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Leveraging a "Catch-Release" Logic Gate Process for the Synthesis and Non-Chromatographic Purification of Thioether or Amine Bridged Macrocyclic Peptides

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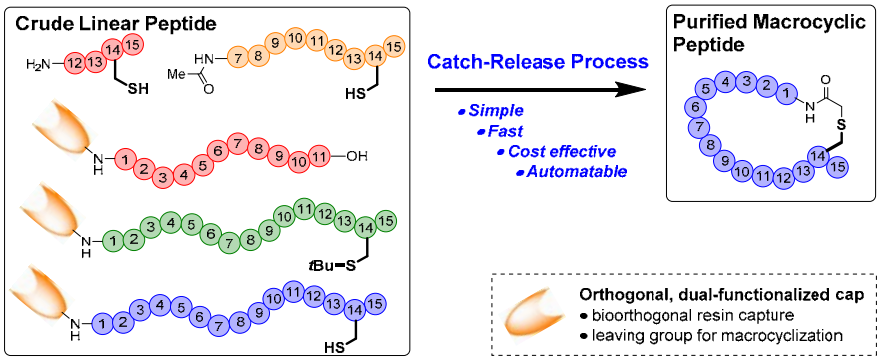


ABSTRACT: Macrocyclic peptides containing *N*-alkylated amino acids have emerged as a promising therapeutic modality, capable of modulating protein-protein interactions and intracellular delivery of hydrophilic payloads. While multi-channel automated Solid Phase Peptide Synthesis (SPPS) is a practical approach for peptide synthesis, the requirement for slow and inefficient chromatographic purification of the product peptides is a significant limitation to exploring these novel compounds. Herein, we invent a "catch-release" strategy for the non-chromatographic purification of macrocyclic peptides. A traceless catch process is enabled by the invention of a dual-functionalized N-terminal acetate analogue, which serves as a handle for capture onto a purification resin and as a leaving group for macrocyclization. Displacement by a C-terminal nucleophilic side-chain thus releases the desired macrocycle from the purification resin. By design, this catch/release process is a logic test for the presence of the key components required for cyclization, thus removing impurities which lack either functionality such as common classes of peptide impurities, including hydrolysis fragments and truncated sequences. The method was shown to be highly effective with three libraries of macrocyclic peptides, containing macrocycles of 5 to 20 amino acids, with either thioether or amine based macrocyclic linkages; in this latter class the reported method represents an enabling technology. In all cases the catch-release protocol afforded significant enrichment of the target peptides purity, in many cases completely obviating the need for chromatography. Importantly, we have adapted this process for automation on a standard multichannel peptide synthesizer, achieving an efficient and completely integrated synthesis and purification platform for the preparation of these important molecules.

Introduction

Macrocyclic peptides are of paramount importance to the discovery of new chemical matter capable of treating human disease.[1] These molecules have the potential to target protein–protein interactions, which have historically been perceived as "undruggable" with traditional small molecules.[2] Besides being a promising chemical modality to treat disease, via either subcutaneous or intravenous administration, collaborative efforts have been initiated to identify cell-permeable and orally bioavailable macrocyclic drugs, targeting intracellular protein-protein interactions.[3] Several cell permeable and orally bioavailable macrocyclic peptides have been developed;[4-8] cyclosporin A, a marketed oral immunosuppressive drug, features multiple sites of *N*-methylation on its amide backbone.[9,10] It should come as no surprise that peptide macrocyclization technology has captured the imagination of academia in recent years.[11-15] Diversity-oriented macrocyclization platforms and technologies have been developed for constructing and screening peptides.[16] PeptiDream's Peptide Discovery Platform System (PDPS) is of particular note, as an *in vitro* mRNA displaying technology, utilizing a thioether displacement for peptide macrocyclization. When this technology is applied to library synthesis, trillions of thioether peptide macrocycles can be generated and screened.[17,18] Importantly, these thioether peptide macrocycles have demonstrated an improved in vivo redox stability relative to disulfide-based analogues,[19] and are easily formed through chemoselective nucleophilic displacement of an N-terminal chloroacetyl "cap" by a resident cysteine.[20,21] Several macrocyclic peptide hits,[16,22-28] as well as an increasing number of disclosed collaborations and partnerships,[29] demonstrate the power of this discovery platform for lead generation.

Despite the aforementioned elegant initial hit identification process, the next step "Hit-to-Lead" in the discovery of macrocyclic peptide drugs remains challenging. Hits need to be validated and optimized into molecules that possess the desired binding affinity, functional activity, stability, physical properties, pharmacokinetic profiles, and efficacy in animal models; all of which requires the rapid synthesis and isolation of hundreds to thousands of distinct macrocyclic peptides, each with high purity. Automated solid phase peptide synthesis (SPPS) is the enabling synthesis platform, providing a powerful tool for the rapid preparation of analogues. However, while synthesis on solid phase is facile, the crude sequences inevitably require significant purification for down-stream applications. The complexity of peptide purification results in this process often being rate-limiting in the drug discovery process. While several potential mechanisms of impurity formation, such as epimerization, insertion of additional amino acids, sequence deletions, aspartimide formation, piperidine adduct formation, and degradation during resin cleavage, can be minimized through optimization, significant levels of impurities are unavoidable as the synthesis of a library requires the use of an unoptimized, though general, synthesis protocol. The presence of backbone *N*-alkylation, often required for activity and improved pharmacokinetic/pharmacodynamics properties, exacerbates impurity formation,[30] due to a significant degree of amide bond hydrolysis during global deprotection.[31] The large number of impurities generated during the synthesis process generally requires multiple chromatographic purifications to reach desired purities, a significant limitation to high-throughput drug discovery and academic discovery alike. Previous approaches to "solid-phase assisted purification" to enrich linear peptide purity[32-34] have resulted in limited success, with specific requirements for each approach: such as requiring modifications of the final peptide, low recoveries, the removal of only truncated failure sequences, and importantly, a lack of ability to automate.

Herein we report our strategy, leveraging a logic gate process for non-chromatographic macrocyclic peptide purification (Figure 1). Full length peptides are functionalized with an orthogonal dual-mode linker, capable of both enabling a solid-phase "capture," and a base-induced "release". We demonstrate that this logic gate catch–release protocol is a scalable and automatable process for construction of thioether macrocycles with different ring sizes (5–20 membered) and amino acid constitutions. The generality of this approach is further explored to enable the formation of amine-linked macrocycles. In all cases, significant purification is observed vs the crude peptides, in many cases

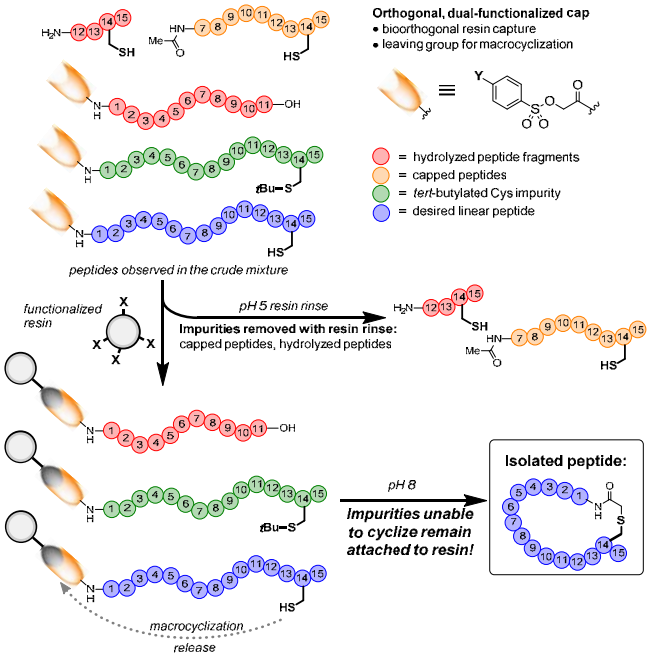


Figure 1. Macrocyclization as a logic test for peptide release: the catch-release strategy for purification. X and Y represent functional groups that can react chemoselectively.

completely obviating the need for chromatography. This method can be automated on a standard peptide synthesizer, demonstrating the first integrated synthesis/purification methodology. To our knowledge, there are no examples of a non-chromatographic purification method capable of removing the full range of peptide impurities, which is also compatible with peptide macrocycles and applicable to automation on standard peptide synthesizers.

