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Direct Peptide Cyclization and One-Pot Modification Using the MeDbz Linker

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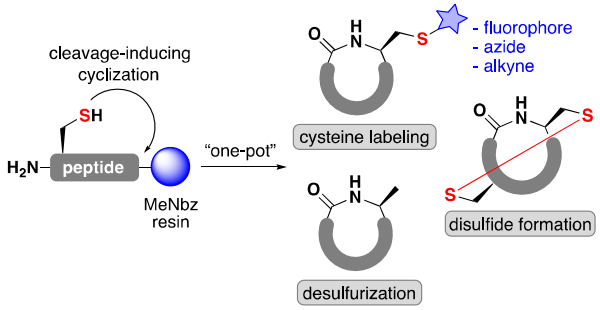


Abstract

The one-pot synthesis and modification of cyclic peptides through a self-cleaving on-resin protocol is described. We apply Dawson's MeDbz linker to achieve direct intramolecular peptide cyclization by thioesterification followed by S→N acyl shift. This native chemical ligation approach requires no activating additive and allows direct modification of the crude cyclic peptides in one-pot. The strategy was applied to synthesize 5 cyclic peptide natural products of varying ring size. Finally, one-pot modifications include desulfurization, fluorophore conjugation, and intramolecular disulfide formation.

Cyclic peptides are receiving increasing interest in drug discovery to fill the lack of promising medium sized therapeutics (500–5000 Da).[1] Their constrained nature may result in predetermined conformations that bind a target protein with high affinity, presumably in part due to lower entropic penalty upon binding. For these reasons, macrocyclic peptides have found interest for development of ligands that target protein–protein interactions.[2,3] Furthermore, improved stability with respect to proteolytic degradation is often achieved by cyclization. Numerous ways have been developed to synthesize macrocyclic peptides, including the commonly applied head-to-tail cyclization of side chain protected linear peptides under high dilution conditions.[4] Alternatively, chemoselective approaches using unprotected peptides for macrocyclization have been developed.[5-7] In particular, native chemical ligation (NCL)[8] and its recent advances[9-16] have enabled the development of diverse strategies based on either Boc solid-phase peptide synthesis (SPPS),[17-21] O→S[22] and N→S[23-26] shift thioester formation, safety-catch linkers,[27,28] C-terminal hydrazides,[29-31] C-terminal *o*-aminoanilides,[32] *N*-acylurea surrogates[33-35] and C-terminal β-thiolactones.[36] Taking advantage of the pseudo-dilution effect on solid

support,[37] on-resin cyclizations[38,39] and cleavage inducing cyclization approaches[40-48] have been developed for various chemistries. We and others have recently exploited Dawson's N^3-Fmoc-3,4-methyl-diaminobenzoic acid (Fmoc-MeDbz) linker[34] using on-resin protocols to synthesize cyclic thiodepsipeptides,[49,50] C-terminally modified peptides[51-53] and homodetic cyclopeptides via attack of the N-terminal amine.[54] Here, we apply this linker for the cleavage-inducing one-pot cyclization and modification of head-to-tail cyclized homodetic peptides by NCL (Figure 1).

In our previously reported protocol, we applied a MeDbz-Gly-resin, which allowed for the global deprotection of the peptides on solid support after formation of the activated *N*-acyl-benzimidazolinone (Nbz) moiety and the triggering of a cleavage-inducing cyclization, upon the addition of pH-controlled buffer to the resin.[50] We envisioned that subjecting N-terminal cysteine-containing peptides to these conditions would lead to a chemoselective cyclization–cleavage event with subsequent S→N shift yielding homodetic peptides **2** (Figure 1). For our initial attempts, we chose a model hexapeptide (**3**) to test our hypothesis (Figure S1A and Table 1).

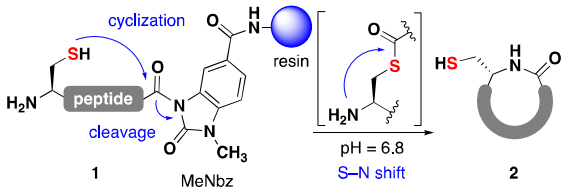
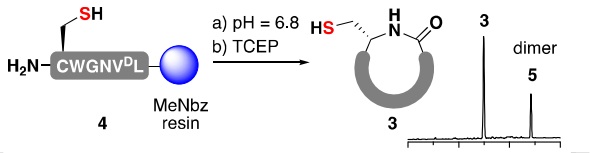


Figure 1 Envisioned strategy for the synthesis of cyclic peptides.

First, the cyclization–cleavage reaction was performed in 0.2 M phosphate buffer–MeCN (1:1) for 2 h at 50 °C on MeDbz-ChemMatrix resin **4**. The resulting crude HPLC chromatogram showed several products, which furnished two peaks upon reduction with TCEP. The major peak corresponded to monomer **3** and the minor peak was isolated and identified as cyclic homodetic peptide dimer **5** by mass spectrometry, ^{1}H NMR spectroscopy, and chemical reactivity tests (Figure S1A–B and Table 1). Since we did not encounter dimerization using the protocol for the synthesis of thiodepsipeptides,[50] we synthesized an N-acetylated version of the hexamer to investigate the dimer formation further (Figure S1C). Here, we only detected the expected thiodepsipeptide, which may suggest a mechanism for the dimer formation involving site–site nucleophilic attack on the resin (Figure S2A) as also previously reported for resin-bound peptides.[39,43,45,48,55] However, it could also be imagined that monomer **3** cleaves another peptide to form an adduct that may cause dimer formation through different mechanisms (Figure S2B and C).

We first envisioned that temporary protection of the N-terminal amine could prevent dimer formation in the cyclization–cleavage step and release a thiodepsipeptide. Removal of the protecting group in buffer would then lead to the desired S→N shift to furnish the desired cyclic peptide. Such a protecting group would need to be stable towards TFA, aqueous buffer at 50 °C, as well as nucleophiles. The propargyloxycarbonyl group (Proc) fulfills these requirements and can be removed using palladium(II) complexes in aqueous media.[56] Thus, we synthesized the Proc-protected hexapeptide and performed the cyclization (Figure S3A). Subsequent LC-MS analysis of the crude material showed excellent purity and confirmed the identity of the Proc-protected thiodepsipeptide. However, we were not able to remove the protecting group using $PdCl_2$ under the reported conditions. We therefore examined the photolabile 6-nitroveratryloxycarbonyl (Nvoc) group,[57] which exhibited stability in both TFA and buffer. Cyclization gave a single peak with the correct *m/z* for the Nvoc-protected thiodepsipeptide, but subsequent UV irradiation led to decomposition (Figure S3B). Instead, we decided to investigate different resins with varying loading and swelling properties to limit dimer formation. We performed test reactions applying MeDbz on PEGA, TentaGel, and TentaGel XV resins and all resulted in improved monomer–dimer ratios compared to the ChemMatrix resin (Table 1). The high-swelling TentaGel XV resin proved superior with an 11% reduction in the dimer formation (Table 1, entry 4). Applying these conditions, we observed satisfying isolated yield of **3** (30%) with only 4% of **5** based on the resin loading.

Table 1: Dimer ratios for different resins



	(mmol/g)	
ChemMatrix[c]	0.50	73:27
PEGA[d]	0.34	80:20
TentaGel[c]	0.22	78:22
TentaGel XV[c]	0.21	84:16

[a]The resin loadings were determined by Fmoc deprotection and subsequent UV measurement. [b]The ratios of **3** to **5** were determined by analytical HPLC (230 nm). [c]Cyclization reactions were performed in 0.2 M phosphate buffer–MeCN (1:1) for 2 h at 50 °C unless otherwise noted. [d]This reaction was performed in 0.1 M phosphate buffer-DMF (7:3) to increase the swelling of the resin

NCL protocols without the use of thiol additives have been used for one-pot ligation–desulfurization[36,58-60] and one-pot ligation–iodine oxidation[61] reactions. We therefore envisioned that the absence of activating additives in our cyclization–cleavage protocol would allow direct modifications of the cysteine residue, including one-pot radical desulfurization to give cyclic alanine-containing peptides.[62] In addition, the use of penicillamine (Pen) instead of Cys would give rise to valine-containing macrocycles.[63] We also expected that our protocol could be used for direct intramolecular disulfide formation by iodine oxidation, subsequent to the cyclization–cleavage step, providing cyclic disulfide containing peptides. Functionalizing cyclic peptides received recent interest[64,65] and we therefore envisioned that the absence of nucleophilic additives would allow us to install functionalities by S_N2 reaction via the cysteine residue and furnish labeled cyclic peptides, requiring just a single purification step. In order to probe the scope, we chose several cyclic peptide natural compounds with varying ring size that contain alanine, valine, or cysteine (Figure 2). We prepared the Cys (**6**) and Ala (**7**) mutants of the hexameric natural peptide destoamide B (**8**)[66] to compare the yields for the one-pot desulfurization. The synthesis of destoamide B V1C (**6**) proceeded in a good isolated yield (23%) and the direct desulfurization using VA-044 and TCEP to provide destoamide B V1A (**7**) proceeded within 2 h under ambient atmosphere to give a similar yield (18%). When performing this one-pot modification for the Pen-containing peptide to give destoamide B (**8**) itself, a considerable amount of hydrolyzed linear peptide was detected (*m/z* +18) together with a peptide with the correct *m/z* (Figure S4A). Analysis of this product by NMR spectroscopy revealed a linear peptide with a C-terminal succinimide moeity (Figure S4B). The steric congestion of the penicillamine side chain presumably slows down the S→N shift, leaving the thioester exposed for hydrolysis or intramolecular nucleophilic attack of the Asn side chain. We then prepared several

alanine-containing natural compounds including stellarin G (5-mer) (**9**),[67] stylostatin 1 (7-mer) (**10**),[68] cyclonellin (8-mer) (**11**),[69] and crotogossamide (9-mer) (**12**)[70] in yields between 13–31% based on the resin loading (Figure 2).

