 2H), 2.79 (s, 2H), 1.27 (s, 9H). $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 156.7 (2C), 143.9, 143.7, 141.3 (2C), 127.7 (2C), 127.1 (2C), 125.2 (2C), 120.0 (2C), 67.4, 63.7, 55.4, 48.0, 47.0, 42.6, 38.8, 29.8 (3C). HRMS (ESI-TOF) m/z: $[M + Na]^+$ calcd for $C_{25}H_{30}N_2O_6S_2Na$, 541.1443; found, 541.1451. IR (cm^{-1}, neat): 3336, 2960, 1697, 1521, 1448, 1413, 1361, 1334, 1247, 1162, 1053, 758, 739, 621. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +13.4.

General Protocol for the Synthesis of Fmoc-Aa(OTbe)-OH. **1d** (197.3 mg, 0.48 mmol) or **1e** (204.0 mg, 0.48 mmol), N,N'-dicyclohexylcarbodiimide (DCC) (125.6 mg, 0.61 mmol), and DMAP (7.5 mg, 0.061 mmol) were dissolved in 15 mL of dry DCM at 0 °C. Tbe-OH (202.2 mg, 1.218 mmol) was added to the reaction system and stirred overnight. After the reaction solution was filtered and evaporated, the crude product was purified by chromatography (petroleum ether/EtOAc 10:1) to obtain compound **1d-1** (230.7 mg, 0.41 mmol, 86%) or **1e-1** (225.5 mg, 0.39 mmol, 82%). To a solution of compound **1d-1** (230.7 mg, 0.41 mmol) or **1e-1** (233.8, 0.41 mmol) in DCM (3 mL) was added TFA (5 mL). After vigorously stirring overnight at 6 h, the reaction solution was evaporated to dryness. After the reaction is completed, residual TFA in the reaction system is removed. The crude product was purified by chromatography (petroleum ether/EtOAc 6:1, with 1% acetic acid) to afford compound **2d−e**.

Fmoc-Asp(OTbe)-O*t*Bu (1d-1). The crude product was purified by flash column chromatography (petroleum ether/EtOAc 10:1) to give **1d-1** as a white powder in 86% yield (230.7 mg). ^1H NMR (400 MHz, chloroform-d): δ 7.77 (d, J = 7.5 Hz, 2H), 7.63−7.59 (m, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.1 Hz, 2H), 5.81 (d, J = 8.6 Hz, 1H), 4.61 (dt, J = 8.7, 4.4 Hz, 1H), 4.47−4.31 (m, 4H), 4.25 (t, J = 7.2 Hz, 1H), 3.02−2.74 (m, 4H), 1.46 (s, 9H), 1.33 (s, 9H). $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 170.6, 169.5, 156.0, 143.9, 143.8, 141.3 (2C), 127.7 (2C), 127.1 (2C), 125.2 (2C), 120.0 (2C), 82.7, 67.3, 63.1, 50.9, 48.1, 47.1, 38.2, 36.9, 29.9 (3C), 27.9 (3C). IR (cm^{-1}, neat): 2970, 1727, 1508, 1450, 1367, 1220, 1151, 1079, 1045, 846, 758, 739. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +16.7.

Fmoc-Glu(OTbe)-O*t*Bu (1e-1). The crude product was purified by flash column chromatography (petroleum ether/EtOAc 10:1) to give **1e-1** as a white powder in 82% yield (225.7 mg). ^1H NMR (400 MHz, chloroform-d): δ 7.76 (d, J = 7.5 Hz, 2H), 7.64−7.58 (m, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.32 (t, J = 7.4 Hz, 2H), 5.51 (d, J = 8.1 Hz, 1H), 4.45−4.33 (m, 5H), 4.22 (t, J = 7.0 Hz, 1H), 2.91 (t, J = 6.7 Hz, 2H), 2.41−2.27 (m, 2H), 2.23−2.16 (m, 1H), 2.03−1.96 (m, 1H), 1.45 (s, 9H), 1.33 (s, 9H).$^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 170.7, 170.0, 156.0, 143.9, 143.7, 141.3 (2C), 127.7 (2C), 127.1 (2C), 125.2, 125.2, 120.0 (2C), 81.9, 67.3, 63.9, 60.7, 50.6, 48.0, 47.1, 38.1, 37.8, 29.8 (3C), 28.1 (3C). IR (cm^{-1}, neat): 3349, 2965, 1725, 1525, 1450, 1363, 1252, 1222, 1152, 1105, 1049, 847, 758, 740, 620. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +8.3.