Methods and optimization

The development of this concept began on a derivative of a representative literature macrocyclic peptide (Scheme 1).[22] We initially chose the azide–alkyne cycloaddition as our capture technology, due to its documented functional group tolerance. Functionalized arylsulfonyls were explored as dual-functional caps, acting as both "capture" and as halo-surrogates for macrocyclization, the "release" strategy (Scheme 1). A series of copper-catalyzed cycloadditions (CuAAC) between a propargylarylsulfonyl capped peptide **1a** and azide functionalized resins (Merrifield/2-chlorotrityl) were investigated. However, no significant peptide capture was observed, presumably due to incompatibility of the cysteine thiol with CuAAC.[35,36] We therefore explored the Strain-Promoted Azide–Alkyne Cycloaddition (SPAAC), capping the linear model peptide with a dibenzocyclooctyl (DBCO) tethered arylsulfonyl, **1b**. Significant hydrolysis of the DBCO amide during TFA peptide cleavage limited the yield of the desired macrocycle. Inverting the system in **1c**, (an azide capped peptide/DBCO functionalized resin), produced minimal degradation during global deprotection/cleavage, however, spontaneous displacement of azidopropoxy arylsulfonyl moiety by HOAt was observed (see the supporting information for more details). We presumed that this was due to the high reactivity of the electron-poor arylsulfonyl towards nucleophiles.[37] A fourth generation linker with an electron-donating ether, attenuated the reactivity of the leaving group. Thus, peptide **1d** exhibited no deleterious side products, had high stability towards TFA cleavage, and was amenable to installation using any standard protocol. Peptide **1d** underwent smooth capture to a DBCO-loaded PEG resin, releasing the desired macrocycle with an unoptimized 82% purity, providing an initial proof-of-concept for this strategy.

With the proof of concept achieved, a comprehensive optimization was performed (Scheme 2, a-e). For optimization of the capture resin, we focused on polyethylene glycol (PEG)-based systems due to their superior swelling properties. Four resins were synthesized (Scheme 2a). To assess resin stability, they were subjected to 0.1M DIPEA in MeOH, the basic conditions being required to facilitate peptide macrocyclization (Scheme 2b). Resins A, B, and C showed significant DBCO-cleavage and methanolysis within 1 h, while the urea resin D was stable. Combining resin D with the azide cap **1d** (Scheme 1), we studied the efficiency of the capture process (Scheme 2c). Based on a quantitative Ellman assay,[38] click reaction in 10% acetic acid-MeOH in the presence of DTT (6-10 mg/mL) produced the best capture yield (68%). With the goal of further increasing yield and reducing on-resin reactions, we evaluated resin equivalents, and introduced a quench of the excess DBCO attached to the resin with benzyl azide, eliminating potential on-resin thiol–yne reactions (Scheme 2d). The highest peptide recovery was observed with 1.5 equiv excess of resin D, and azide-quench prior to release. To further improve the conditions, we optimized the bases and solvents used for the "release", quantifying the resulting purity (by HPLC) and recovery of the resulting macrocycle (Scheme 2e). MeOH generally gave the highest recoveries and purities for both resins A and D. Among all the combinations explored, 0.1 M NH_4OAc-NH_3/MeOH/resin D provided the best results.

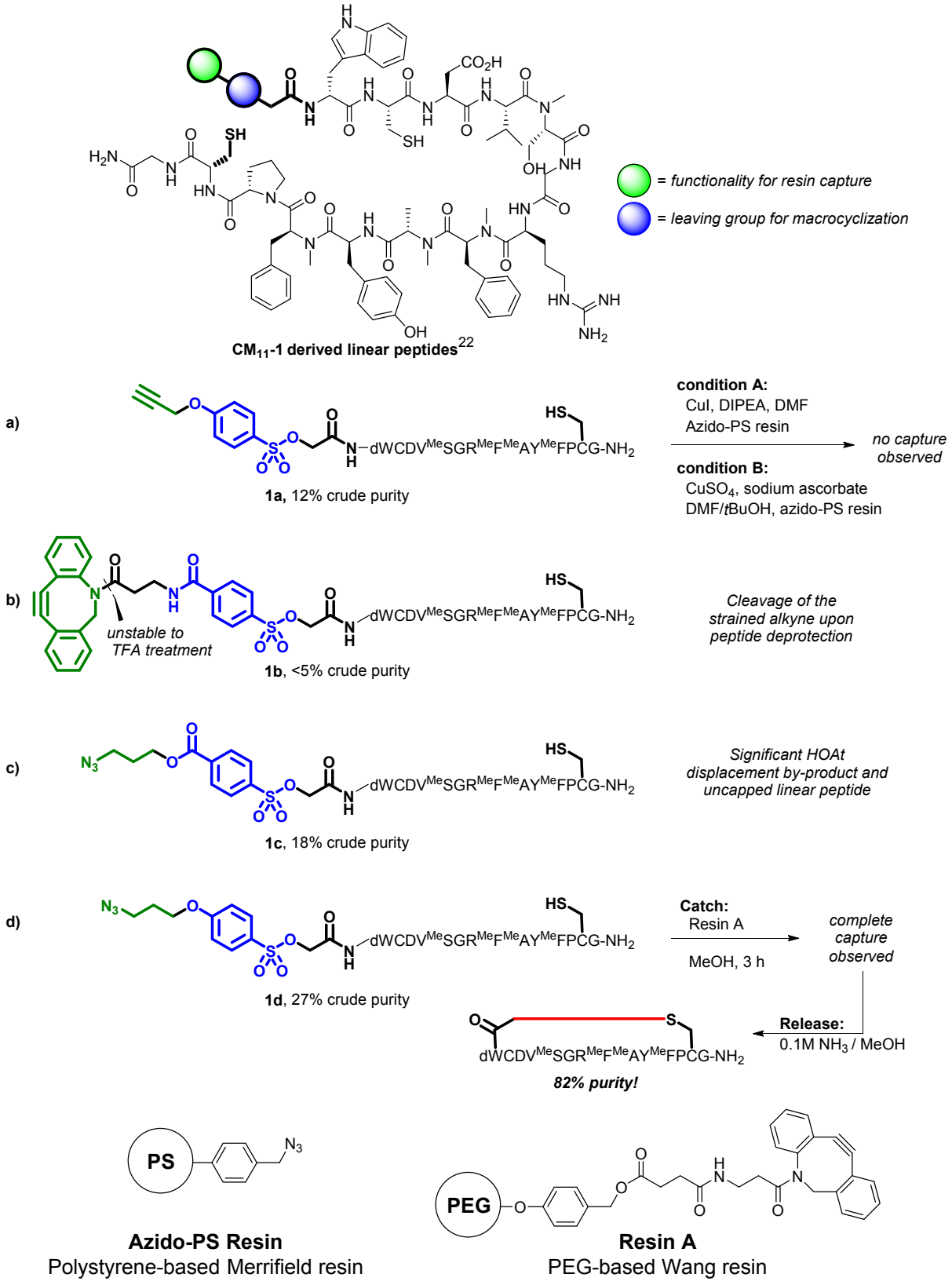
Results and Discussion

To evaluate the efficiency of the purification through catch release (CR), we performed a head-to-head comparison between the two methods. Traditional chloro-acetate displacement and CR (Table **1**). To avoid the ambiguity created by the extensive handling, use of an unoptimized (general) peptide synthesis procedure and the chromatography needed to purify these peptides, we elected to compare the relative recoveries between the two cyclization methods (e.g. directly comparing crude solution concentrations of the macrocycles from CR to those from chloroactetate cyclization(Cl). Thus, the CR results are post-purification while comparison is made to the crude unpurified peptide from chloro-acetate cyclization. Peptide CM_{11}-1, a known literature macrocyclic peptide (Entry **10**, table **1**)[22] was chosen as a model analogue. A library of peptides from 5 to 20 amino acids were synthesized on a standard peptide synthesizer. The chloroacetate-capped and azide-tethered sulfonyl-capped linear peptides were subjected to optimized cyclization protocols. It is worth mentioning that many of these systems, especially those featuring N-methylation, are difficult to prepare (e.g., reported yield for CM_{11}-1 was 5%, (see supplemental information of ref 22) and the SPPS synthesis of the linear peptides was left intentionally unoptimized in order to mimic a real drug-discovery effort. Full experimental details are outlined in the experimental section.