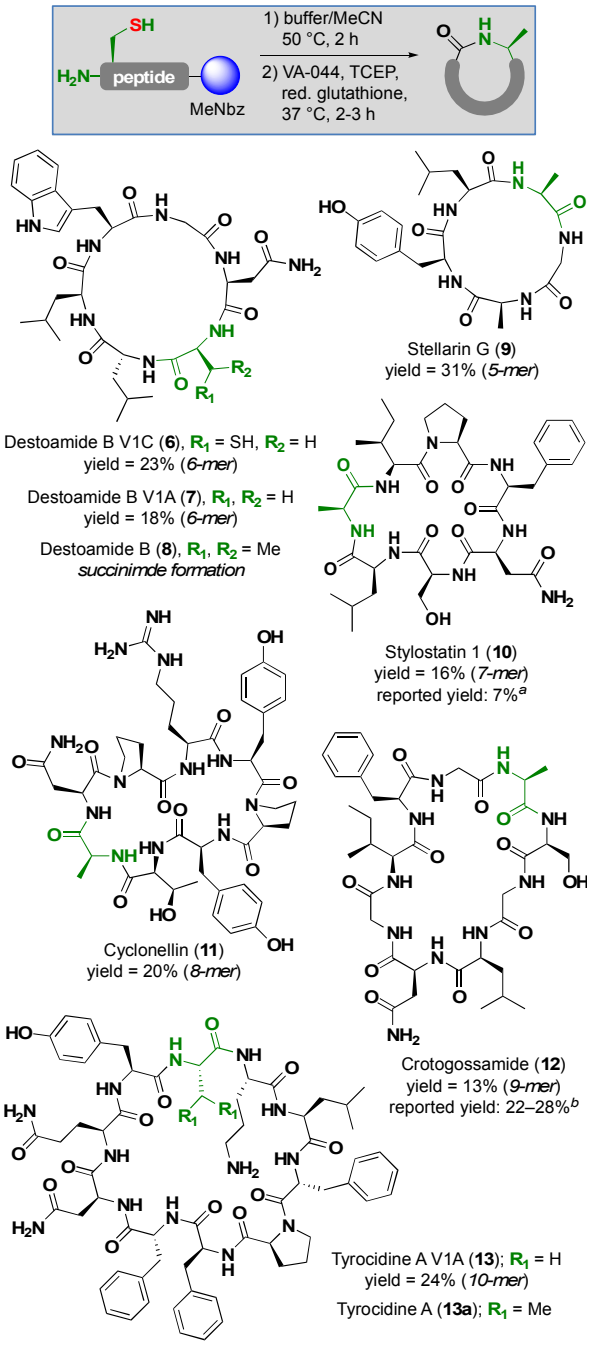


Figure 2 Scope of cyclization–one-pot desulfurization. [a]Yield as stated in the manuscript.[42] [b]Yield as stated in the manuscript.[62]

Finally, we attempted the syntheses of the antibiotic tyrocidine A (**13a**),[71] a 10-mer valine-containing peptide, and its alanine mutant tyrocidine A V1A (**13**). The latter was obtained in good yield but when employing Pen as the N-terminal amino acid, we again encountered hydrolysis and were not able to synthesize tyrocidine A. Thus, applying penicillamine to obtain valine-containing peptides appears to be a limitation of the protocol based on the two examples attempted here. The cyclization of tetrameric peptides is challenging due to conformational constraints and could not be achieved using our protocol.[72-75]

Next, we tested our protocol for the synthesis of a peptide with an internal disulfide bond by synthesizing sunflower trypsin inhibitor 1 (SFTI-1, **14**) (Figure 3A).[76] In contrast to the previously applied conditions, TCEP was added to the cyclization reaction to prevent disulfide formation while the peptide is still attached to the resin. The presence of an internal cysteine can lead to the formation of a thiolactone intermediate in the cyclization–cleavage step, which has been shown to effectively promote thiol-additive free NCL and should therefore lead to the final peptide.[77] Indeed, the cyclization reaction gave a major peak, which corresponded to the reduced form of SFTI-1. Addition of an iodine solution quickly oxidized the peptide and gave bicyclic form of SFTI-1 (**14**) in a yield of 11% after HPLC purification.

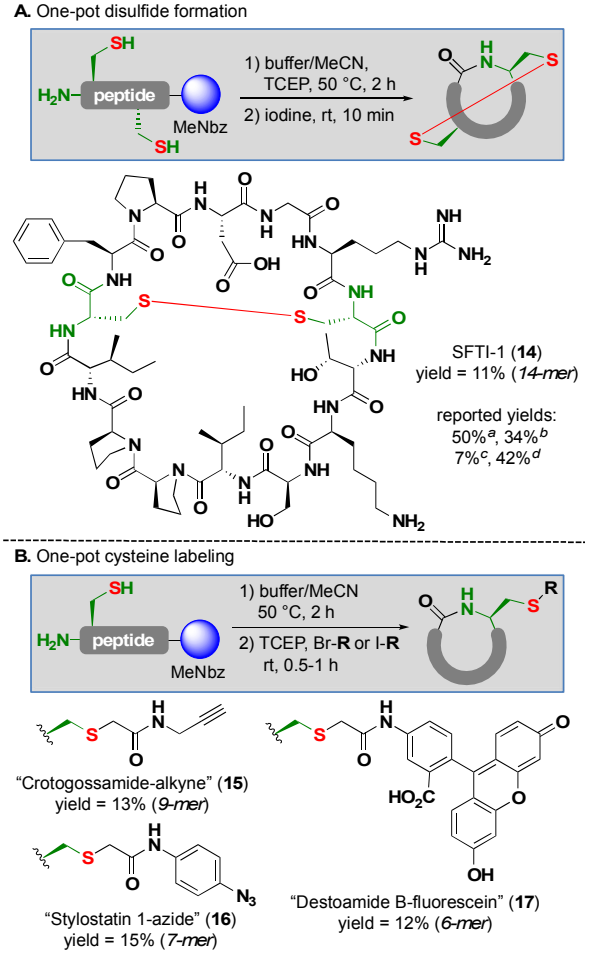


Figure 3 One-pot, postcyclization modification. [a]Yield based on resin loading.[33] [b]Yield based on purified linear precursor.[30] [c]Yield based on resin loading.[23] [d]Yield as stated in the manuscript.[47]

Lastly, we prepared cyclic peptides containing handles for copper-catalyzed azide–alkyne click chemistry[78,79] or a fluorophore (Figure 3B). We chose haloacetamides as electrophiles due the ease of preparation. First, we introduced an alkyne handle into crotogossamide (**12**) by addition of *N*-propargyl-iodoacetamide directly to the reaction mixture after the cyclization. The reaction went to completion in 30 min, yielding the modified peptide **15** in a similar yield (13%) to natural product **12**. Next, we prepared an azide-modified version of stylostatin 1 (**10**) using a *N*-(4-azidophenyl)-bromoacetamide. The alkylation proceeded in 1 h and provided the modified peptide **16** in a yield of 15%. Encouraged by these results, we subjected destoamide B V1C (**6**) to labeling with bromoacetamide-functionalized fluorescein under the same conditions. The fluorescent peptide **17** was obtained within 1 h in only slightly decreased yield (12%) compared to destoamide B V1A (**7**) (Figure 3B). The ease of synthesis and the variety of commercial haloacetamides offer access to a vast number of possible modifications in a single step that does not require additional purification steps.

In conclusion, we have developed a procedure for the cleavage, cyclization, and modification of homodetic cyclopeptides using the MeDbz linker in one-pot fashion. The absence of activating additives allowed us to perform direct desulfurization subsequent to the cyclization, enabling efficient preparation of 4 natural cyclic peptides together with 3 analogues thereof. Further, we demonstrated one-pot intramolecular disulfide formation in the preparation of SFTI-1 (**14**) as well as installation of various handles for bioconjugation using haloacetamide reagents. The developed chemistry offers rapid access to cyclic peptides of varying ring size and, importantly, enables a variety of post-cyclization modifications without requiring additional purification steps.