Fmoc-Asp(OTbe)-OH (2d). The crude product was purified by flash column chromatography (petroleum ether/EtOAc 6:1, with 1% acetic acid) to give **2d** as a white powder in 90% yield (186.0 mg). ^1H NMR (400 MHz, chloroform-d): δ 7.73 (d, J = 7.5 Hz, 2H), 7.56 (s, 2H), 7.36 (t, J = 7.4 Hz, 2H), 7.28 (d, J = 7.3 Hz, 2H), 6.08 (s, 1H), 4.74−4.15 (m, 6H), 3.14−2.77 (m, 4H), 1.29 (s, 9H). $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 171.1 (2C), 156.3, 143.8, 143.7, 141.3 (2C), 127.7 (2C), 127.1 (2C), 125.2 (2C), 120.0 (2C), 67.4, 63.4, 48.0, 47.0, 38.2 (2C), 29.8 (3C). HRMS (ESI-TOF) m/z: $[M + Na]^+$ calcd for $C_{25}H_{29}NO_6S_2Na$, 526.1334; found, 526.1347. IR (cm^{-1}, neat): 3401, 2960, 1756, 1737, 1714, 1536, 1515, 1450, 1409, 1384, 1353, 1257, 1189, 1091, 1039, 975, 860, 756, 737, 621. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +24.73.

Fmoc-Glu(OTbe)-OH (2e). The crude product was purified by flash column chromatography (petroleum ether/EtOAc 6:1, with 1% acetic acid) to give **2e** as a white powder in 88% yield (177.4 mg). ^1H NMR (400 MHz, chloroform-d): δ 7.71 (d, J = 7.5 Hz, 2H), 7.55 (s, 2H), 7.35 (t, J = 7.1 Hz, 2H), 7.30−7.25 (m, 2H), 5.83 (s, 1H), 4.57−4.06 (m, 6H), 2.84 (t, J = 5.9 Hz, 2H), 2.53−1.92 (m, 4H), 1.29 (s, 9H). $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 174.4, 173.1, 156.6, 143.9, 143.7, 141.3 (2C), 127.7 (2C), 127.1 (2C), 125.2 (2C), 120.0 (2C), 67.2, 63.1, 53.6, 48.0, 47.1, 38.3, 30.3, 29.8 (3C), 27.2. HRMS (ESI-TOF) m/z: $[M + Na]^+$ calcd for $C_{26}H_{31}NO_6S_2Na$, 540.1490; found, 540.1486. IR (cm^{-1}, neat): 3340, 2960, 1716, 1523, 1450, 1162, 1054, 760, 739, 621. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +14.30.

General Protocol for the Synthesis of Fmoc-Aa-OTbe. **1f** (0.48 mmol) or **1g** (0.48 mmol), DCC (125.6 mg, 0.61 mmol), and DMAP (7.5 mg, 0.061 mmol) were dissolved in 15 mL of dry DCM at 0 °C. Tbe-OH (202.2 mg, 1.218 mmol) was added to the reaction system and stirred overnight. After the reaction solution was filtered and evaporated, the crude product was purified by chromatography (petroleum ether/EtOAc 10:1) to obtain compound **1f-1** or **1g-1**. To a solution of compound **1f-1** (203 mg, 0.36 mmol) or **1g-1** (241.5 g, 0.42 mmol) in DCM (3 mL) was added TFA (5 mL). After vigorously stirring overnight at 6 h, the reaction solution was evaporated to dryness. After the reaction is completed, residual TFA in the reaction system is removed. The crude product was purified by chromatography (petroleum ether/EtOAc 6:1, with 1% Acetic acid) to afford compound **2f−g**.

Fmoc-Asp(Otbu)-OTbe (1f-1). The crude product was purified by flash column chromatography (petroleum ether/EtOAc 10:1) to give **1f-1** as a white powder in 76% yield (203.0 mg). ^1H NMR (400 MHz, chloroform-d): δ 7.77 (d, J = 7.5 Hz, 2H), 7.63−7.59 (m, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.1 Hz, 2H), 5.81 (d, J = 8.6 Hz, 1H), 4.61 (dt, J = 8.7, 4.4 Hz, 1H), 4.47−4.31 (m, 4H), 4.25 (t, J = 7.2 Hz, 1H), 3.02−2.74 (m, 5H), 1.46 (s, 9H), 1.33 (s, 9H). $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 170.7, 170.0, 156.0, 143.9, 143.7, 141.3 (2C), 127.7 (2C), 127.1 (2C), 125.2, 125.2, 120.0 (2C), 81.9, 67.3, 63.9, 50.6, 48.0, 47.1, 38.1, 37.8, 29.8 (3C), 28.1 (3C). IR (cm^{-1}, neat): 3340, 2960, 1712, 1508, 1450, 1336, 1154, 1105, 1052, 758, 739, 621. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +9.90.