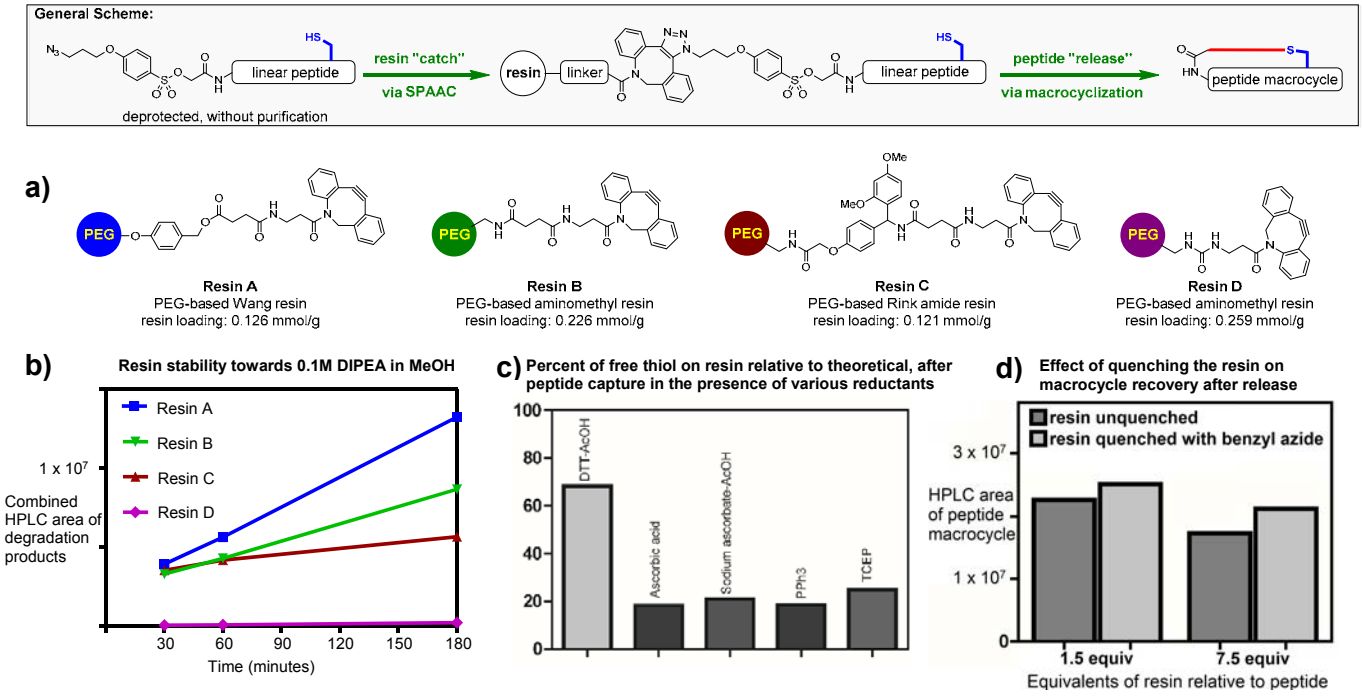
The resulting purity and relative recovery ratio (CR/Cl, CR = catch–release; Cl = chloroacetate) are summarized (Table **1**). In all cases, catch–release resulted in significantly increased purities (60–95%) in comparison to solution phase macrocyclization (ranging 3–89%). Comparing the

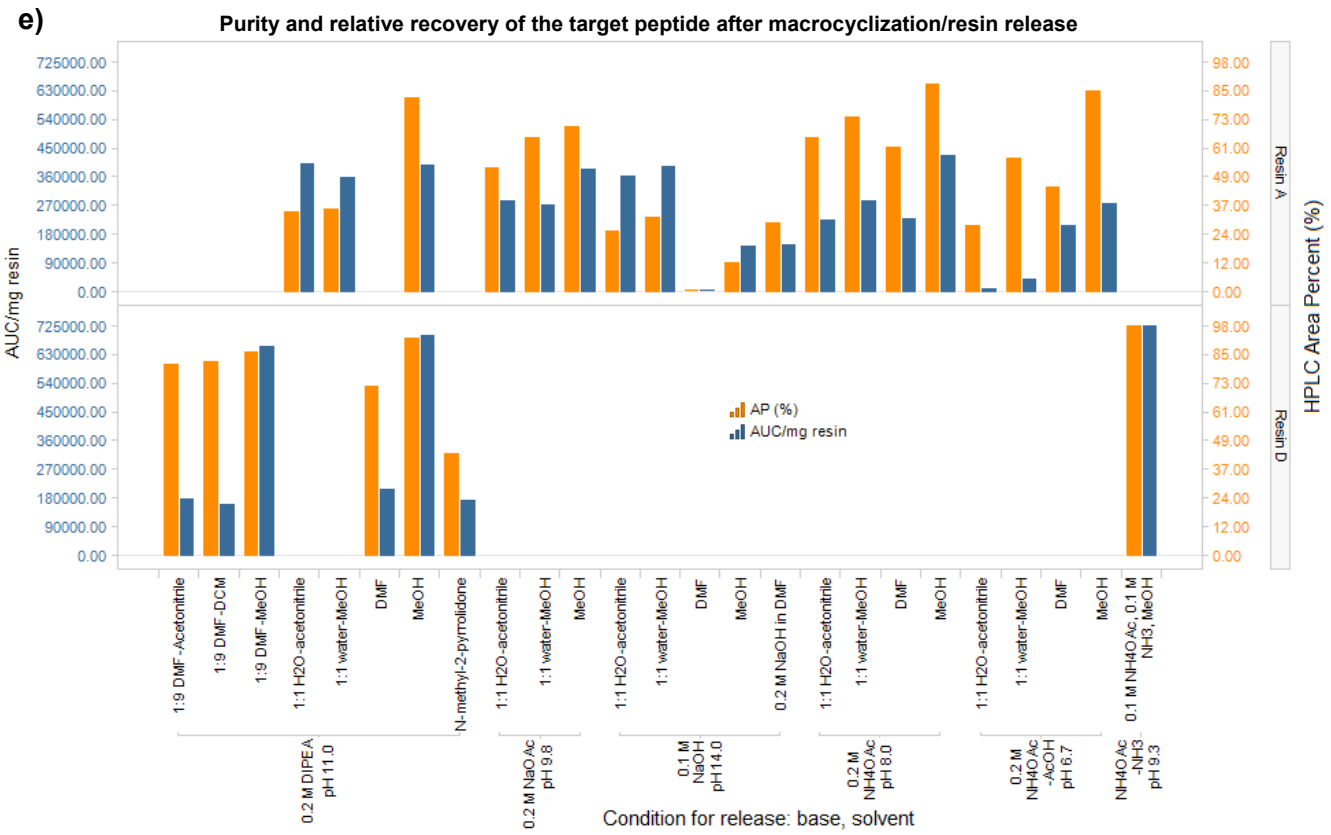
LCMS traces for both cyclization strategies, it was evident that the cysteine *tert*-butylation byproducts are successfully captured by the DBCO-functionalized resin D and remained attached upon release of the desired macrocycle, effectively removing the impurity below HPLC detection limits. In entries **6–17**, the main impurities are truncated

Scheme 1. Proof-of-concept for the catch-release purification



a) Propargyloxy arylsulfonyl capped peptide **1a**. b) Dibenzocyclooctyl (DBCO) capped peptide **1b**. c) Azidopropyl benzo-ylsulfonyl capped peptide **1c**. d) Azidopropoxy arylsulfonyl capped peptide **1d**. dAA: D-amino acids; ^{Me}AA: N-methylated amino acids





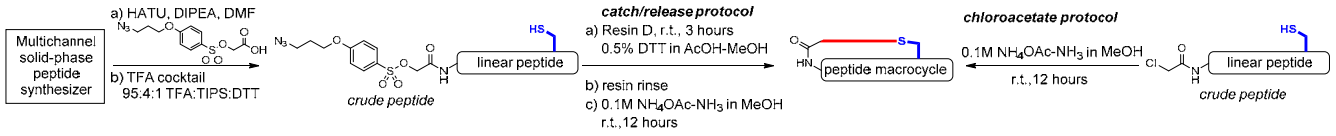
Scheme 2. Optimization of the catch–release protocol for peptide purification. a) Loading of the strained alkyne onto PEG-based resins using four different linker chemistries. b) Exploration of the stability of the strained alkyne linker under basic conditions. c) The free-thiol present on the peptide–resin quantified with the Ellman test[38] with various reductants. d) Impact of resin D equivalents and "quenching" post-capture, on the recovery of the peptide macrocycle post release. e) Survey of bases and solvents in the release. Refer to Supporting Information for full experimental details. DTT = 1,4-dithiothreitol; TCEP = tris(2-carboxyethyl) phosphine; AUC= area under curve; HPLC Area Percent (AP%) refers to percent purity

products due to the presence of *N*-methylated amino acids which arise from both hydrolysis of the amide backbone and incomplete couplings between ^{Me}Phe–^{Me}Gly, ^{Me}Ser–Gly and Val–^{Me}Ser. Based on LCMS traces, the catch–release approach demonstrated complete removal of acetate-capped truncated byproducts in addition to the hydrolyzed sequences. The unique capabilities of catch–release for removing these undesirable byproducts combine to result in high purity of the final macrocyclic peptides. Additionally, the macrocyclization strategy is remarkably effective for all the ring sizes evaluated (5 to 20 amino acids).