Experimental Section

General Methods and Materials

All reagents and solvents were obtained from commercial suppliers. Flash column chromatography was performed on silica gel 60 (particle size 35–70 μm). Analytical high-performance liquid chromatography (HPLC) was performed on a C18 column (2.6 μm, 100 Å, 150 × 4.60 mm) using a diode array ultraviolet detector. A gradient with eluent A (water–MeCN–TFA, 95:5:0.1) and eluent B (0.1% TFA in MeCN) rising linearly from 0 to 95% of B over 15.0 min was applied at a flow rate of 1 mL min^{-1} to monitor reactions and a gradient over 30 min was applied to determine the purity of peptides (λ = 210 nm). Ultra-high-performance liquid chromatography (UPLC) mass spectrometry (MS) analyses were performed on a C18 column (1.7 μm, 100 Å, 50 × 2.10 mm). A gradient with eluent C (0.1% HCOOH in water) and eluent D (0.1% HCOOH in MeCN) rising linearly from 0 to 95% of D over 5.20 min at a flow rate of 0.6 mL min^{-1} was applied. Preparative HPLC purification was performed on a C18 column (5 μm, 100 Å, 250 × 21.2 mm) or a C8 column (5 μm, 100 Å, 250 × 21.2 mm) using a diode array ultraviolet detector. Fractions containing the purified target peptide were identified using UPLC-MS or matrix assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Selected fractions were pooled and lyophilized. MALDI-TOF mass spectra were recorded using a matrix of alpha-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid in water/MeCN (1:1) containing 0.1% TFA. High-resolution mass spectra (HRMS) were recorded using either MALDI, or electrospray ionization (ESI). ^{1}H NMR spectra were recorded at 400 MHz or 600 MHz at 298 K. ^{13}C NMR spectra were recorded at 101 MHz or 150 MHz at 298 K. Chemical shifts are reported in parts per million (ppm) relative to the deuterated solvent peak of DMSO-d_6 (δ_H = 2.50 ppm; δ_C = 39.52 ppm) or $CDCl_3$ (δ_H = 7.26 ppm; δ_C = 77.16 ppm) as internal standard.

General procedure for preparation of MeDbz resins

Aminomethyl resin (0.30 mmol) was placed in a polypropylene syringe equipped with a fritted disk, swelled in DMF for 30 min and washed with DMF (5 × 5 mL).

Coupling of the first residue. Fmoc-Gly-OH or Fmoc-Rink amide linker (1.20 mmol, 4.0 equiv), HATU (445 mg, 1.17 mmol, 3.9 equiv), and *i*-Pr_2NEt (417 μL, 2.40 mmol, 8.0 equiv) were pre-incubated in DMF (6.0 mL, 0.2 M) for 2 min and then added to the resin. After 2 h, the resin was washed with DMF (3 × 5 mL), MeOH (3 × 5 mL), and CH_2Cl_2 (3 × 5 mL) and treated with a capping solution (Ac_2O–*i*-Pr_2NEt–CH_2Cl_2, 2:2:6, v/v/v, 5.0 mL). After 2 h, the resin was washed with DMF (3 × 5 mL), MeOH (3 × 5 mL), and CH_2Cl_2 (3 × 5 mL).

Fmoc deprotection. The resin was treated with piperidine in DMF (1:4, v/v, 5.0 mL) (1 × 2 min, 1 × 20 min) and washed with DMF (3 × 5 mL), MeOH (3 × 5 mL), and CH_2Cl_2 (3 × 5 mL).

Coupling of second residue. Fmoc-MeDbz-OH (**S14**) (466 mg, 1.20 mmol, 4.0 equiv), HATU (445 mg, 1.17 mmol, 3.9 equiv), and *i*-Pr_2NEt (417 μL, 2.40 mmol, 8.0 equiv) were pre-incubated in DMF (6.0 mL, 0.2 M) for 2 min and then added to the resin. After 2 h, the resin was washed with DMF (3 × 5 mL), MeOH (3 × 5 mL), and CH_2Cl_2 (3 × 5 mL). After coupling of the second residue, the resin was dried under high vacuum for 16 h and the loading was determined using the outlined procedure.

The following resins were prepared using the general procedure:

MeDbz-Rink-ChemMatrix resin (0.43 mmol/g)

MeDbz-Gly-ChemMatrix resin (0.50 mmol/g)

MeDbz-Gly-TentaGel resin (0.22 mmol/g)

MeDbz-Gly-TentaGel XV resin (0.21 mmol/g)

MeDbz-Gly-PEGA resin (0.33 mmol/g)

Resin-loading determination

Vacuum-dried resin (10 mg) was agitated in DMF (2.0 mL) containing 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (40.0 μL). After 30 min, the resin was allowed to settle and 16.0 μL of the solution were diluted with MeCN (984 μL) and the absorption at 294 nm or 304 nm was determined. The loading was calculated using the following equations:

$$\text{loading}\left(\frac{\text{mmol}}{\text{g}}\right) = \frac{14.2 \times \text{A294 nm}}{\text{weight of resin (mg)}} \quad \text{or} \quad \text{loading}\left(\frac{\text{mmol}}{\text{g}}\right) = \frac{13.3 \times \text{A304 nm}}{\text{weight of resin (mg)}}$$

General protocol for automated peptide synthesis

Automated peptide synthesis was carried out on an automated peptide synthesizer using standard Fmoc SPPS chemistry. The following Fmoc-protected amino acids with side chain protecting groups were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*-Bu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-Gly(Dmb)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-D-

Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Orn(Boc)-OH, Fmoc-Phe-OH, Fmoc-D-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*-Bu)-OH, Fmoc-Thr(*t*-Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*-Bu)-OH, and Fmoc-Val-OH. The following amino acids were used as *N*-terminal amino acids: Boc-Cys(Trt)-OH, Proc-Cys(Trt)-OH (**S10**), Nvoc-Cys(Trt)-OH (**S11**) and Boc-Pen(Trt)-OH. SPPS was performed on 0.02 mmol scale using MeDbz-Gly-resins resin containing additional 5% MeDbz-Rink-ChemMatrix. Fmoc deprotection was performed in two stages: 1) piperidine in DMF (2:3, v/v) for 3 min and 2) piperidine in DMF (1:4, v/v) for 12 min. The deprotection was followed by washing with DMF (5 × 45 s). The first coupling reaction was performed as double coupling using Fmoc-Xaa-OH (6.0 equiv to the resin loading), HATU (5.9 equiv) and *i*-Pr_2NEt in NMP (12 equiv, 2.0 M) in DMF (final concentration = 0.15 M) for 90 min for each coupling. The remaining coupling reactions were performed as double couplings with Fmoc-Xaa-OH (6.0 equiv to the resin loading), HBTU (5.9 equiv) and *i*-Pr_2NEt in NMP (12 equiv, 2.0 M) in DMF (final concentration = 0.15 M) for 40 min for each coupling. Couplings reactions for Fmoc-Gly(Dmb)-OH, Proc-Cys(Trt)-OH (**S10**), Nvoc-Cys(Trt)-OH (**S11**) and Boc-Pen(Trt)-OH were performed manually in a polypropylene syringe equipped with a fritted disk using 2.5 equiv of amino acid, HATU (2.5 equiv) and *i*-Pr_2NEt (5 equiv) in DMF (final concentration = 0.10 M) for 120 min.

General procedure for *N*-acyl-benzimidazolinone (Nbz) formation

After automated peptide elongation, the resin (0.02 mmol, 1.0 equiv) was transferred into a polypropylene syringe equipped with a fritted disk using CH_2Cl_2 and the resin was washed with CH_2Cl_2 (5 × 1 min). A solution of 4-nitrophenyl-chloroformate (20.1 mg, 0.10 mmol, 5.0 equiv) in CH_2Cl_2 (1.0 mL) was added to the resin and the suspension was agitated for 30 min. The resin was then washed with CH_2Cl_2 (2 × 1 min) and the procedure was repeated. The resin was then washed with CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min) and a solution of *i*-Pr_2NEt (87 μL, 0.50 mmol, 25.0 equiv) in DMF (1.0 mL) was added to the resin. After 15 min, the resin was washed with DMF (3 × 1 min) and the procedure was repeated. The resin was then washed with DMF (3 × 1 min), *i*-Pr_2NEt in DMF (5%, v/v) (3 × 1 min), DMF (3 × 1 min), MeOH (3 × 1 min), and CH_2Cl_2 (3 × 1 min) and dried under high vacuum.

General procedures for one-pot peptide cyclization

Dried peptidyl MeNbz-Gly-TentaGel XV resin (0.02 mmol, 1.0 equiv) containing additional 5% MeNbz-Rink-ChemMatrix was placed in a polypropylene syringe equipped with a fritted disk and treated with a deprotection/cleavage cocktail (2.0 mL, TFA–*i*-Pr_3SiH–water, 94:3:3, v/v/v). After 1 h [2 h for peptides containing Arg(Pbf)], the cleavage solution was collected for assessment of the peptide synthesis and the resin was washed with CH_2Cl_2 (3 × 1 min), DMF (3 × 1 min), and CH_2Cl_2 (3 × 1 min)

and dried under suction for several minutes. The cyclization buffer [5.0 mL, phosphate buffer (0.2 M, pH = 6.8)–MeCN, 1:1, v/v; for procedure D, a TCEP solution (0.5 M, pH = 6.8, 0.2 mL, 0.10 mmol, 5.0 equiv) was added to the buffer] was added to the resin (final concentration = 4.0 mM) and the suspension was agitated at 50 °C.

Procedure A: For peptides containing a cysteine. After 2 h, a TCEP solution (0.5 M, pH = 6.8) (0.2 mL, 0.10 mmol, 5.0 equiv) was added and the syringe was agitated for 15 min at room temperature. The solution was removed from the resin and the resin was rinsed with fresh cyclization buffer. The combined peptide-containing washings were pooled and purified by preparative HPLC. Fractions containing pure peptide were lyophilized to afford the desired cyclized peptide.

Procedure B: For thiodepsipeptides. After 2 h, the solution was removed from the resin and the resin was rinsed with fresh cyclization buffer. The combined peptide-containing washings were pooled and purified by preparative HPLC. Fractions containing pure peptide were lyophilized to afford the desired cyclized peptide.