Fmoc-Glu(Otbu)-OTbe (1g-1). The crude product was purified by flash column chromatography (petroleum ether/EtOAc 10:1) to give **1g-1** as a white powder in 88% yield (241.5 mg). ^1H NMR (400 MHz, chloroform-d): δ 7.76 (d, J = 7.5 Hz, 2H), 7.64−7.58 (m, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.32 (t, J = 7.4 Hz, 2H), 5.51 (d, J = 8.1 Hz, 1H), 4.45−4.33 (m, 4H), 4.22 (t, J = 7.0 Hz, 1H), 2.91 (t, J = 6.7 Hz, 2H), 2.41−2.27 (m, 2H), 2.23−2.16 (m, 1H), 2.03−1.96 (m, 1H), 1.45 (s, 9H), 1.33 (s, 9H). $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 172.1, 171.8, 156.0, 143.9, 143.7, 141.3, 141.3, 127.7 (2C), 127.1 (2C), 125.2 (2C), 125.1 (2C), 120.0 (2C), 80.9, 67.1, 63.7, 53.6, 48.1, 47.2, 38.7, 31.5, 29.9 (3C), 28.1 (3C), 27.5. IR (cm^{-1}, neat): 3359, 2965, 1724, 1525, 1452, 1363, 1149, 1047, 846, 758, 739, 621. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +3.00.

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Fmoc-Asp-OTbe (2f). The crude product was purified by flash column chromatography (petroleum ether/EtOAc 6:1, with 1% Acetic acid) to give **2f** as a white powder in 91% yield (164.7 mg). ^{1}H NMR (400 MHz, chloroform-*d*): δ 7.76 (d, J = 7.5 Hz, 2H), 7.60 (d, J = 7.4 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.4 Hz, 2H), 5.83 (d, J = 8.4 Hz, 1H), 4.71−4.64 (m, 1H), 4.46−4.30 (m, 4H), 4.24 (t, J = 7.0 Hz, 1H), 3.17−2.86 (m, 4H), 1.32 (s, 9H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$): δ 175.7, 170.5, 156.1, 143.8, 143.7, 141.3 (2C), 127.8 (2C), 127.1 (2C), 125.2 (2C), 120.0 (2C), 67.4, 64.1, 50.3, 48.1, 47.1, 38.0, 36.5, 29.8 (3C). HRMS (ESI-TOF) m/z: $[M + Na]^+$ calcd for $C_{25}H_{29}NO_6S_2Na$, 526.1334; found, 526.1332. IR (cm^{-1}, neat): 3340, 2958, 1712, 1513, 1450, 1334, 1189, 1162, 1105, 1054, 757, 739, 621. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +10.23.

Fmoc-Glu-OTbe (2g). The crude product was purified by flash column chromatography (petroleum ether/EtOAc 6:1, with 1% acetic acid) to give **2g** as a white powder in 88% yield (191.1 mg). ^{1}H NMR (400 MHz, chloroform-*d*): δ 7.76 (d, J = 7.5 Hz, 2H), 7.59 (d, J = 7.1 Hz, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.4 Hz, 2H), 5.48 (d, J = 8.2 Hz, 1H), 4.54−4.29 (m, 5H), 4.21 (t, J = 6.8 Hz, 1H), 2.90 (t, J = 6.6 Hz, 2H), 2.45 (q, J = 7.2, 6.7 Hz, 2H), 2.24 (dd, J = 14.9, 6.9 Hz, 1H), 1.98 (dd, J = 14.2, 6.8 Hz, 1H), 1.33 (s, 9H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$): δ 177.9, 171.7, 156.1, 143.7 (2C), 141.3 (2C), 127.8 (2C), 127.1 (2C), 125.1 (2C), 120.0 (2C), 67.2, 63.8, 53.3, 48.1, 47.2, 38.1, 31.0, 29.9 (3C), 27.42. HRMS (ESI-TOF) m/z: $[M + Na]^+$ calcd for $C_{26}H_{31}NO_6S_2Na$, 540.1490; found, 540.1486. IR (cm^{-1}, neat): 3340, 2958, 1712, 1513, 1450, 1334, 1164, 1105, 1053, 758, 739, 621. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +3.43.