Careful evaluation of LCMS spectra of the starting linear peptides and the isolated macrocycles from the catch–release protocol (Table **1**) revealed several liabilities that limited final purities. For example, those derived from backbone isomerization through aspartimide formation were observed in entries **12–17**, and Arg side chain degradants generated during TFA cleavage were observed in entries **9** and **11**. Additionally, in entries **2–17**, impurities were tracked back to the commercially available starting material Fmoc-^{Me}Ser(tBu)-OH, which contained a deformylated impurity (Fmoc-^{Me}Gly-OH), which was not effectively removed in the catch–release protocol. While the starting peptides were synthesized with generic peptide coupling conditions, in principle, the occurrence of these deleterious impurities can be minimized by judicious selection of starting materials, SPPS and TFA cleavage conditions. It is noteworthy to mention that all the impurities described above were also observed in comparable levels in the chloroacetate cyclizations and are not generated during the catch–release protocol itself.

With an optimized protocol in hand, encouraging empirical efficiency in a head-to-head comparison with the chloro-acetate cyclization (Table **1**) and an understanding of limitations to the protocol from thorough analysis of impurities (*vide supra*), we sought to employ the strategy

Table 1. A head-to-head comparison of the catch–release to chloroacetate cyclization



Entry	*Linear Peptide Sequence*	*Ring Size (in amino acids)*	*Relative recovery (%, CR/Cl)*[a]	*Chloroacetate Macrocycle Purity (%)*[b]	*Catch–Release (CR) Macrocycle Purity (%)*[b]
1	dW^{Me}ADVCG-NH$_2$	5	83	89	95
2	dW^{Me}ADV^{Me}SCG-NH$_2$	6	66	49	88
3	dW^{Me}ADV^{Me}SGCG-NH$_2$	7	N/O	70	88
4	dW^{Me}ADV^{Me}SGRCG-NH$_2$	8	98	64	91
5	dW^{Me}ADV^{Me}SGR ^{Me}FCG-NH$_2$	9	146[39]	17	80
6	dW^{Me}ADV^{Me}SGR ^{Me}F^{Me}GCG-NH$_2$	10	64	30	78
7	dW^{Me}ADV^{Me}SGR^{Me}F^{Me}GYCG-NH$_2$	11	72	20	69
8	dW^{Me}ADV^{Me}SGR^{Me}F^{Me}GY^{Me}FCG-NH$_2$	12	105[39]	7	69
9	dW^{Me}ADV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG-NH$_2$	13	50	13	61
10	CM$_{11}$-1: dWCDV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG-NH$_2$	13	28	12	91
11	dWP^{Me}ADV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG-NH$_2$	14	33	6	60

12	dWDP^{Me}ADV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG-NH$_2$	15	74	8	81
13	dWGDP^{Me}ADV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG-NH$_2$	16	51	7	74
14	dW^{Me}SGDP^{Me}ADV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG-NH$_2$	17	29	6	70
15	dWD^{Me}SGDP^{Me}ADV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG-NH$_2$	18	50	5	75
16	dWYD^{Me}SGDP^{Me}ADV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG-NH$_2$	19	57	6	75
17	dW^{Me}AYD^{Me}SGDP ^{Me}ADV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG-NH$_2$	20	44	3	70

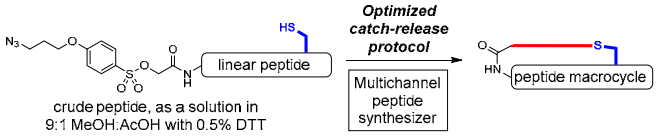
[a]The relative recovery of the catch–release process vs. the chloroacetate process is analogous to the yield, and was determined by quantitative HPLC. [b]Purity was determined by HPLC. ^{Me}AA: *N*-methylated amino acids; dAA: *D*-amino acids.

in an automated, high-throughput format.

Automation of the catch–release protocol

The ongoing interest in evaluating cyclic peptides as potential therapeutic agents requires the development of automated platforms, which enable parallel synthesis technologies. Having obtained optimized conditions and demonstrated efficiency in a head-to-head comparison, the solid-phase cyclization strategy appeared well suited for automation. We prepared a twelve member library of macrocyclic peptides based on G7-18NATE,[40] (Table **2**). We then utilized the "cleavage" functionality of a standard multichannel peptide synthesizer to demonstrate the catch–release protocol in an automated library setting.

Table 2. Automation of the optimized catch–release protocol on a multichannel peptide synthesizer



Entry	*Linear Peptide Sequence*	*Linear Purity (%)*[a]	*Macrocycle Purity (%)*[a]
1	FVE^{Me}GCG-NH$_2$	72	93
2	FVE^{Me}GYCG-NH$_2$	62	98
3	FVE^{Me}GY^{Me}FCG-NH$_2$	64	92
4	FVE^{Me}GY^{Me}F^{Me}GCG-NH$_2$	62	97
5	FVE^{Me}GY^{Me}F^{Me}GTCG-NH$_2$	60	96
6	FVE^{Me}GY^{Me}F^{Me}GT^{Me}FCG-NH$_2$	12	96
7	FVE^{Me}GY^{Me}F^{Me}GT^{Me}FPCG-NH$_2$	31	92
8	FVE^{Me}GY^{Me}F^{Me}GWT^{Me}FPCG-NH$_2$	12	96
9	FVE^{Me}GY^{Me}F^{Me}GRWT^{Me}FPCG-NH$_2$	18	98
10	FVE^{Me}GY^{Me}F^{Me}GRAWT^{Me}FPCG-NH$_2$	8	97
11	FVE^{Me}GY^{Me}F^{Me}GRAYWT^{Me}FPCG-NH$_2$	11	93
12	FVE^{Me}GY^{Me}F^{Me}GRNAYWT^{Me}FPCG-NH$_2$	8	95

[a] Purity: conversion by HPLC.

Resin D was loaded into the reaction vessels, utilizing the extra amino acid positions for the solutions of crude linear peptide, and the basic "release solution" (0.1M NH_4OAc/NH_3 in MeOH) replaced the TFA cleavage solution. In this fashion, the instrument was programmed for consistent and reproducible peptide and solvent delivery, with automated macrocyclization and product collection in the cleavage vessels (workflow and synthesis program available in SI). The model peptide library, composed of sequences 5–16 amino acids in length, was delivered through library automation as summarized (Table **2**). While the HPLC purity of the linear peptides ranged from 8–72%, the subsequent automated catch–release protocol produced cyclic peptides with exceptional purity (higher than 92%) for *all* sequences, a purity level generally acceptable for direct evaluation in biological screening assay without further manipulation.

Extension of the scope to amine bridged macrocycles

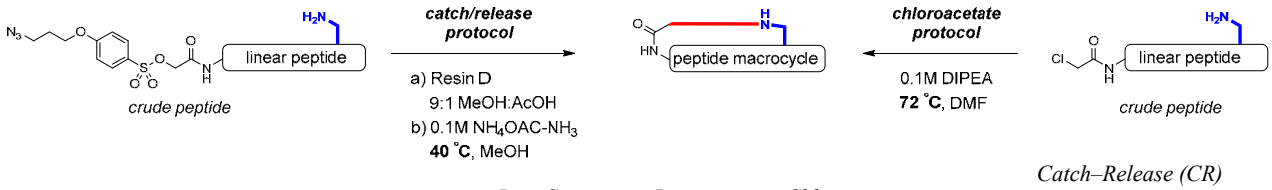
With the success of the catch–release protocol demonstrated, we sought to pursue an extension of the substrate scope to include a primary amine as nucleophile. While medicinally promising for several reasons (among them are an increased chemical stability, increased hydrophilicity, and an additional functional handle for further elaboration), there are a paucity of reports of macrocyclic peptides featuring a nitrogen bridge due to problems with their synthesis.[41] To this end, we replaced the nucleophilic cysteine with an amine analogue, 2,3-diaminopropionic acid (Dap). Initially we observed that achieving macrocyclization of the Dap containing peptides via chloroacetate displacement required elevated temperature, presumably due to the reduced nucleophilicity of nitrogen vs sulfur. Under optimized conditions, the chloroacetate solution-phase cyclization used DMF and 0.1M DIPEA, reaching completion in 12 h at 72 °C. In contrast to chloride, efficient cyclizations with the aforementioned arylsulfonate leaving groups used in catch-release were observed in 1 h at just 40 °C in 0.1 M NH4OAc-NH3/MeOH for the analogous substrates.