Procedure C: For desulfurization of peptides. After 2 h, the solution was removed from the resin into a 50 mL centrifugal tube containing a magnetic stir bar and the resin was rinsed with fresh cyclization buffer. To the solution was added a TCEP solution (0.5 M, pH = 6.8, 3.6 mL, final concentration = 0.2 M), reduced glutathione (123 mg, 0.40 mmol, 20.0 equiv) and VA-044·HCl (64.6 mg, 0.20 mmol, 10.0 equiv) and the reaction mixture was stirred for 2-3 h at 37 °C. The reaction mixture was monitored by analytic HPLC and upon complete desulfurization purified by preparative HPLC. Fractions containing pure peptide were lyophilized to afford the desired cyclized peptide.

Procedure D: For peptides containing an internal disulfide bond. After 2 h, the solution was removed from the resin into a 50 mL centrifugal tube containing a magnetic stir bar and the resin was rinsed with fresh cyclization buffer. An iodine solution in MeCN (0.2 M, ca. 0.40 mL, 4.0 equiv) was added dropwise to the solution until the decoloring of the iodine solution stopped. Excess iodine was quenched with a drop of saturated $Na_2S_2O_3$ solution and the reaction mixture was purified by preparative HPLC. Fractions containing pure peptide were lyophilized to afford the desired cyclized peptide.

Procedure E: For cysteine labeling of peptides using bromo/iodoacetamides. After 2 h, a TCEP solution (0.5 M, pH = 6.8, 0.2 mL, 0.10 mmol, 5.0 equiv) was added and the syringe was agitated for 15 min at room temperature. The solution was removed from the resin into a 50 mL centrifugal tube containing a magnetic stir bar and the resin was rinsed with fresh cyclization buffer. A solution of bromo/iodoacetamides (3.0 equiv) in DMF (0.1 mL) was added to the solution and the reaction mixture was stirred for 0.5–1.0 h at room temperature. The reaction mixture was monitored by analytic HPLC

and upon complete alkylation purified by preparative HPLC. Fractions containing pure peptide were lyophilized to afford the desired cyclized peptide.

*Model peptide (**3**).* The peptide was synthesized on a 10 μmol scale using procedure A. Preparative RP-HPLC (C18, 05–95% B in 30 min) afforded model peptide (**3**) as a fluffy white solid after lyophilization (2.0 mg, 30%). Analytical HPLC t_R = 19.1 min, purity 95% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{31}H_{45}N_8O_7S^+$ 673.3126; found 673.3123. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.81–0.87 (m, 9H), 0.91 (d, J = 6.5 Hz, 3H), 1.42 (dt, J = 13.7, 7.1 Hz, 1H), 1.46–1.52 (m, 1H), 1.67 (dp, J = 13.4, 6.6 Hz, 1H), 1.90–2.00 (m, 1H), 2.30 (t, J = 8.6 Hz, 1H), 2.51–2.55 (m, 4H), 2.66 (dt, J = 13.8, 8.9 Hz, 1H), 2.89–2.98 (m, 1H), 3.04 (dd, J = 14.5, 6.2 Hz, 1H), 3.21–3.28 (m, 2H), 3.80 (dd, J = 14.8, 5.7 Hz, 1H), 4.14 (dt, J = 8.9, 6.2 Hz, 1H), 4.18–4.21 (m, 1H), 4.21–4.26 (m, 1H), 4.38 (td, J = 7.3, 5.3 Hz, 1H), 4.58 (q, J = 6.8 Hz, 1H), 6.95 (s, 1H), 6.97 (t, J = 7.5 Hz, 1H), 7.03–7.08 (m, 3H), 7.28 (d, J = 8.1 Hz, 1H), 7.31 (d, J = 8.1 Hz, 1H), 7.35 (s, 1H), 7.52 (d, J = 7.9 Hz, 1H), 7.72 (d, J = 7.4 Hz, 1H), 8.39 (d, J = 7.8 Hz, 1H), 8.44 (d, J = 5.6 Hz, 1H), 8.53 (t, J = 5.7 Hz, 1H), 8.58 (d, J = 8.0 Hz, 1H), 10.80 (d, J = 2.4 Hz, 1H).

*Peptide dimer (**5**).* The peptide was isolated in the synthesis of model peptide (**3**) on a 10 μmol scale using procedure A. Preparative RP-HPLC (C18, 05–95% B in 30 min) afforded peptide dimer (**5**) as a fluffy white solid after lyophilization (0.5 mg, 8%). Analytical HPLC t_R = 22.4 min, purity 88% (λ = 210 nm). UPLC-MS *m/z*: $[M+H]^+$ Calcd for $C_{62}H_{89}N_{16}O_{14}S_2^+$ 1345.62; found 1345.70. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.79–0.87 (m, 18H), 0.87 (d, J = 6.6 Hz, 6H), 1.43–1.54 (m, 4H), 1.56–1.63 (m, 2H), 1.99–2.06 (m, 2H), 2.14 (t, J = 8.6 Hz, 2H), 2.44–2.50 (m, 2H) 2.52–2.60 (m, 2H), 2.60–2.69 (m, 2H), 2.75–2.81 (m, 2H), 3.01 (dd, J = 14.9, 9.3 Hz, 2H), 3.14–3.18 (m, 2H), 3.69 (dd, J = 16.6, 5.2 Hz, 2H), 3.79 (dd, J = 16.6, 5.9 Hz, 2H), 4.11–4.17 (m, 2H), 4.19–4.26 (m, 2H), 4.34–4.40 (m, 2H), 4.50–4.57 (m, 2H), 4.60 (q, J = 7.0 Hz, 2H), 6.92 (s, 2H), 6.95 (t, J = 7.5 Hz, 2H), 7.04 (t, J = 7.3 Hz, 2H), 7.15–7.21 (m, 2H), 7.31 (d, J = 8.0 Hz, 2H), 7.35 (s, 2H), 7.57 (d, J = 7.9 Hz, 2H), 7.77 (d, J = 8.1 Hz, 2H), 8.02 (d, J = 7.4 Hz, 2H), 8.07 (d, J = 7.4 Hz, 2H), 8.15 (d, J = 7.4 Hz, 2H), 8.23 (d, J = 7.1 Hz, 2H), 8.25–8.30 (m, 2H), 10.79 (d, J = 2.3 Hz, 2H).

*Destoamide B V1C (**6**).* The peptide was synthesized using procedure A. Preparative RP-HPLC (C8, 05–95% B in 30 min) afforded destoamide B V1C (**6**) as a fluffy white solid after lyophilization (3.2 mg, 23%). Analytical HPLC t_R = 20.0 min, purity 96% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{32}H_{47}N_8O_7S^+$ 687.3283; found 687.3278. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.77 (d, J = 6.4 Hz, 3H), 0.82 (d, J = 6.6 Hz, 3H), 0.84–0.90 (m, 6H), 1.35–1.62 (m, 6H), 2.10 (t, J = 8.4 Hz, 1H), 2.55 (d, J = 6.2 Hz, 2H), 2.69–2.78 (m, 2H), 3.06 (dd, J = 14.7, 5.9 Hz, 1H), 3.27 (dd, J = 14.7, 5.9 Hz, 1H), 3.33 (dd, J = 15.1, 5.4 Hz, 1H), 3.78 (dd, J = 15.1, 5.4 Hz, 1H), 4.00 (ddd, J = 11.4, 7.5, 3.9

Hz, 1H), 4.28 (q, *J* = 7.4 Hz, 1H), 4.34–4.40 (m, 2H), 4.55 (q, *J* = 6.1 Hz, 1H), 6.90 (s, 1H), 6.96 (ddd, *J* = 7.9, 6.9, 1.0 Hz, 1H), 6.99 (d, *J* = 2.4 Hz, 1H), 7.05 (ddd, *J* = 8.1, 6.9, 1.2 Hz, 1H), 7.28–7.33 (m, 3H), 7.35 (d, *J* = 7.1 Hz, 1H), 7.47 (d, *J* = 7.9 Hz, 1H), 8.31 (d, *J* = 6.8 Hz, 1H), 8.49 (d, *J* = 7.6 Hz, 1H), 8.65 (d, *J* = 7.5 Hz, 1H), 8.67 (t, *J* = 5.5 Hz, 1H), 10.82 (d, *J* = 2.4 Hz, 1H).

Destoamide B V1A (7). The peptide was synthesized using procedure C. Preparative RP-HPLC (C18, 05–95% B in 30 min) afforded destoamide B V1A (**7**) as a fluffy white solid after lyophilization (2.4 mg, 18%). Analytical HPLC t_R = 19.2 min, purity 96% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{32}H_{47}N_8O_7^+$ 655.3562; found 655.3558. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.78 (d, *J* = 6.3 Hz, 3H), 0.81 (d, *J* = 6.5 Hz, 3H), 0.84–0.90 (m, 6H), 1.18 (d, *J* = 6.7 Hz, 3H), 1.36–1.42 (m, 2H), 1.43–1.49 (m, 2H), 1.50–1.62 (m, 2H), 2.45–2.50 (m, 1H), 2.51–2.57 (m, 1H), 3.07 (dd, *J* = 14.6, 5.6 Hz, 1H), 3.28 (dd, *J* = 14.6, 6.1 Hz, 1H), 3.32 (dd, *J* = 15.0, 5.8 Hz, 1H), 3.77 (dd, *J* = 15.0, 5.3 Hz, 1H), 4.01 (ddd, *J* = 11.3, 7.5, 3.8 Hz, 1H), 4.22 (q, *J* = 7.5 Hz, 1H), 4.27 (p, *J* = 6.7 Hz, 1H), 4.32–4.37 (m, 1H), 4.53 (q, *J* = 6.1 Hz, 1H), 6.89 (s, 1H), 6.96 (ddd, *J* = 8.0, 6.9, 1.0 Hz, 1H), 6.98 (d, *J* = 2.4 Hz, 1H), 7.05 (ddd, *J* = 8.2, 7.0, 1.2 Hz, 1H), 7.22 (d, *J* = 6.9 Hz, 1H), 7.28 (s, 1H), 7.29–7.32 (m, 2H), 7.48 (d, *J* = 7.9 Hz, 1H), 8.09 (d, *J* = 6.8 Hz, 1H), 8.53 (d, *J* = 7.5 Hz, 1H), 8.60 (d, *J* = 7.5 Hz, 1H), 8.69 (t, *J* = 5.6 Hz, 1H), 10.82 (d, *J* = 2.4 Hz, 1H).