Synthesis of Fmoc-3-carboxy-Phe(O*t*Bu)-OAllyl. Sodium bicarbonate (46.0 mg, 0.54 mmol) and allyl bromide (0.048 mL, 0.55 mmol) were dissolved in DMF (10 mL). Fmoc-3-carboxy-Phe(O*t*Bu)-OH (200 mg, 0.46 mmol) was added to the reaction system and stirred overnight. The mixture was extracted with EtOAc. The combined organic phase was washed with water (three times) and brine (three times) and dried over Na_2SO_4. The crude product was purified by chromatography (petroleum ether/EtOAc 4:1) to afford the desired product (219.6 mg, 0.416 mmol, 90%).

Fmoc-3-carboxy-Phe(O*t*Bu)-OAllyl. White powder. Yield 90% (219.6 mg).^{1}H NMR (400 MHz, chloroform-*d*): δ 7.88 (d, J = 7.6 Hz, 1H), 7.80−7.74 (m, 3H), 7.56 (t, J = 7.6 Hz, 2H), 7.39 (t, J = 7.5 Hz, 2H), 7.31 (m, 4H), 5.88 (m, 1H), 5.37−5.23 (m, 3H), 4.71 (dt, J = 8.0, 5.9 Hz, 1H), 4.63 (d, J = 5.9 Hz, 2H), 4.47−4.29 (m, 2H), 4.21 (t, J = 7.2 Hz, 1H), 3.19 (qd, J = 13.9, 5.9 Hz, 2H), 1.57 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 171.1, 165.5, 155.6, 143.8, 143.7, 141.3 (2C), 135.9, 133.4, 132.4, 131.3, 130.3, 128.5, 128.3, 127.7 (2C), 127.1 (2C), 125.1, 125.1, 120.0, 120.0, 119.4, 81.1, 67.1, 66.3, 54.8, 47.1, 38.1, 28.2 (3C). IR (cm^{-1}, neat): 3345, 2977, 1709, 1522, 1448, 1367, 1292, 1249, 1159, 1145, 1083, 1049, 989, 933, 849, 756, 739, 621. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +25.78.

Synthesis of 1h. Fmoc-3-carboxy-Phe(O*t*Bu)-OAllyl was dissolved in DCM/TFA (1:1, v/v). The reaction solution was stirred for 12 h at room temperature, and the solvent was evaporated under vacuum, affording **1h** without further purification.

Fmoc-3-acrboxy-Phe(OTbe)-O*t*Bu (1h). White powder. ^{1}H NMR (400 MHz, chloroform-*d*): δ 8.02−7.90 (m, 2H), 7.74 (d, J = 7.5 Hz, 2H), 7.56 (t, J = 7.2 Hz, 2H), 7.42−7.26 (m, 6H), 5.88 (m, 1H), 5.48 (d, J = 8.1 Hz, 1H), 5.29 (m, 2H), 4.74 (q, J = 6.6 Hz, 1H), 4.68−4.54 (m, 2H), 4.45 (dd, J = 10.9, 7.0 Hz, 1H), 4.33 (dd, J = 10.6, 7.2 Hz, 1H), 4.18 (t, J = 7.1 Hz, 1H), 3.21 (qd, J = 13.9, 5.8 Hz, 2H). ^{13}C{^{1}H} (101 MHz, $CDCl_3$): δ 171.5, 171.1, 155.7, 143.8, 143.7, 141.3 (2C), 136.4, 134.8, 131.2, 131.1, 129.7, 129.1, 128.8, 127.7 (2C), 127.1 (2C), 125.1, 125.1, 120.0 (2C), 120.0, 67.2, 66.5, 54.8, 47.1, 38.1. IR (cm^{-1}, neat): 3322, 2919, 1741, 1520, 1450, 1164, 1087, 1045, 754, 735, 620. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +17.78.

Synthesis of Fmoc-3-carboxy-Phe(OTbe)-OH. Compound **1h** (381.5 mg, 0.81 mmol), DCC (215.6 mg, 1.05 mmol), and DMAP (12.8 mg, 0.1045 mmol) were dissolved in 15 mL of DCM at 0 °C. Tbe-OH (202.2 mg, 1.218 mmol) was added to the reaction system and stirred overnight. After the reaction mixture was filtrated and evaporated, the crude product was purified by chromatography (petroleum ether/EtOAc 6:1) to afford the compound, which was dissolved in THF (10 mL) solution. We added $Pd(PPh_3)_4$ (163.5 mg, 0.141 mmol) and N-methylaniline (155.3 μL, 1.42 mmol) to the abovementioned system. After vigorously stirring for 2 h, the reaction solution is directly concentrated in vacuo. The crude product was purified by chromatography (petroleum ether/EtOAc 5:1, with 1% acetic acid) to obtain compound **2h** (215.7 mg, 0.373 mmol, 46%).