A broad screen of amine bridged analogues derived from the CM_{11}-1 was therefore prepared, with a head-to-head

comparison of the solution phase chloro-displacement and the analogous catch–release macrocyclization (Table **3**, *vide infra*). It is worth noting that the linear peptides were identical in each case prior to capping with the functionalized acetate, allowing a direct comparison of the two methodologies. The target *N*-backbone macrocycles were obtained in significantly higher purity from the catch–release protocol than those from the chloroacetate protocol in all cases, forming macrocycles 5–17 amino acids in size. For substrates from 5–9 amino acids in size (entries **1–5**), purities from 86 to >98% were obtained via the catch–release protocol, whereas the purity of the solution-phase chloro-cyclization rapidly dropped off to <10% (entries **3–7**) with the increase of the ring size. In entries **7–9**, the purity of the peptides obtained from the catch-release protocol was slightly diminished with the observation of two alternate cyclization pathways: cyclization of the indole side chain of the *N*-terminal tryptophan residues (entries **8, 9**) and cyclization of inter-sequence aspartic acid as a competitive nucleophile (entries **7, 9**).

Despite these alternative pathways, the purity remained good (55–87%), and dramatically improved versus the solution-phase cyclization (6–17%). In larger ring-sized macrocycles (13 to 17 amino acids, entries **11–15**), the purity ob

Table 3. Catch–release with nitrogen-bridged nucleophilic macrocyclization



Entry	*Linear Peptide Sequence*	*Ring Size (in amino acids)*	*Recovery (CR/Cl, %)*[a]	*Chloroacetate Macrocycle Purity (%)*[b]	*Catch–Release (CR) Macrocycle Purity (%)*[c]
1	^{Me}GY^{Me}FPDapG-NH$_2$	5	46	70	96
2	^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	6	87	49	93
3	K(Alloc)^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	7	30	8	>98
4	GK(Alloc)^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	8	47	5	>98
5	SGK(Alloc)^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	9	58	6	86
6	VSGK(Alloc)^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	10	71	6	76
7	^{Me}ADVSGK(Alloc)^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	12	87	6	55
8	dW^{Me}AYVSGK(Alloc)^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	13	86	17	68
9	dW^{Me}ADVSGR^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	13	232[39]	7	87
10	F^{Me}AYVSGK(Alloc)^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	13	54	5	76
11	F^{Me}AYVSGR^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	13	40	7	>98
12	FP^{Me}AYVSGK(Alloc)^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	14	60	<5	>98
13	F^{Me}GP^{Me}AYVSGK(Alloc)^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	15	37	<5	>98
14	FL^{Me}GP^{Me}AYVSGK(Alloc)^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	16	36	<5c	>98
15	FYL^{Me}GP^{Me}AYVSGK(Alloc)^{Me}F^{Me}GY^{Me}FPDapG-NH$_2$	17	28	<5	>98

[a] Relative recovery is analogous to the yield, determined by quantitative HPLC. [b] Purity was determined by HPLC. [c] This experiment was done in MeOH. Alloc = allyloxycarbonyl, Dap = 2,3-diaminopropionic acid.

tained from the catch–release protocol was dramatically higher than the chloroacetate macrocyclization; in the chloroacetate protocol, it became difficult to discern the product from the complex reaction mixture. In this sense, the solid-phase assisted purification was observed to be an enabling technology for the synthesis of backbone amine peptide macrocycles.

Conclusion

We have invented a protocol for the non-chromatographic purification of macrocyclic peptides that's fundamentally orthogonal to standard purification techniques. The utilization of a dual-functionalized capping agent serves as the lynchpin for both purification and macrocyclization. Impurities that lack the capping reagent are rinsed away during the "catch" with the "release" macrocyclization removing impurities lacking a nucleophile. Thus, throughput method using standard equipment. This has the potential to dramatically increase the throughput of peptide discovery. Eliminating the requirements for expensive and inefficient prep-chromatography, significantly reduces the barrier to entry for researchers interested in exploring this fascinating field. Finally, this method enables new nucleophiles to be utilized in the key macrocyclization, as demonstrated by the formation of amine bridged macrocycles. As such, the catch-release protocol represents an enabling technology for non-sulfur derived macrocycles, where traditional approaches fail. The further development of processes that enable the simple, cheap and efficient purification of this important chemical class will facilitate further advances in health care.

EXPERIMENTAL

Methods for LC-MS and HRMS analysis: Waters CORTECS C18 (2.7 µm, 4.6 x 150 mm) analytical column using mobile phase water–acetonitrile with 0.05% TFA (v/v) modifier, with a flow rate 1.3 mL/min, 60 °C column oven temperature, and monitoring at 220 nm wavelength. ESI-MS was used for peptide characterization. The solvent gradients employed, are summarized in the Supporting Information. The HRMS analysis was performed on all the final macrocyclic peptides on an LTQ Orbitrap mass spectrometer (positive electrospray ionization, 4.5 kV) in line with UPLC, which allowed collection of molecular ion data with accuracy of <5 ppm.

General procedure for SPPS

Linear peptides were assembled on a 100 µmol scale by standard Fmoc chemistry using HATU/NMM systems on an automated peptide synthesizer (Symphony X, Protein Technologies), with 1.5 equive of amino acid relative to the resin loading. Rink amide AM resin with 0.54 mmol/g loading was used. The concentrations of reagents were as follows: 0.075 M Fmoc-protected amino acid (delivered 2 mL), 0.15 M HATU (delivered 1 mL), and 0.15 M NMM (delivered 2 mL) in DMF. Coupling time was adopted to 20 min across all couplings. Double coupling was performed for hindered, unnatural, and *N*-methylated amino acids. Each coupling was followed by double treatment with Ac_2O-DIPEA with 5 mL, 10 to 2 ratio in DMF for 10 min. Fmoc deprotections were carried out as double treatments with 20% piperidine in DMF (5 mL) for 5 min each. After each coupling, Ac2O capping, and deprotection step, the resin was washed with DMF (5 mL, 5 x 30 sec).

General procedure for chloroacetate and azidosulfonylacetate D capping: Swelling of the Fmoc-protected peptide-bound resin was conducted as pre-treatment with DMF (3 x 10 min). After draining of DMF, the Fmoc group was deprotected by adding 20% piperidine in DMF solution (2 x 4 mL, 5 min each). The solution was drained, and the resin was washed with DMF-DCM sequentially (5 x 4 mL). Chloroacetate capping on a 25-umol scale: in two separate vials were weighed chloroacetic acid (24 mg, 0.25 mmol) and DIC (32 mg, 0.25 mmol). Each compound was dissolved in 1 mL of DMF. The two solutions were mixed for 1 min and immediately added to the Fmoc-deprotected resin, with nitrogen sparge to mix for 1 h, followed by draining the solution. The resin was washed sequentially with DMF-DCM (5 x 4 mL), and finally with diethyl ether, then was dried under vacuum for 2 h. Azidosulfonylacetate **D** peptide capping on a 75-umol scale: In three separate vials were weighed the azidosulfonylacetate capping reagent **D** (71 mg, 0.23 mmol), HATU (86 mg, 0.23 mmol), and DIPEA (30 mg, 0.23 mmol). Each compound was dissolved in 700 µL of DMF. To the Fmoc-deprotected resin was added sequentially DIPEA, the azidosulfonylacetate capping reagent, and finally HATU. The solution was mixed with nitrogen sparge for 1 h, followed by draining the solution. The resin was washed sequentially with DMF-DCM (5 x 4 mL), and finally with diethyl ether, then was dried under vacuum for 2 h.

General procedure for peptide cleavage: To the dry resin was added 25 mL/g of the cleavage cocktail: 97% TFA, 2.5% TIS, 0.5% DTT; 3 mL for 120 mg of resin. Resins were stirred for 1 h at room temperature. After which, the resin was filtered, and the solution was dripped into 30 mL of cold diethyl ether. Peptide was precipitated as a white solid. The mixture was centrifuged (5 min, 3000 rpm, 0 °C), decanted and the remaining peptide pellet was washed twice with 15 mL of cold ether as described above. The crude peptide was dissolved in 10-20% water-acetonitrile, and a sample solution was injected in LCMS for analysis of the corresponding linear peptide. The above water-acetonitrile solution was then lyophilized overnight, and the

this protocol serves as a logic test for the presence of both the *N*-terminal lynchpin and the tethered nucleophile, a logic test which eliminated most observed classes of peptide impurities. In practice, we have demonstrated that the most common impurities observed are removed, with the final peptide purities frequently meeting the criteria for direct evaluation of biological activity. Importantly, the current procedure has been fully automated in a high

weight of the obtained linear peptide was recorded and the ratio of the obtained peptide (mg) vs. the Fmoc resin (mg) was calculated.