*Stellarin G (**9**)*. The peptide was synthesized using procedure C. Preparative RP-HPLC (C18, 05–50% B in 30 min) afforded stellarin G (**9**) as a fluffy white solid after lyophilization (2.9 mg, 31%). Analytical HPLC t_R = 15.7 min, purity 99% (λ = 210 nm). UPLC-MS *m/z*: $[M+H]^+$ Calcd for $C_{23}H_{34}N_5O_6^+$ 476.25; found 476.23. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.78 (d, *J* = 6.5 Hz, 3H), 0.85 (d, *J* = 6.5 Hz, 3H), 1.17 (d, *J* = 7.3 Hz, 3H), 1.21 (d, *J* = 6.9 Hz, 3H), 1.31–1.40 (m, 1H), 1.39–1.47 (m, 1H), 1.71 (ddd, *J* = 13.1, 10.1, 5.0 Hz, 1H), 2.80–2.89 (m, 2H), 3.34 (dd, *J* = 14.5, 5.1 Hz, 1H), 3.89–3.97 (m, 2H), 4.06 (p, *J* = 7.3 Hz, 1H), 4.17 (p, *J* = 7.1 Hz, 1H), 4.27 (q, *J* = 8.1 Hz, 1H), 6.64 (d, *J* = 8.4 Hz, 2H), 6.97 (d, *J* = 8.4 Hz, 2H), 7.65 (d, *J* = 8.6 Hz, 1H), 8.06 (d, *J* = 8.0 Hz, 1H), 8.14 (d, *J* = 7.3 Hz, 1H), 8.20 (d, *J* = 7.9 Hz, 1H), 8.37 (t, *J* = 5.6 Hz, 1H), 9.17 (s, 1H). The spectral data is in accordance with the literature.[67]

*Stylostatin 1 (**10**)*. The peptide was synthesized using procedure C. Preparative RP-HPLC (C18, 05–95% B in 30 min) afforded stylostatin 1 (**10**) as a fluffy white solid after lyophilization (2.4 mg, 16%). Analytical HPLC t_R = 18.0 min, purity 95% (λ = 210 nm). UPLC-MS *m/z*: $[M+H]^+$ Calcd for $C_{36}H_{55}N_8O_9^+$ 743.40; found 743.32. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.71–0.76 (m, 1H) 0.77–0.83 (m, 9H), 0.85 (d, *J* = 6.1 Hz, 3H), 1.14 (d, *J* = 6.5 Hz, 3H), 1.21–1.32 (m, 1H), 1.50–1.57 (m, 4H), 1.57–1.63 (m, 2H), 1.68–1.76 (m, 1H), 2.17 (dd, *J* = 11.8, 6.5 Hz, 1H), 2.59–2.63 (m, 1H), 3.01–3.11 (m, 3H), 3.15 (dd, *J* = 13.8, 4.2 Hz, 1H), 3.20–3.28 (m, 1H), 3.68–3.70 (m, 2H), 3.84–3.89 (m, 1H),

4.03 (dd, *J* = 8.6, 4.9 Hz, 1H), 4.16 (ddd, *J* = 12.1, 7.7, 4.3 Hz, 1H), 4.28–4.33 (m, 2H), 4.43 (d, *J* = 7.4 Hz, 1H), 4.44–4.50 (m, 1H), 7.12–7.16 (m, 2H), 7.19–7.23 (m, 1H), 7.26–7.31 (m, 3H), 7.34 (d, *J* = 7.4 Hz, 1H), 7.76 (d, *J* = 5.4 Hz, 1H), 7.78 (s, 1H), 8.04 (d, *J* = 9.6 Hz, 1H), 8.47 (d, *J* = 3.8 Hz, 1H), 8.59 (d, *J* = 7.7 Hz, 1H), 8.65 (d, *J* = 4.8 Hz, 1H). The spectral data is in accordance with the literature.[68]

*Cyclonellin (**11**).* The peptide was synthesized using procedure C. Preparative RP-HPLC (C18, 05–50% B in 30 min) afforded cyclonellin (**11**) as a fluffy white solid after lyophilization (3.8 mg, 20%). Analytical HPLC t_R = 13.9 min, purity 95% (λ = 210 nm). UPLC-MS *m/z*: $[M+H]^+$ Calcd for $C_{45}H_{63}N_{12}O_{12}^+$ 963.47; found 963.44. ^{1}H NMR (600 MHz, DMSO-d_6): δ 1.05 (d, *J* = 6.4 Hz, 3H), 1.25–1.30 (m, 1H), 1.31 (d, *J* = 7.4 Hz, 3H), 1.34–1.41 (m, 1H), 1.47–1.54 (m, 1H), 1.61–1.70 (m, 1H), 1.70–1.78 (m, 1H), 1.78–1.85 (m, 2H), 1.85–1.92 (m, 2H), 1.92–2.00 (m, 1H), 2.17–2.24 (m, 1H), 2.48–2.53 (m, 1H), 2.65–2.72 (m, 1H), 2.75 (dd, *J* = 14.0, 4.6 Hz, 1H), 2.95–3.01 (m, 3H), 3.15 (dd, *J* = 13.9, 4.2 Hz, 1H), 3.22–3.28 (m, 1H), 3.52–3.61 (m, 2H), 3.62–3.72 (m, 2H), 3.83 (dt, *J* = 9.9, 6.9 Hz, 1H), 3.86–3.90 (m, 1H), 4.05–4.10 (m, 2H), 4.11–4.14 (m, 1H), 4.36–4.41 (m, 1H), 4.71 (dd, *J* = 9.9, 3.7 Hz, 1H), 4.79 (ddd, *J* = 12.6, 9.1, 3.8 Hz, 1H), 4.94 (td, *J* = 9.5, 4.7 Hz, 1H), 5.49 (d, *J* = 12.4 Hz, 1H), 6.61 (d, *J* = 8.4 Hz, 2H), 6.66 (d, *J* = 8.5 Hz, 2H), 6.89–6.96 (m, 4H), 7.35 (s, 1H), 7.37 (d, *J* = 9.9 Hz, 1H), 7.50 (t, *J* = 5.5 Hz, 1H), 7.74 (d, *J* = 9.6 Hz, 1H), 7.98 (s, 1H), 8.00 (d, *J* = 8.2 Hz, 1H), 8.34 (d, *J* = 6.6 Hz, 1H), 8.52 (d, *J* = 8.9 Hz, 1H), 8.62 (d, *J* = 6.8 Hz, 1H), 9.13 (br s, 2H). The spectral data is in accordance with the literature.[69]

*Crotogossamide (**12**).* The peptide was synthesized using procedure C. Preparative RP-HPLC (C8, 05–50% B in 30 min) afforded crotogossamide (**12**) as a fluffy white solid after lyophilization (2.1 mg, 13%). Analytical HPLC t_R = 17.3 min, purity 96% (λ = 210 nm). UPLC-MS *m/z*: $[M+H]^+$ Calcd for $C_{37}H_{57}N_{10}O_{11}^+$ 817.42; found 817.36. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.59 (d, *J* = 6.8 Hz, 3H), 0.73 (t, *J* = 7.4 Hz, 3H), 0.82 (d, *J* = 5.9 Hz, 3H), 0.87 (d, *J* = 6.0 Hz, 3H), 0.98–1.06 (m, 1H), 1.27 (d, *J* = 7.1 Hz, 3H), 1.29–1.33 (m, 1H), 1.48–1.55 (m, 2H), 1.47–1.62 (m, 1H), 1.70–1.79 (m, 1H), 2.48–2.53 (m, 1H), 2.72 (dd, *J* = 15.6, 5.2 Hz, 1H), 2.97 (dd, *J* = 14.2, 9.8 Hz, 1H), 3.17 (dd, *J* = 14.2, 5.5 Hz, 1H), 3.37–3.43 (m, 1H), 3.62–3.69 (m, 3H), 3.75 (dd, *J* = 16.8, 6.2 Hz, 1H), 3.85–3.95 (m, 3H), 4.04 (q, *J* = 5.8 Hz, 1H), 4.24–4.34 (m, 3H), 4.38 (p, *J* = 7.2 Hz, 1H), 4.99–5.07 (m, 1H), 6.85 (s, 1H), 7.16–7.21 (m, 3H), 7.22–7.28 (m, 2H), 7.36 (d, *J* = 7.2 Hz, 1H), 7.39 (s, 1H), 7.52 (d, *J* = 7.8 Hz, 1H), 7.72 (d, *J* = 7.4 Hz, 1H), 8.06 (t, *J* = 6.1 Hz, 1H), 8.16 (t, *J* = 6.1 Hz, 1H), 8.23 (d, *J* = 7.2 Hz, 1H), 8.31 (s, 1H), 8.35 (s, 1H), 8.51 (s, 1H). The spectral data is in accordance with the literature.[70]