Fmoc-3-acrboxy-Phe(OTbe)-OH (2h). White powder. Yield 46% (215.7 mg). ^{1}H NMR (400 MHz, chloroform-*d*): δ 7.94−7.84 (m, 2H), 7.68 (d, J = 7.5 Hz, 2H), 7.46−7.38 (m, 2H), 7.31 (dd, J = 14.3, 6.5 Hz, 4H), 7.23−7.14 (m, 2H), 5.69 (s, 1H), 4.57 (s, 1H), 4.43 (t, J = 6.4 Hz, 2H), 4.34−4.27 (m, 1H), 4.18−4.11 (m, 1H), 4.09−4.03 (m, 1H), 3.26 (d, J = 12.0 Hz, 1H), 3.08 (d, J = 7.3 Hz, 1H), 1.30 (s, 9H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$): δ 176.3, 166.0, 156.2, 143.7, 143.7, 141.2 (2C), 137.3, 134.2, 130.7, 130.1, 128.6, 128.2, 127.6 (2C), 127.0 (2C), 125.1, 125.1, 119.9 (2C), 67.1, 63.4, 55.7, 48.0, 46.9, 38.5, 37.6, 29.8 (3C). HRMS (ESI-TOF) m/z: $[M + Na]^+$ calcd for $C_{31}H_{33}NO_6S_2Na$, 602.1647; found, 602.1656. IR (cm^{-1}, neat): 3372, 2971, 1714, 1519, 1450, 1277, 1193, 1083, 1045, 758, 739, 620. $[\alpha]_D^{20}$ (c = 1.00, $CHCl_3$): +35.97.

Optimization of Deprotection Conditions of the *tert*-Butyl Disulfide Group in SPPS. *General Protocol for the Synthesis of the Model Peptide.* AM Rink amide resin (156 mg, 0.05 mmol, 0.32 mmol g^{-1}) was weighed and placed in a synthesis container. The resin was swelled in mixed solvent of DCM and DMF (5 and 5 mL) for 30 min and then drained. The Fmoc group of the resin was removed by using piperidine (20% in DMF). Fmoc-Gly-OH (5.0 equiv, 0.25 mmol) was preactivated with HATU (5.0 equiv), HOAT (5.0 equiv), and DIPEA (10.0 equiv) in DMF for 0.5 min. Then, the mixture was added to the resin for coupling for 1 h. Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg-(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys-(Boc)-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Lys(Boc)-OH were assembled to the resin-bound peptide to obtain the model peptide by the standard Fmoc-based SPPS protocol.

Optimization of Deprotection Conditions of the Tbeoc Group of Lys. Fmoc-Lys(Tbeoc)-OH is coupled to the model peptide. Using different reaction solvents on the solid phase, the conditions for the highest deprotection efficiency of the Tbeoc-protecting group of the lysine side-chain amino group were screened. The reaction mixture was analyzed by HPLC (gradient 10−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 15.8 min.

Deprotection of the Tbeoc Group of Orn and Dap. Fmoc-Orn(Tbeoc)-OH and Fmoc-Dap(Tbeoc)-OH are coupled to the model peptide. The mixed-solvent deprotection condition [2-mercaptoethanol/DIPEA dissolved in 40% H_2O/DMF (v/v)] can efficiently remove the Tbeoc groups on the side chains of ornithine and diaminopropionic acid on the model peptide. Model peptides containing Orn were analyzed by HPLC (gradient 10−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 15.0 min. Model peptides containing Dap were analyzed by HPLC (gradient 10−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), tR = 15.8 min.

Deprotection of Tbeoc and Tbe Groups. AM Rink amide resin (156 mg, 0.05 mmol, 0.32 mmol g^{-1}) was weighed and placed in a synthesis container. The resin was swelled in mixed solvent of DCM and DMF (5 and 5 mL) for 30 min and then drained. Fmoc-Lys(Tbeoc)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Glu(OTbe)-OH were assembled to the amino group of the resin to obtain **3** by the standard Fmoc-based SPPS protocol. The mixed-solvent deprotection condition [2-mercaptoethanol/DIPEA dissolved in 40% H_2O/DMF (v/v)] can efficiently remove the Tbeoc/Tbe groups on the model peptide to obtain **4**. The crude reaction mixtures were analyzed by HPLC (gradient 10−80% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 15.7 min.