General procedure for solution phase cyclization of linear peptides: With MeOH as the solvent. The lyophilized peptide (20–30 mg) was dissolved in MeOH (4.5 mL) in a scintillation vial. Then a solution of 0.2 M NH_4OAc-NH_3 (pH=9.3, 4.5 mL) was added slowly to the MeOH solution. For the cyclizations with nucleophilic sulfur, the mixture was stirred at room temperature for 12-18 h. For the cyclizations with nucleophilic amine, the mixture was stirred at 72 °C for 12–18 h. The reaction mixture was transferred into a specific volumetric flask, the vial was washed, and the solutions were combined and diluted with MeOH to the desired volume. The resulting solution was directly injected in LCMS for analysis of area under curve (AUC) and percent purity values.

With DMF as the solvent. The lyophilized peptide (20–30 mg) was dissolved in DMF (4.5 mL) in a scintillation vial. Then a solution of 0.2 M DIPEA in DMF (pH=11, 4.5 mL) was added slowly to the DMF solution. For the cyclizations with nucleophilic sulfur, the mixture was stirred at room temperature for 12–18 h. For the cyclizations with nucleophilic amine, the mixture was stirred at 72 °C for 12–18 h. The solvent was removed using Genevac and the peptide residue was redissolved in MeOH. The solution was transferred into a specific volumetric flask, the vial was washed, and the solutions were combined and diluted with MeOH to the desired volume. The resulting solution was directly injected in LCMS for analysis of area under curve (AUC) and percent purity values.

Optimized procedure for catch–release purification of crude linear peptides:

a) Catch of the linear peptide. A fresh stock solution of 0.5% DTT and 10% acetic acid in MeOH was prepared. In a 12-mL syringe equipped with a polypropylene frit, the anchored strained cyclooctyne resin, DBCO resin D for most experiments (4 equiv with respect to the empirically derived DBCO loading and the crude peptide), was weighed and swelled with DCM for 30 min. The solution was drained. The lyophilized peptide (20–30 mg) was dissolved in 250 µL of MeOH and sonicated. The resin was suspended in 750 µL of the stock solution. The above peptide solution was added slowly to the resin suspension. The peptide vial was washed twice with 250 µL of the stock solution and added to the resin. Final concentration of the peptide was in the range of 10–13 mg/mL. The resulting mixture was stirred at room temperature for 3–5 h and monitored by LCMS to confirm the completion of the reaction. For reactions that were run more dilute, click reaction was allowed to proceed 18 h. After the click reaction was completed, the solution was drained, and the resin was washed with the stock solution (3 x 4 mL). The resin was suspended in the stock solution (1 mL) and the excess alkyne resin D was quenched with 20 µL of benzyl azide for 30 min. The solution was drained, and the resin was washed successively with the stock solution (5 x 4 mL), then MeOH (2 x 4 mL).

b) Macrocyclization release of the peptide macrocycle. The resin was suspended in MeOH (4.5 mL), then a solution of 0.2 M NH_4OAc-NH_3 in MeOH (4.5 mL) was added. For the cyclizations with nucleophilic sulfur, the reaction mixture was stirred at room temperature for 18 h. For the cyclizations with nucleophilic amines, the reaction mixture was stirred at 40 °C for 18 h. Then the resin was filtered, and the solution was collected in a 100-mL round-bottomed flask. The resin was washed with 0.1 M NH_4OAc-NH_3 solution in MeOH (7 x 5 mL), then MeOH (2 x 2 mL), and finally 50% acetonitrile-water (2x 2 mL). The solvents were reduced using a rotavap at 23 °C and the peptide residue was re-dissolved in MeOH. The resulting solution was transferred into a volumetric flask (if performing a head-to-head comparison with the solution phase reaction, then the same size as that was used in the solution phase cyclization, and diluted to the desired volume. The solution was directly injected in LCMS for further analysis and direct comparison with chloroacetate solution phase cyclization.

Calculation of the macrocycle relative recovery: The overall relative recovery of macrocyclic peptides via solution phase chloroacetate cyclization and catch-release was calculated as following. Starting from the same Fmoc-capped linear peptide-bound resin, the resin was split into two equal portions. The first portion was capped with chloroacetate, denoted as R_{Cl} in the following formula and the second portion was capped with Azidosulfonylacetate **D**, denoted as R_{CR}. Resins were subjected to cleavage cocktail, peptides were precipitated and dried to obtain chloroacetate capped linear peptide, denoted as P_{Cl} and Azidosulfonylacetate capped linear peptide, denoted as P_{CR}. For comparison studies, specific amount of each crude linear peptide was weighed, S_{Cl} for chloroacetate capped and S_{CR} for the catch-release and proceed for cyclization using the above-mentioned procedure to obtain the area under the curve of the desired macrocycle, denoted as AUC_{Cl} and AUC_{CR}. As described above, the injection and final dilution volumes are the same for the chloroacetate solution cyclization and catch–release protocol, therefore the relative recovery ratio of cyclized product was calculated for each sample using the following equation:

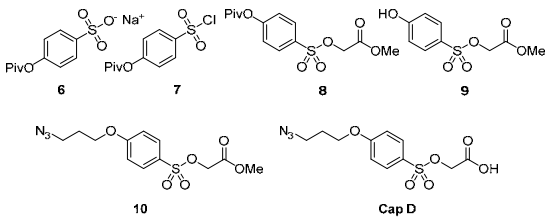
$$Relative\ Recovery = \left(\frac{AUC_{CR} \times P_{CR}}{R_{CR} \times S_{CR}}\right) \div \left(\frac{AUC_{Cl} \times P_{Cl}}{R_{Cl} \times S_{Cl}}\right)$$

AUC = HPLC area under the curve
R: mg Fmoc resin that was capped
S: mg linear peptide for each head-to-head experiment
P: total mg crude linear peptide obtained after cleavage
Subscript CR: values for the catch–release experiment
Subscript Cl: values for the chloroacetate experiment

General procedure for automated catch-release: First, the amino acid file for automated catch-release was created and defined as single shots. The solvent file was created for the catch-release process. The linear peptide solutions were prepared in 0.5% DTT and 10% acetic acid in MeOH (6 mL, average concentration 10 mg/mL) and placed in the single shot spots of the instrument. The solution of 0.1 M benzyl azide in 0.5% DTT and 10% acetic acid in MeOH was placed in one of the positions for unnatural amino acids (positions 1-8). The solution of 0.1 M NH_4OAc-NH_3 in MeOH was placed in the cleavage solvent position and MeOH solvent for intermittent washes was placed in one of the unused solvent places. The DBCO-tethered resin D (low loading) was weighed out (4 equiv to peptide) and swelled in 5 mL of DCM. For each peptide, a sequence of single shot was created. The following preswelling and synthesis program were used for automated catch-release. The pause in the synthesis program was added to allow the opportunity to check for completion of the click reaction. The final solutions were collected in cleavage vessels and analyzed by LCMS.

NMR Spectroscopy: NMR data were acquired on a 600 MHz Bruker AVANCE III HD NMR spectrometer with a TCI (1H/19F,13C,15N,2H) cryprobe equipped with actively shielded z-gradient coils. Samples were prepared in DMSO-d_6 (99.9% deuteration, Cambridge isotope Lab) and placed in 5 mm tubes. The sample temperature was 25 °C. The proton spectral dimensions were referenced to set the DMSO- d_5 1H peak to 2.50 ppm and the carbon spectral dimensions were referenced by setting the DMSO-d_6 peak at 39.52 ppm. Spectra data are reported in the format: chemical shift (multiplicity, coupling constants, and number of hydrogens). ^{13}C NMR spectra were proton decoupled using WALTZ16-decoupling. Detailed conditions for 2D NMR and structure elucidation of Table 1, Entry 1 and Table 3, Entry 1 are reported in SI.

Synthesis of 2-(((4-(3-Azidopropoxy)phenyl)sulfonyl)oxy)acetic acid (cap D)



To a 500-mL round bottom flask was added a magnetic stir bar, sodium 4-hydroxybenzenesulfonate (limiting reagent, 25.02 g, 126.3 mmol), and pivalic acid (1.1 equiv, 14.2 g, 139 mmol); the flask was capped with a septum and positive pressure nitrogen line. To this flask was added trifluoroacetic acid (3 mL/g, 75 mL), which formed a thick slurry upon stirring. Upon the addition of trifluoroacetic anhydride (4 equiv, 71.4 mL, 505 mmol), dropwise, the solution became hot, and went homogenous. Then, the stir bar was removed, and the solution concentrated *in vacuo* in a 40 °C water bath to a white powder (crude mass of 54.7 g). Acetonitrile (100 mL) was added, and the solution was evaporated again *in vacuo*, then placed on high vacuum for 3 h. The final crude **6**, as a white powder, had a mass of 38.66 g.