*Tyrocidine A V1A (**13**).* The peptide was synthesized using procedure C. Preparative RP-HPLC (C8, 05–95% B in 30 min) afforded tyrocidine A V1A (**13**) as a fluffy white solid after lyophilization (5.9 mg, 24%). Analytical HPLC t_R = 25.4 min, purity 97% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*:

$[M+H]^+$ Calcd for $C_{64}H_{84}N_{13}O_{13}^+$ 1242.6306; found 1242.6302. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.35–0.47 (m, 1H), 0.92 (d, J = 6.6 Hz, 3H), 0.94 (d, J = 6.5 Hz, 3H), 1.00–1.06 (m, 1H), 1.21–1.28 (m, 5H), 1.34–1.40 (m, 1H), 1.41–1.50 (m, 2H), 1.61–1.70 (m, 5H), 1.74–1.81 (m, 1H), 1.85–1.91 (m, 1H), 1.95–2.04 (m, 1H), 2.16 (q, J = 9.0 Hz, 1H), 2.25 (t, J = 12.9 Hz, 1H), 2.37–2.41 (m, 1H), 2.67–2.89 (m, 5H), 2.91–2.96 (m, 2H), 2.98–3.03 (m, 1H), 3.05–3.12 (m, 1H), 3.27–3.35 (m, 2H), 3.79–3.84 (m, 1H), 4.07 (d, J = 8.0 Hz, 1H), 4.26–4.35 (m, 2H), 4.47–4.55 (m, 3H), 4.65 (p, J = 6.7 Hz, 1H), 5.19–5.25 (m, 1H), 5.51–5.57 (m, 1H), 6.63 (d, J = 8.5 Hz, 2H), 6.89 (s, 1H), 6.92 (d, J = 8.5 Hz, 2H), 7.03–7.16 (m, 6H), 7.17–7.27 (m, 11H), 7.35 (d, J = 7.1 Hz, 1H), 7.45 (m, 3H), 7.54 (s, 1H), 7.88 (d, J = 8.5 Hz, 1H), 7.98 (s, 1H), 8.31 (d, J = 9.5 Hz, 1H), 8.74 (d, J = 4.5 Hz, 1H), 8.91–8.97 (m, 2H), 9.06 (d, J = 9.8 Hz, 1H), 9.18 (br s, 1H), 9.26 (d, J = 3.7 Hz, 1H).

*SFTI-1 (**14**).* Fmoc-Gly(Dmb)-OH was used to prevent aspartimide formation during SPPS. The peptide was synthesized using procedure D. Preparative RP-HPLC (C18, 20–50% B in 30 min) afforded SFTI-1 (**14**) as a fluffy white solid after lyophilization (3.2 mg, 11%). Analytical HPLC t_R = 17.7 min, purity 95% (λ = 210 nm). UPLC-MS *m/z*: $[M+H]^+$ Calcd for $C_{67}H_{105}N_{18}O_{18}S_2^+$ 1513.73; found 1513.55; $[M+2H]^{2+}$ Calcd for $C_{67}H_{106}N_{18}O_{18}S_2^{2+}$ 757.37; found 757.53. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.54 (d, J = 6.7 Hz, 3H), 0.69 (t, J = 7.4 Hz, 3H), 0.76–0.86 (m, 6H), 0.91–1.05 (m, 2H), 1.21–1.34 (m, 7H) 1.35–1.40 (m, 1H), 1.41–1.50 (m, 1H), 1.51–1.57 (m, 2H), 1.62–1.79 (m, 6H), 1.80–1.90 (m, 4H), 1.91–2.03 (m, 3H), 2.04–2.09 (m, 1H), 2.17–2.23 (m, 1H), 2.23–2.29 (m, 1H), 2.59–2.66 (m, 1H), 2.70–2.84 (m, 5H), 2.90–3.01 (m, 2H), 3.02–3.12 (m, 2H), 3.22 (d, J = 13.4 Hz, 1H), 3.44–3.55 (m, 5H), 3.65 (dd, J = 17.0, 5.8 Hz, 1H), 3.71–3.84 (m, 4H), 3.81 (d, J = 7.7 Hz, 1H), 3.87 (dd, J = 17.0, 6.4 Hz, 1H), 4.13 (d, J = 6.4 Hz 1H), 4.16–4.25 (m, 5H), 4.25–4.34 (m, 4H), 4.54–4.64 (m, 2H), 4.95 (d, J = 8.5 Hz, 1H), 5.20 (d, J = 6.4 Hz, 1H), 5.26–5.35 (m, 2H), 5.72–5.75 (m, 1H), 7.10–7.14 (m, 1H), 7.19 (t, J = 7.6 Hz, 2H), 7.22 –7.27 (m, 1H), 7.29 (d, J = 8.5 Hz, 1H), 7.35 (d, J = 7.6 Hz, 2H), 7.49–7.52 (m, 2H), 7.63–7.72 (m, 3H), 7.80–7.85 (m, 1H), 8.18 (t, J = 6.3 Hz, 1H), 8.36 (d, J = 8.8 Hz, 1H), 8.47 (br s, 1H), 8.64 (d, J = 9.6 Hz, 1H), 8.70 (d, J = 7.0 Hz, 1H), 8.81 (d, J = 9.4 Hz, 1H), 12.40 (br s, 1H). The spectral data is in accordance with the literature.[76]

*Crotogossamide-alkyne (**15**).* The peptide was synthesized using procedure E and 2-iodo-*N*-(prop-2-yn-1-yl)acetamide (**S16**) (13.3 mg, 0.06 mmol, 3.0 equiv) as alkylating reagent. Preparative RP-HPLC (C18, 05–95% B in 30 min) afforded crotogossamide-alkyne (**15**) as a fluffy off-white solid after lyophilization (2.4 mg, 13%). Analytical HPLC t_R = 18.9 min, purity 95% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{42}H_{62}N_{11}O_{12}S^+$ 944.4295; found 944.4282. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.51 (d, J = 6.8 Hz, 3H), 0.74 (t, J = 7.4 Hz, 3H), 0.82 (d, J = 5.9 Hz, 3H), 0.88 (d, J = 6.0 Hz, 3H), 0.99–1.08 (m, 1H), 1.31–1.38 (m, 1H), 1.48–1.64 (m, 3H), 1.70–1.78 (m, 1H), 2.48–2.53 (1H

signal not fully visible due to DMSO-d_6 signal), 2.79–2.84 (m, 2H), 3.02–3.10 (m, 2H), 3.10 (t, J = 2.5 Hz, 1H), 3.22–3.29 (m, 3H), 3.36 (d, J = 14.4 Hz, 1H), 3.43 (dd, J = 16.6, 4.9 Hz, 1H), 3.66–3.76 (m, 4H), 3.83–3.86 (m, 1H), 3.88 (dd, J = 5.6, 2.5 Hz, 1H), 3.90 (dd, J = 5.6, 2.5 Hz, 1H), 3.91–3.94 (m, 1H), 3.94–3.97 (m, 1H) 4.02 (q, J = 5.6 Hz, 1H), 4.22–4.36 (m, 3H), 4.62 (q, J = 7.8 Hz, 1H), 6.85 (s, 1H), 7.15–7.20 (m, 3H), 7.22–7.26 (m, 2H), 7.30 (d, J = 6.6 Hz, 1H), 7.37–7.44 (m, 2H), 7.67–7.74 (m, 1H), 7.99 (t, J = 6.3 Hz, 1H), 8.13–8.22 (m, 2H), 8.32 (t, J = 5.4 Hz, 1H), 8.50 (t, J = 5.5 Hz, 1H), 8.68 (br s, 1H), 8.77 (br s, 1H).

*Stylostatin 1-azide (**16**)*. The peptide was synthesized using procedure E and *N*-(4-azidophenyl)-2-bromoacetamide (**S17**) (15.3 mg, 0.06 mmol, 3.0 equiv) as alkylating reagent. Preparative RP-HPLC (C18, 20–60% B in 30 min) afforded stylostatin 1-azide (**16**) as a fluffy off-white solid after lyophilization (2.9 mg, 15%). Analytical HPLC t_R = 21.8 min, purity 98% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{44}H_{61}N_{12}O_{10}S^+$ 949.4349; found 949.4334. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.67–0.74 (m, 1H), 0.77–0.82 (m, 9H), 0.82–0.85 (m, 3H), 0.85–0.91 (m, 1H), 1.26–1.33 (m, 1H), 1.50–1.57 (m, 5H), 1.58–1.65 (m, 1H), 1.73–1.81 (m, 1H), 2.17 (dd, J = 11.8, 6.4 Hz, 1H), 2.58–2.63 (m, 1H), 2.75 (dd, J = 13.3, 4.3 Hz, 1H), 2.95–3.00 (m, 2H), 3.03 (dd, J = 15.5, 3.2 Hz, 1H), 3.09 (dd, J = 15.5, 4.6 Hz, 1H), 3.15 (dd, J = 13.8, 4.0 Hz, 1H), 3.23–3.29 (m, 1H), 3.36 (s, 2H), 3.68–3.70 (m, 2H), 3.84–3.87 (m, 1H), 4.07 (dd, J = 8.7, 5.1 Hz, 1H), 4.13 (ddd, J = 12.0, 7.6, 4.1 Hz, 1H), 4.30–4.36 (m, 2H), 4.44 (d, J = 7.2 Hz, 1H), 4.64 (ddd, J = 9.8, 7.7, 4.5 Hz, 1H), 7.04 (d, J = 8.9 Hz, 2H), 7.14–7.20 (m, 3H), 7.21–7.25 (m, 2H), 7.26 (s, 1H), 7.45 (d, J = 7.8 Hz, 1H), 7.62 (d, J = 8.9 Hz, 2H), 7.79 (d, J = 5.5 Hz, 1H), 7.82 (s, 1H), 7.99 (d, J = 9.7 Hz, 1H), 8.41 (d, J = 7.5 Hz, 1H), 8.52 (d, J = 3.8 Hz, 1H), 8.87 (d, J = 5.0 Hz, 1H), 10.05 (s, 1H).