Solid-phase peptide synthesis of Lactam Cyclic Peptide. *General Procedure for the Synthesis of SFTI-1 and SFTI-2.* 2-Cl resin (441 mg, 0.15 mmol, 0.34 mmol g^{-1}) was weighed and placed in a

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synthesis container. The resin was swelled in mixed solvent of DCM and DMF (3 and 3 mL) for 30 min and then drained. Fmoc-Asp-OTbe (0.6 mmol, 4 equiv) was dissolved in DMF solution containing NMM (1.5 mmol, 132 μL) and reacted with the resin overnight. Four milliliters of 5% MeOH/DMF (vol/vol) was added to the resin in order to cover the unreacted sites on the Cl resin and reacted for 10 min. Fmoc-Pro-OH and Fmoc-Phe-OH were sequentially coupled to the resin-bound peptide to obtain **pre SFTI-1 I** or **pre SFTI-2 I** by the standard protocol. Fmoc-Cys(Trt)-OH, Fmoc-Ile-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Ile-OH, Fmoc-Ser-OH, Fmoc-Lys(Boc)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Gly-OH were coupled to the resin-bound peptide to obtain **pre SFTI-1 II** or **pre SFTI-2 II** by the standard Fmoc-SPPS protocol. The Tbe-protecting group was removed by treating with solution of 2-mercaptoethanol (5 mmol)/DIPEA (2.6 mmol) in a mixed solvent system [40% H_2O/DMF (v/v)] for 6 h. The side-chain carboxyl group of Asp was efficiently cyclized with the N-terminal amino group of Gly using PyAOP(5.0 equiv)/HOAT(5.0 equiv)/NMM(10.0 equiv) to give **pre SFTI-1 III** or **pre SFTI-2 III**. Then, the resin was drained with DMF (three times), DCM (three times), DMF (three times), and DCM (three times).

Cleavage from the Resin and Purification. The TFA cleavage cocktails (TFA/phenol/thioanisole/EDT/H_2O = 85:5:5:2.5:2.5) were added to the dried peptide-containing resin for 3 h at 37 °C. TFA was removed by blowing N_2 over the mixture in a fume hood. After the peptides were precipitated with cold Et_2O, they were collected by centrifugation and repeated twice. The peptide was purified by semipreparative HPLC and acquired as a white powder to obtain **pre SFTI-1 IV** or **pre SFTI-2 IV**. **pre SFTI-1 IV** was analyzed by HPLC (gradient 10−70% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 25.5 min(major). **pre SFTI-2 IV** was analyzed by HPLC (gradient 10−70% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 25.4 min. **pre SFTI-1 IV** or **pre SFTI-2 IV** was refolded under neutral conditions (20:1 GSSG/GSH, pH 7.5). The solution was concentrated using a freeze dryer, and final folded **SFTI-1** or **SFTI-2** was lyophilized and purified by semipreparative HPLC. **SFTI-1** was analyzed by HPLC (gradient 1−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 22.5 min. **SFTI-2** was analyzed by HPLC (gradient 1−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 20.5 min.

General Procedure for the Synthesis of Site-Specific Modified SFTI. 2-Cl resin (441 mg, 0.15 mmol, 0.34 mmol g^{-1}) was weighed and placed in a synthesis container. The resin was swelled in mixed solvent of DCM and DMF (3 and 3 mL) for 30 min and then drained. Fmoc-Asp(O*t*Bu)-OH (0.6 mmol, 4 equiv) was dissolved in DMF solution containing NMM (1.5 mmol, 132 μL) and reacted with the resin overnight. Four milliliters of 5% MeOH/DMF (vol/vol) was added to the resin in order to cover the unreacted sites on the Cl resin and reacted for 10 min. Fmoc-Pro-OH and Fmoc-3 carboxy-Phe(OTbe)-OH were sequentially coupled to the resin-bound peptide to obtain **9** by the standard protocol. Fmoc-Cys(Trt)-OH, Fmoc-Ile-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Ile-OH, Fmoc-Ser-OH, Fmoc-Lys(Boc)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Arg(Pbf)-OH, and Boc-Gly-OH were coupled to obtain **10** by the standard Fmoc-SPPS protocol. It is worth mentioning that the last amino acid in the synthetic sequence is Boc-Gly-OH.

*Synthesis Route of **11a**.* React for 6 h under deprotection conditions 2-mercaptoethanol (5 mmol)/DIPEA (2.6 mmol) in a mixed solvent system [40% H_2O/DMF (v/v)] to obtain **11a**. **11a** was analyzed by HPLC (gradient 1−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 24.0 min.