To the flask containing **6** (126 mmol, MW 280.27, crude), as a white powder, was added a magnetic stir bar and capped with a septum and positive pressure nitrogen line. To this flask was added dichloromethane (5 mL/g, 177 mL), DMF (0.05 mL/mmol, 1.76 mL) through the septum. The solution formed a thick slurry upon stirring. To this solution was added oxalyl chloride (2.0 mol/L solution in DCM, 158 mL, 316 mmol). The solution was allowed to stir at ambient temperature overnight. Once complete, the crude reaction mixture was poured into ethyl acetate (20 mL/g, 697 mL), and washed with water (8 mL/g, 279 mL), then 20% brine (8 mL/g, 279 mL), then saturated brine (4 mL/g, 139 mL). The rich organic layer was dried over magnesium sulfate, filtered, and concentrated *in vacuo* to a solid. The crude solid was isolated by crystallization from 1.5:1 heptane: toluene (v/v, 4 mL/g, 139 mL) for a first crop, then a second crop of crystals was obtained from 3:1 heptane: toluene (v/v, 1.15 mL/g, 40 mL). The combined isolated yield of **7** was 76% (26.46 g, 95.6 mmol). The spectroscopic data of **7** matches that of the literature and commercial compound.[43] ^1H NMR (500 MHz, $CDCl_3$) δ 8.09 (d, J = 8.7 Hz, 2H), 7.37 (d, J = 8.7 Hz, 2H), 1.41 (s, 9H) ppm.

To a 300-mL round bottom flask was added a magnetic stir bar, 4-(chlorosulfonyl)phenyl pivalate (**7**, 1.05 equiv, 26.02 g, 94.0 mmol), and the flask was capped with a septum with positive pressure nitrogen line. To the flask was added tetrahydrofuran (25 g/mL relative to methyl glycolate, 202 mL), added via cannula through the septum, and the flask was cooled in a 0 °C ice bath. Upon equilibrating to temperature, methyl glycolate (limiting reagent, 8.06 g, 89.49 mmol) was added in a single portion, followed by dropwise addition of triethylamine (1.1 equiv, 9.74 g, 96.3 mmol) over a minute. The reaction was left to warm to room temperature overnight. Progress was monitored by HPLC, and the reaction was complete after 24 h. To work up, the process stream was diluted with MTBE (10 mL/g relative to the input sulfonyl chloride, 260 mL), and subsequently washed

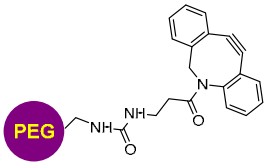
with the following: twice with half-saturated aqueous ammonium chloride (8 mL/g, 208 mL each wash), then water (8 mL/g, 208 mL), then brine (4 mL/g, 104 mL). The rich organic stream was then dried over magnesium sulfate, filtered, and concentrated to dryness *in vacuo* to form a thick oil that solidified upon standing. The crude **8** was crystallized from hot 3:1 heptane: MTBE (v/v, 4 mL/g of input sulfonyl chloride). Pure **8**, as off-white crystals, was isolated upon cooling to 0 °C with rapid stirring. The isolated yield of **8** was 84% (24.73 g, 74.9 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 (d, *J* = 8.7 Hz, 2H), 7.27 (d, *J* = 8.7 Hz, 2H), 4.64 (s, 2H), 3.73 (s, 3H), 1.37 (s, 9H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.2, 166.3, 155.6, 132.6, 129.8, 122.6, 64.8, 52.7, 39.3, 27.0 ppm; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calc'd for $C_{14}H_{19}O_7S$ 331.0846; Found 331.0845.

To a 500-mL flask with **8** (limiting reagent, 24.33 g, 73.64 mmol) was added a magnetic stir bar and MeOH (5 mL/g, 122 mL). The flask was cooled to 0 °C in an ice bath with moderate stirring, and formed a thin slurry upon cooling. Upon equilibration to temperature, sodium methoxide (25 wt% solution in MeOH, 1.05 equiv, 17.7 mL, 77.3 mmol) was added slowly via syringe pump over 30 min. The reaction reached homogeneity 10 min after completion of the sodium methoxide addition, and reaction completion was observed by HPLC. The reaction was quenched with the addition of aqueous hydrochloric acid (1 mol/L, 147 mL, 147 mmol). The process stream was extracted three times with dichloromethane (8 mL/g, 195 mL each), and the combined organic layers were washed with brine (5 mL/g, 122 mL), dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude product was then azeotroped with toluene to yield an off-white crystal. The solid **9** recovered, 17.13 g, was telescoped forward as is. ^{1}H NMR (500 MHz, CD_3OD) δ 7.78 (d, *J* = 7.5 Hz, 2H), 6.97 (d, *J* = 7.6 Hz, 2H), 6.48 (s, 1H), 4.63 (s, 2H), 3.71 (s, 3H) ppm; ^{13}C NMR (126 MHz, CD_3OD) δ 167.1, 163.0, 130.3, 125.1, 115.6, 64.4, 51.5 ppm; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calc'd for $C_9H_{11}O_6S$ 247.0271; Found 247.0279.

To the flask containing the crude **9** (limiting reagent, 15.20 g, 61.73 mmol) was added a magnetic stir bar, 2-methyltetrahydrofuran (10 mL/g, 152 mL), and cooled in a 0 °C bath. Upon equilibration to temperature, 3-azidopropan-1-ol (1.1 equiv, 7.04 g, 69.6 mmol) was added. Then, triphenylphosphine (1.3 equiv, 21.02 g, 80.14 mmol) was added in one portion, followed immediately by the dropwise addition of diisopropyl azodicarboxylate (1.3 equiv, 16.32 g, 80.71 mmol) over 1 min. The reaction was complete after 1 h. The crude process stream was diluted in MTBE (10 mL/g, 152 mL), washed with half-saturated ammonium chloride (5 mL/g, 76 mL), then water (5 mL/g, 76 mL), and finally brine (5 mL/g, 76 mL). The rich organic layer was dried over magnesium sulfate, filtered, and concentrated *in vacuo*. The oil thus obtained was purified by column chromatography (silica, hexane/ethyl acetate, eluted at 35% ethyl acetate). **10** was obtained after concentration as a solid with a yield of 75% (26.13 g, 58% potency by qNMR (CD_3OD, fumaric acid internal standard), with 41% reduced-DIAD). This material was used without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.88 (d, *J* = 8.5 Hz, 2H), 7.02 (d, *J* = 8.4 Hz, 2H), 4.59 (s, 2H), 4.14 (t, *J* = 5.2 Hz, 2H), 3.74 (s, 3H), 3.54 (t, *J* = 6.0 Hz, 2H), 2.09 (quin, *J* = 5.9 Hz, 2H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.6, 163.2, 130.4, 127.1, 114.9, 65.1, 64.5, 52.6, 48.0, 28.5 ppm; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calc'd for $C_{12}H_{19}N_4O_6S$ 347.1020; Found 347.1013.

To a 500-mL reaction flask was added **10** (limiting reagent, 25.58 g, 45.3 mmol, 58.3% potency) and a magnetic stir bar. To this flask was added MeOH (8 mL/g, 205 mL), which was heated gently while stirring. After reached homogeneity at a solution temperature of 32 °C, the solution was then allowed to cool and placed in a 0 °C ice bath. The solution formed a thick slurry at a solution temp of 12 °C. At a solution temperature of 10 °C, aqueous sodium hydroxide (1.0 mol/L, 1.5 equiv, 68 mL, 68 mmol) was added dropwise over 2 min, maintaining an internal solution temperature below 12 °C. Upon completion of the addition, the solution was homogenous, and reaction completion was observed. The solution was warmed to room temperature, and aqueous hydrochloric acid (1 mol/L, 2 equiv, 90 mL, 90 mmol) was added in one portion. A thick slurry rapidly formed, and the reaction was gently warmed to an internal solution temperature of 32 °C to reach homogeneity. Then, the solution was cooled slowly over 1 h to 0 °C to form a thick slurry, which was subsequently aged at 0 °C for 1 h with stirring. The thick slurry was filtered, and the wet cake was rinsed with 1:1 water: MeOH (v/v, 1mL/mmol, 45 mL). The isolated **cap D** thus obtained was collected and further dried over vacuum. The isolated yield of **cap D** was 86% (12.26 g, 38.9 mmol). The potency of the solid obtained was 100% by qNMR (CD_3OD, fumaric acid internal standard). Note: The ^{13}C NMR spectrum was acquired in $CDCl_3$ despite limited solubility; in CD_3OD, a signal was obscured by the solvent peak at 49 ppm. ^{1}H NMR (500 MHz, CD_3OD) δ 7.88 (d, *J* = 8.9 Hz, 2H), 7.14 (d, *J* = 9.0 Hz, 2H), 4.58 (s, 2H), 4.18 (t, *J* = 6.0 Hz, 2H), 3.53 (t, *J* = 6.6 Hz, 2H), 2.07 (quin, *J* = 6.3 Hz, 2H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.0, 163.4, 130.5, 126.8, 115.0, 65.2, 63.8, 48.0, 28.5 ppm; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calc'd for $C_{11}H_{14}N_3O_6S$ 316.0598; Found 316.0598.