*Destoamide B-fluorescein (**17**)*. The peptide was synthesized using procedure E and 5-(bromoacetamido)fluorescein (**S18**) (28.1 mg, 0.06 mmol, 3.0 equiv) as alkylating reagent. Preparative RP-HPLC (C18, 05–95% B in 30 min) afforded destoamide B-fluorescein (**17**) as a fluffy yellow solid after lyophilization (2.5 mg, 12%). Analytical HPLC t_R = 21.3 min, purity 96% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+Na]^+$ Calcd for $C_{54}H_{59}N_9NaO_{13}S^+$ 1096.3845; found 1096.3840. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.77 (d, J = 6.4 Hz, 3H), 0.80 (d, J = 6.6 Hz, 3H), 0.84 (d, J = 6.6 Hz, 3H), 0.86 (d, J = 6.4 Hz, 3H), 1.32–1.39 (m, 1H), 1.41–1.49 (m, 2H), 1.50–1.61 (m, 3H), 2.52–2.59 (m, 2H), 2.86–2.96 (m, 2H), 3.06 (dd, J = 14.7, 5.9 Hz, 1H), 3.27 (dd, J = 14.7, 5.9 Hz, 1H), 3.33 (dd, J = 14.9, 5.8 Hz, 1H), 3.41 (s, 2H), 3.78 (dd, J = 14.9, 5.1 Hz, 1H), 3.99 (ddd, J = 11.3, 7.4, 3.8 Hz, 1H), 4.25–4.31 (m, 1H), 4.43 (td, J = 8.1, 4.1 Hz, 1H), 4.50–4.57 (m, 2H), 6.51–6.55 (m, 2H), 6.58 (dd, J = 8.7, 2.3 Hz, 2H), 6.67 (d, J = 2.3 Hz, 2H), 6.89 (s, 1H), 6.95 (ddd, J = 7.9, 6.9, 1.0 Hz, 1H), 6.99 (d, J = 2.4 Hz, 1H), 7.04 (ddd, J = 8.1, 6.9, 1.2 Hz, 1H), 7.21 (d, J = 8.3 Hz, 1H), 7.28–7.32 (m, 2H), 7.33–7.39 (m, 2H),

7.47 (d, *J* = 7.9 Hz, 1H), 7.80 (dd, *J* = 8.3, 2.0 Hz, 1H), 8.32 (d, *J* = 2.0 Hz, 1H), 8.41 (d, *J* = 6.8 Hz, 1H), 8.51 (d, *J* = 7.7 Hz, 1H), 8.63–8.69 (m, 2H), 10.11 (br s, 2H), 10.49 (s, 1H), 10.83 (d, *J* = 2.4 Hz, 1H).

*Model thiodepsipeptide (**S1**).* The peptide was synthesized on a 10 μmol scale using procedure B. Preparative RP-HPLC (C18, 05–95% B in 30 min) afforded model thiodepsipeptide (**S1**) as a fluffy white solid after lyophilization (3.6 mg, 50%). Analytical HPLC t_R = 18.1 min, purity 98% (λ = 210 nm). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{33}H_{47}N_8O_8S^+$ 715.3232; found 715.3229. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.77 (d, *J* = 6.5 Hz, 3H), 0.85 (d, *J* = 6.5 Hz, 3H), 0.88–0.93 (m, 6H), 1.48 (t, *J* = 11.9 Hz, 1H), 1.52–1.63 (m, 2H), 1.79 (s, 3H), 2.12 (dq, *J* = 13.7, 6.8 Hz, 1H), 2.54–2.57 (m, 1H), 2.59–2.63 (m, 1H), 2.88 (dd, *J* = 12.7, 3.5 Hz, 1H), 2.90–2.98 (m, 1H), 2.97–3.03 (m, 1H), 3.13 (dd, *J* = 14.8, 5.0 Hz, 1H), 3.62 (dd, *J* = 16.7, 5.9 Hz, 1H), 3.76 (dd, *J* = 16.7, 4.8 Hz, 1H), 4.01–4.06 (m, 1H), 4.28 (ddd, *J* = 8.3, 6.5, 4.5 Hz, 1H), 4.34–4.39 (m, 1H), 4.39–4.44 (m, 1H), 4.46–4.51 (m, 1H), 6.92–6.97 (m, 2H), 7.04 (t, *J* = 7.5 Hz, 1H), 7.12 (d, *J* = 2.3 Hz, 1H), 7.25 (d, *J* = 8.3 Hz, 1H), 7.31 (d, *J* = 8.1 Hz, 1H), 7.41 (s, 1H), 7.52 (d, *J* = 7.9 Hz, 1H), 7.93 (d, *J* = 8.3 Hz, 1H), 8.10 (d, *J* = 7.3 Hz, 1H), 8.18 (d, *J* = 7.9 Hz, 1H), 8.24–8.31 (m, 2H), 10.83 (d, *J* = 2.3 Hz, 1H).

*N-((prop-2-yn-1-yloxy)carbonyl)-S-trityl-L-cysteine (Proc-Cys(Trt)-OH) (**S10**).* The compound was prepared using a procedure adapted from the literature.[56] H-Cys(Trt)-OH (0.50 g, 1.38 mmol, 1.0 equiv) was suspended in NaOH (2 M, 25 mL) and the pH was adjusted to 8.5. Propargyl chloroformate (0.68 mL, 7.00 mmol, 5.0 equiv) was added dropwise to the suspension and the reaction mixture was stirred for 4 h. The reaction mixture was then diluted with EtOAc (50 mL) and acidified with saturated citric acid (10 mL). The acidic phase was extracted with EtOAc (3 × 50 mL) and the combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. Purification of the crude product by flash column chromatography (SiO_2, CH_2Cl_2/MeOH/AcOH = 95/5/0.5) afforded the title compound **S10** as white solid (487 mg, 1.09 mmol, 79%). UPLC-MS *m/z*: $[M-H]^-$ Calcd for $C_{26}H_{22}NO_4S^-$ 444.13; found 444.30. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.46–2.50 (m, 1H), 2.64–2.76 (m, 2H), 4.28 (dt, *J* = 8.1, 5.6 Hz, 1H), 4.58–4.73 (m, 2H), 5.22 (d, *J* = 8.1 Hz, 1H), 7.17–7.46 (m, 15H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 33.7, 52.9, 53.2, 67.5, 75.2, 77.9, 127.1 (3C), 128.2 (6C), 129.6 (6C), 144.3 (3C), 155.0, 175.0. The spectral data is in accordance with the literature.[56]

*N-(((4,5-dimethoxy-2-nitrobenzyl)oxy)carbonyl)-S-trityl-L-cysteine (Nvoc-Cys(Trt)-OH) (**S11**).* A solution of 6-nitroveratryloxycarbonyl chloride (Nvoc-Cl, 152 mg, 0.55 mmol, 1.1 equiv) in dioxane (1.0 mL) was added dropwise to a solution of H-Cys(Trt)-OH (182 mg, 0.50 mmol, 1.0 equiv) and Na_2CO_3 (117 mg, 1.10 mmol, 2.2 equiv) in dioxane/water (1:1, 10 mL, v/v) at 0 °C. After 30 min was the reaction mixture allowed to warm to room temperature and stirred for 20 h. The reaction mixture

was then evaporated to dryness, redissolved in water (10 mL) and washed with EtOAc (3 × 10 mL). The aqueous phase was acidified with saturated citric acid (5 mL), extracted with EtOAc (3 × 10 mL) and the combined organic layers dried over Na_2SO_4 and concentrated under reduced pressure. Purification of the crude product by flash column chromatography (SiO_2, CH_2Cl_2/MeOH/AcOH = 90/10/0.5) afforded the title compound **S11** as yellow solid (169 mg, 0.28 mmol, 56%). HRMS (ESI/Q-TOF) *m/z*: $[M+Na]^+$ Calcd for $C_{32}H_{29}N_2NaO_8S^+$ 625.1615; found 625.1610. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.41–2.47 (m, 1H), 2.56–2.60 (m, 1H), 3.80–3.92 (m, 7H), 5.37 (s, 1H) 7.17 (s, 1H), 7.20–7.38 (m, 16H), 7.71 (s, 1H), 7.77 (br s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 33.5, 48.6, 56.1, 56.2, 62.3, 66.0, 108.1, 109.7, 126.7 (3C), 128.0 (6C), 128.4, 129.1 (6C), 138.9, 144.3 (3C), 147.6, 153.5, 155.3.