*Synthesis Route of **11b**.* React for 6 h under deprotection conditions 2-mercaptoethanol (5 mmol)/DIPEA (2.6 mmol) in a mixed solvent system [40% H_2O/DMF (v/v)] to obtain **11a**. Activate the carboxyl group of peptides by diisopropylcarbodiimide (DIC) (10 equiv, 1.5 mmol) and HOAT (10 equiv, 1.5 mmol) 0.5−1 min at room temperature. After adding $NH_3 \cdot H_2O$ (10 equiv, 1.5 mmol) to the resin, react overnight to obtain **11b**. **11b** was analyzed by HPLC (gradient 1−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 23.7 min.

*Synthesis Route of **11c**.* React for 6 h under deprotection conditions 2-mercaptoethanol/DIPEA dissolved in 40% H_2O/DMF (v/v) to obtain **11a**. Activate the carboxyl group of peptides by DIC (10 equiv, 1.5 mmol) and HOAT (10 equiv, 1.5 mmol) 0.5−1 min at room temperature. After adding diethylamine (10 equiv, 1.5 mmol) to the resin, react overnight to obtain **11c**. **11c** was analyzed by HPLC (gradient 1−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 24.5 min.

*Synthesis Route of **11d**.* React for 6 h under deprotection conditions 2-mercaptoethanol (5 mmol)/DIPEA (2.6 mmol) in a mixed solvent system [40% H_2O/DMF (v/v)] to obtain **11a**. Activate the carboxyl group of peptides by DIC (10 equiv, 1.5 mmol) and HOAT (10 equiv, 1.5 mmol) 0.5−1 min at room temperature. After adding pyrrolidone (10 equiv, 1.5 mmol) to the resin, react overnight to obtain **11d**. **11d** was analyzed by HPLC (gradient 1−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 24.1 min.

Cleavage from the Resin and Purification. The TFA cleavage cocktails (TFA/phenol/thioanisole/EDT/H_2O = 85:5:5:2.5:2.5) were added to the dried peptide-containing resin for 3 h at 37 °C. TFA was removed by blowing N_2 over the mixture in a fume hood. Diethyl ether was cooled to 0 °C. After the peptides were precipitated with cold Et_2O, they were collected by centrifugation and repeated twice. The resulting peptide **11a−d** was purified by semipreparative HPLC, which was refolded under neutral conditions (20:1 GSSG/GSH, pH 7.5) to give product **12a−d**. The solution was concentrated using a freeze dryer, and final folded **12a−d** was lyophilized and purified by semipreparative HPLC. **12a** was analyzed by HPLC (gradient 1−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 24.0 min. **12b** was analyzed by HPLC (gradient 1−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 23.4 min. **12c** was analyzed by HPLC (gradient 1−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 24.6 min. **12d** was analyzed by HPLC (gradient 1−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 24.0 min.

General Procedure for the Synthesis of Tyrocidine A. AM Rink amide resin (156 mg, 0.05 mmol, 0.32 mmol g^{-1}) was weighed and placed in a synthesis container. The resin was swelled in mixed solvent of DCM and DMF (3 and 3 mL) for 30 min and then drained. Fmoc-Glu-OTbe, Fmoc-Asn(Trt)-OH, and Fmoc-D-Phe-OH were assembled to the amino group of the resin to obtain **pre tyrocidine A I** by the standard Fmoc-based SPPS protocol. Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-D-Phe-OH, Fmoc-Leu-OH, Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, and Fmoc-Tyr(*t*Bu)-OH were coupled to the resin-bound peptide to obtain **pre tyrocidine A II** by the standard protocol. After removing the Tbe protecting group, **pre tyrocidine A III** was obtained by cyclization.

Cleavage from the Resin and Purification. The TFA cleavage cocktails (TFA/phenol/thioanisole/EDT/H_2O = 85:5:5:2.5:2.5) were add to the dried peptide-containing resin for 3 h at 37 °C. TFA was removed by blowing N_2 over the mixture in a fume hood. After the peptides were precipitated with cold Et_2O, they were collected by centrifugation and repeated twice. The resulting peptide was analyzed by HPLC (gradient 30−99% B in A over 40 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 24.0 min.

Synthesis of the Human Lactoferrin Derivative. AM Rink amide resin (156 mg, 0.05 mmol, 0.32 mmol g^{-1}) was weighed and placed in a synthesis container. The resin (0.05 mmol) was swelled in mixed solvent of DCM and DMF (5 and 5 mL) for 30 min and then drained.

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Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, and Fmoc-Ile-OH were sequentially coupled to the resin-bound peptide to obtain **pre HLBD1 I** by the standard protocol. Fmoc-Cys(Trt)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Val-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Met-OH, Fmoc-Lys(Tbeoc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Glu-(Tbe)-OH, Fmoc-Phe-OH, Fmoc-Cys(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Ala-OH, and Fmoc-Glu(O*t*Bu)-OH were coupled to the resin-bound peptide by the standard protocol. After removal of the Fmoc group, solution of Ac_2O/DIPEA/DMF (v/v/v = 2:3:5) (4 mL) was added to the resin, and the reaction mixture was shaken for 20 min to obtain **pre HLBD1 II**. After removing the Tbeoc and Tbe group, **pre HLBD1 III** was obtained by cyclization. The peptide was obtained by cleavage from the resin and analyzed by HPLC (gradient 1−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 23.2 min. The resulting peptide was purified by semipreparative HPLC, which was refolded under neutral conditions to give product **16** (**HLBD1**). HLBD1 was analyzed by HPLC (gradient 10−80% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 17.2 min.

Minimum Inhibitory Concentration. *B. subtilis* and *S. aureus* were cultivated to 10^8 CFU with LB medium at 37 °C. Tyrocidine A was dissolved in 30% ethanol solution. The MIC was determined by the standard serial dilution method. Detailed data of the antimicrobial assay are provided in Supporting Information.

NMR evidence for deprotected Tbe-AAs and Tbeoc-Aas.

Tbeoc-Aas Deprotection. **2a** is dissolved in mixed solution of DMF and water. After adding 2-mercaptoethanol (5 mmol) and DIPEA (2.6 mmol), the reaction was carried out for 6 h. The product was separated by HPLC to obtain Fmoc-Lys-OH (gradient 10−90% B in A over 30 min with, A, water + 0.1% TFA and, B, MeCN + 0.08% TFA, flow rate = 1.0 mL/min, λ = 214 nm), t_R = 23.1 min.

Fmoc-Lys-OH. White powder. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.68 (s, 1H), 7.90 (d, J = 7.5 Hz, 2H), 7.73 (dd, J = 7.5, 3.7 Hz, 3H), 7.64 (d, J = 8.0 Hz, 1H), 7.43 (t, J = 7.4 Hz, 2H), 7.33 (t, J = 7.4 Hz, 2H), 4.34−4.20 (m, 3H), 3.97−3.89 (m, 1H), 2.77 (s, 2H), 1.72 (d, J = 7.4 Hz, 1H), 1.65−1.47 (m, 3H), 1.37 (d, J = 8.1 Hz, 2H).

Tbe-Aas Deprotection. **1a-1** is dissolved in mixed solution of DMF and water. After adding 2-mercaptoethanol (5 mmol) and DIPEA (2.6 mmol), the reaction was carried out for 6 h. The product was separated by flash column chromatography (petroleum ether/EtOAc 6:1, with 1% acetic acid) to obtain Fmoc-Asp-O*t*Bu.

Fmoc-Asp-OtBu. White powder. ^{1}H NMR (400 MHz, chloroform-d): δ 7.76 (d, J = 7.5 Hz, 2H), 7.60 (d, J = 7.6 Hz, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.4 Hz, 2H), 5.79 (d, J = 8.1 Hz, 1H), 4.55 (dt, J = 8.7, 4.6 Hz, 1H), 4.39 (qd, J = 10.6, 7.2 Hz, 2H), 4.23 (t, J = 7.2 Hz, 1H), 2.98 (ddd, J = 63.1, 17.3, 4.6 Hz, 2H), 1.47 (s, 9H).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.joc.1c00255.

General protocol for the synthesis of Fmoc-Aa(Tbeoc)-OH, Fmoc-Aa(OTbe)-OH, and Fmoc-Aa-OTbe, synthesis of Fmoc-3-carboxy-Phe(OtBu)-OAllyl, 1h, and Fmoc-3-carboxy-Phe(OTbe)-OH, ESI-MS analysis of I, intermediate product, and II, deprotection of the Tbeoc group on Orn and Dap, deprotection of Tbeoc and Tbe group, chemical structure, HPLC traces, and ESI-MS analysis of pre SFTI-1 IV, pre SFTI-1, pre SFTI-2 IV, SFTI-2, 11a, 12a, 11b, 12b, 11c, 12c, 11d, 12d, Tyrocidine A, pre HLBD1 IV, and 16(HLBD1), compound characterization, NMR spectra, CD spectra, and HPLC, MS, HRMS, and MIC data (PDF)

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Notes

The authors declare no competing financial interest.

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