*4-(Methylamino)-3-nitrobenzoic acid (**S12**).* The compound was prepared using a procedure adapted from the literature.[50] A solution of methylamine (30 mL, 33% in MeOH, 320 mmol, 12.0 equiv) was added to a solution of 4-fluoro-3-nitrobenzoic acid (5.00 g, 27.0 mmol, 1.0 equiv) in MeOH (66 mL) and the reaction mixture was stirred at room temperature. After 18 h, the reaction mixture was poured into water (75 mL), acidified with HCl (35%, ~18 mL) to reach pH = 5, and the resulting yellow precipitate was collected by filtration and washed with water. Drying for 24 h under high vacuum gave the title compound **S12** (5.20 g, 26.5 mmol, 98%) as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6): δ 3.00 (d, J = 4.9 Hz, 3H), 7.03 (d, J = 9.1 Hz, 1H), 7.97 (dd, J = 9.1 Hz, 2.0 Hz, 1H), 8.50−8.56 (m, 1H), 8.60 (d, J = 2.0 Hz, 1H), 12.8 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 29.9, 114.4, 116.8, 128.3, 130.4, 136.0, 147.9, 166.0. The spectral data is in accordance with the literature.[50]

*3-Amino-4-(methylamino)benzoic acid (**S13**).* The compound was prepared using a procedure adapted from the literature.[50] 4-(Methylamino)-3-nitrobenzoic acid (**S12**) (5.20 g, 26.5 mmol, 1.0 equiv) was hydrogenated over Pd/C (250 mg, 10% wt) at atmospheric pressure in MeOH (250 mL) at room temperature. After 16 h, the catalyst was removed by filtration and evaporation of the solvent under reduced pressure gave the title compound **S13** (4.31 g, 25.9 mmol 98%) as a dark solid. ^{1}H NMR (600 MHz, DMSO-d_6): δ 2.76 (s, 3H), 5.26 (br s, 1H), 6.37 (d, J = 8.3 Hz, 1H), 7.15 (d, J = 2.0 Hz, 1H), 7.22 (dd, J = 8.3 Hz, 2.0 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 29.8, 107.3, 114.3, 118.1, 120.9, 133.9, 141.2, 168.4. The spectral data is in accordance with the literature.[50]

*Fmoc-3-amino-4-(methylamino)benzoic acid (Fmoc-MeDbz-OH) (**S14**).* The compound was prepared using a procedure adapted from the literature.[50] A solution of Fmoc-chloride (6.00 g, 23.2 mmol, 0.9 equiv) in MeCN (48 mL) was added dropwise to a solution of 3-amino-4-(methylamino)-benzoic acid (**S13**) (4.31 g, 25.9 mmol, 1.0 equiv) and *i*-Pr_2NEt (4.27 mL, 24.5 mmol, 0.95 equiv) in a mixture of MeCN in water (96 mL, 1:1, v/v) until all starting material was consumed. The solvent was evaporated under reduced pressure and the precipitate collected by filtration and washed with MeCN.

Drying for 24 h under high vacuum gave the title compound **S14** (6.16 g, 15.9 mmol, 61%) as a grey solid. UPLC-MS *m/z*: $[M+H]^+$ Calcd for $C_{23}H_{21}N_2O_4^+$ 389.15; found 389.17. ^{1}H NMR (600 MHz, DMSO-d_6): δ 2.78 (d, J = 3.6 Hz, 3H), 4.30−4.40 (m, 3H), 5.87 (br s, 1H), 6.62 (d, J = 8.5 Hz, 1H), 7.28−7.38 (m, 2H), 7.45 (t, J = 7.4 Hz, 2H), 7.61−7.80 (m, 4H), 7.92 (d, J = 7.4 Hz, 2H), 8.71 (br s, 1H), 12.15 (br s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 29.5, 46.7, 66.0 (2C), 108.8, 116.7, 120.1 (2C), 122.0, 125.3 (2C), 127.1 (2C), 127.6 (2C), 140.7 (2C), 143.8 (2C), 154.7, 167.3. The spectral data is in agreement with the literature.[50]

*2-Chloro-N-(prop-2-yn-1-yl)acetamide (**S15**).* The compound was prepared using a procedure adapted from the literature.[80] A solution of chloroacetyl chloride (0.38 mL, 4.8 mmol, 1.2 equiv) in CH_2Cl_2 (5 mL) was added dropwise to a solution of propargyl amine (0.26 □L, 4.00 mmol, 1.0 equiv) and *i*-Pr_2NEt (0.84 mL, 4.8 mmol, 1.2 equiv) in CH_2Cl_2 (27 mL) at 0 °C. The ice-bath was removed and the reaction stirred for 30 min. The reaction mixture was then diluted with CH_2Cl_2 (20 mL), washed with saturated $NaHCO_3$ (3 × 20 mL), 2 M HCl (3 × 20 mL) and brine (1 × 20 mL) and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure gave the title compound **S15** (421 mg, 3.2 mmol, 80%) as a brown oil. UPLC-MS *m/z*: $[M+H]^+$ Calcd for $C_5H_7ClNO^+$ 132.02; found 132.12. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.28 (t, J = 2.6 Hz, 1H), 4.07 (s, 2H), 4.10 (dd, J = 5.4, 2.6 Hz, 2H), 6.78 (br s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 29.7, 42.5, 72.3, 78.7, 165.8. The spectral data is in accordance with the literature.[81]

*2-Iodo-N-(prop-2-yn-1-yl)acetamide (**S16**).* The compound was prepared using a procedure adapted from the literature.[80] To a solution of chloroacetamide **S15** (180 mg, 1.37 mmol, 1.0 equiv) in acetone (15 mL) was added NaI (614 mg, 4.10 mmol, 3.0 equiv) and the reaction mixture was refluxed for 18 h. The solvent was removed under reduced pressure and the remaining residue was partitioned between EtOAc (20 mL) and water (20 mL). The organic layer was separated, washed with saturated $Na_2S_2O_3$ (3 × 20 mL), water (1 × 20 mL) and brine (1 × 20 mL) and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure gave the title compound **S16** (151 mg, 0.68 mmol, 50%) as a brown solid. UPLC-MS *m/z*: $[M+H]^+$ Calcd for $C_5H_7INO^+$ 223.96; found 223.95. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.28 (t, J = 2.6 Hz, 1H), 3.71 (s, 2H), 4.07 (dd, J = 5.3, 2.6 Hz, 2H), 6.22 (br s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ -1.3, 30.4, 72.4, 78.7, 166.5.

*N-(4-azidophenyl)-2-bromoacetamide (**S17**).* A solution of bromoacetyl bromide (0.13 mL, 1.49 mmol, 1.0 equiv) in anhydrous CH_2Cl_2 (1 mL) was added dropwise to a solution of 4-azidoaniline hydrochloride (253 mg, 1.49 mmol, 1.0 equiv) and *i*-Pr_2NEt (0.52 mL, 2.98 mmol, 2.0 equiv) in anhydrous CH_2Cl_2 (3.7 mL) at 0 °C for 10 min. The ice-bath was removed and the reaction stirred for 30 min. The reaction mixture was then diluted with CH_2Cl_2 (20 mL), washed with saturated $NaHCO_3$

(3 × 20 mL), 2 M HCl (3 × 20 mL) and brine (1 × 20 mL) and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure gave the title compound **S17** (353 mg, 1.38 mmol, 93%) as a brown solid. HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_8H_8BrN_4O^+$ 254.9876; found 254.9877. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.02 (s, 2H), 7.02 (d, J = 8.8 Hz, 2H), 7.53 (d, J = 8.8 Hz, 2H), 8.12 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 29.5, 119.8 (2C), 121.7 (2C), 134.0, 137.0, 163.4.

*5-(Bromoacetamido)fluorescein (**S18**).* To a suspension of 5-aminofluorescein (isomer I, 100 mg, 0.29 mmol, 1.0 equiv) and $NaHCO_3$ (73.0 mg, 0.87 mmol, 3.0 equiv) in anhydrous THF (10 mL) at 0 °C was added dropwise a solution of bromoacetyl bromide (30.2 µL, 0.35 mmol, 1.2 equiv) in anhydrous THF (1 mL) over 10 min. The ice-bath was removed and the reaction stirred for 2 h. The reaction mixture was then partitioned between with EtOAc (20 mL) and water (10 mL). The aqueous phase was acidified with saturated citric acid (5 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (20 mL), dried over Na_2SO_4 and concentrated under reduced pressure. Purification of the crude product by flash column chromatography (SiO_2, CH_2Cl_2/MeOH/ = 9/1) afforded the title compound **S18** as an orange solid (70 mg, 0.15 mmol, 52%). HRMS (ESI/Q-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{22}H_{15}BrNO_6^+$ 468.0077; found 468.0074. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.10 (s, 2H), 6.53–6.62 (m, 4H), 6.67 (d, J = 2.3 Hz, 2H), 7.24 (d, J = 8.3 Hz, 1H), 7.82 (dd, J = 8.3, 2.0 Hz, 1H), 8.25–8.33 (m, 1H), 10.10 (s, 2H), 10.85 (s, 1H). (Signals reported for the open acid conformation) ^{13}C NMR (101 MHz, DMSO-d_6): δ 30.1, 83.1, 102.2 (2C), 109.6 (2C), 112.5 (2C), 113.6, 124.6, 126.4, 127.0, 129.1 (2C), 140.1, 147.4, 151.9 (2C), 159.4 (2C), 165.5, 168.4.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Supporting figures, copies of HPLC traces as well as ^{1}H and ^{13}C NMR spectra.

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Notes

The authors declare no competing financial interest.

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