Synthesis of resin D urea linker



resin D
SPAAC resin, urea linker

The resins were synthesized via the following procedures. Resin loading was then quantified by HPLC against an internal standard.

a) Preparation of the amino-PEG resin. To a 500-mL fritted bottom reactor was added Aminomethyl-PEG resin (0.56 mmol/g, 15.0 g, 8.4 mmol). The resin was swollen in DMF with nitrogen bubbling through the frit for agitation. After 5 min of agitation, the DMF was drained through the frit by vacuum. Then, the resin was washed similarly, in the following protocol [5 min per wash, with nitrogen bubbling for agitation]: DMF, DCM (twice), DMF, 2% DIPEA in DMF (v/v) (twice), and finally DMF. After draining the last rinse, the swollen resin was suspended in DMF to prepare for the loading.

b) Activation of the DBCO-amine. The dibenzocyclooctyne (DBCO) reagent was purchased commercially as a trifluoroacetate salt, which was freebased before activation. The DBCO-amine trifluoroacetate salt (95% purity, 1.64 g, 4.2 mmol) was added to a separatory funnel, followed by ethyl acetate (50 mL/g, 82 mL). A slurry formed, to which was added 0.4M aqueous potassium carbonate (25 mL/g, 41 mL, 16.4 mmol, 4 equiv), and then saturated sodium chloride (25 mL/g, 41 mL). The separatory funnel was shaken, and a homogenous bilayer rapidly formed. The lean aqueous was drained, and the rich organic layer was dried over magnesium sulfate, filtered, concentrated *in vacuo* to a thick oil. The thick oil was azeotroped with toluene (25 mL/g, 41 mL). The oil thus obtained was used directly, without purification. To the freebase DBCO-amine oil (4.2 mmol) was added dichloromethane (10 mL/g, 16.4 mL), and a magnetic stir bar, and the solution was cooled in a 0 °C ice bath. Upon equilibrating to temperature, DIPEA (1.1 equiv, 0.80 mL, 4.6 mmol) was added, followed by 4-nitrophenyl chloroformate (1.0 equiv, 0.88 g, 4.2 mmol) in one portion. The solution turned yellow immediately. Activation was verified by HPLC, to observe the 4-nitrophenyl carbonate intermediate.

c) Loading of the DBCO-amine. To the solution of activated DBCO-amine, was added 4-dimethylaminopyridine (0.10 equiv, 0.052 g, 0.42 mmol). The amino-PEG resin, which had been washed and suspended in DMF, was agitated by nitrogen bubbling. The activated DBCO-amine solution was added directly to the resin, and the solution was allowed to mix overnight with nitrogen bubbling for agitation. After agitation overnight, HPLC showed complete consumption of the 4-nitrophenyl carbonate intermediate. The solvent was drained from the resin through the frit with vacuum, and the resin was washed with DMF, DCM, and DMF. Then, an acetate capping solution was prepared in an Erlenmeyer flask by the addition of DMF (66 mL), DIPEA (22 mL, 126 mmol), and acetic anhydride (12 mL, 127 mmol). The resin was suspended in minimal DMF, and the capping solution was added in one portion, and allowed to mix with nitrogen agitation for 30 min. Then, the capping solution was drained, and the resin was washed extensively with DMF, then DCM, and finally MTBE. The resin was dried on vacuum with a slow nitrogen bleed for 16h. The mass of resin after drying was 15.30 grams. The resin loading was quantified following the general procedure above.

General procedure for the quantitation of the resin loading: The resin loading was determined by HPLC disappearance of an azide reagent relative to a known internal standard, as follows. Azide reagent **D**, prepared above, was massed into a 10 mL volumetric flask (MW 315.30, 40.0–50.0 mg), followed by Fmoc-proline-OH (internal standard, about 30 mg). This standard solution prepared with a diluent of MeOH, to prepare a solution of 12.7 to 15.9 millimolar concentration of azide. An HPLC sample of the standard solution was taken as a reference (100 µL of standard solution diluted in 500 µL of MeOH). Two samples of the resin (40.0–50.0 mg) were massed into small vials, to run the quantitation in duplicate. The resin was swollen in MeOH (0.20 mL), and then an aliquot of the standard azide solution (0.800 mL) was added to each vial. The vials were then vortexed and placed on a shaker to react. Samples of the supernatant of each vial were prepared after 6 h (100 µL of supernatant diluted in 500 µL of MeOH, then filtered for HPLC analysis). The loading for each sample and time point can then be calculated with the following equation:

$$loading\,\frac{mmol}{gram} = \left(1 - \frac{A_4 \times \frac{A_1}{A_3}}{A_2}\right) \times \frac{0.80\,mL \times B}{C}$$

A_1 = HPLC area of Fmoc-Pro-OH in the standard
A_2 = HPLC area of reagent **D** in the standard
A_3 = HPLC area of Fmoc-Pro-OH in the sample
A_4 = HPLC area of reagent **D** in the sample
B = mmol/mL of reagent **D** in the standard
C = g of resin massed in the sample

NMR data of Table 1, Entry 1. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.84 (d, J = 2.4 Hz, 1H), 8.63 (d, J = 6.9 Hz, 1H), 8.13 (d, J = 7.5 Hz, 1H), 7.97 (t, J = 5.8 Hz, 1H), 7.64 (s, 1H), 7.52 (d, J = 7.9 Hz, 1H), 7.31 (d, J = 8.0 Hz, 1H), 7.14 (d, J = 2.3 Hz,

1H), 7.11 (d, *J* = 4.4 Hz, 2H), 7.04 (t, *J* = 7.5 Hz, 1H), 6.96 (t, *J* = 7.4 Hz, 1H), 4.89 (q, *J* = 7.2 Hz, 1H), 4.83 (q, *J* = 6.8 Hz, 1H), 4.38 – 4.28 (m, 2H), 3.95 (t, *J* = 7.6 Hz, 1H), 3.70 – 3.57 (m, 2H), 3.41 (d, *J* = 14.4 Hz, 1H), 3.17 (d, *J* = 14.4 Hz, 1H), 3.12 (dd, *J* = 14.0, 8.7 Hz, 1H), 3.02 – 2.94 (m, 2H), 2.79 (dd, *J* = 13.8, 8.0 Hz, 1H), 2.76 – 2.60 (m, 2H), 2.51 (s, 3H), 1.99 (h, *J* = 6.8 Hz, 1H), 0.91 (d, *J* = 7.2 Hz, 3H), 0.83 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 172.2, 172.0, 171.0, 170.6, 170.4, 170.1, 170.0, 169.4, 136.0, 127.2, 123.9, 120.9, 118.4, 118.1, 111.4, 109.3, 59.0, 53.4, 52.0, 50.7, 50.5, 42.0, 36.1, 35.2, 33.9, 30.2, 29.2, 27.1, 19.3, 18.6, 12.9.

NMR data of Table 3, Entry 1. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.65 (d, *J* = 4.8 Hz, 1H), 9.11 (s, 1H), 8.86 (d, *J* = 9.8 Hz, 1H), 7.19 – 7.14 (m, 2H), 7.14 – 7.08 (m, 4H), 6.93 (t, *J* = 6.4 Hz, 1H), 6.79 (d, *J* = 8.5 Hz, 2H), 6.65 (s, 1H), 6.57 (d, *J* = 8.5 Hz, 2H), 4.79 (dd, *J* = 8.7, 6.1 Hz, 1H), 4.52 (dt, *J* = 9.9, 7.3 Hz, 1H), 4.32 (ddd, *J* = 10.3, 7.3, 2.3 Hz, 1H), 4.19 (dd, *J* = 9.7, 2.4 Hz, 1H), 4.08 (d, *J* = 15.8 Hz, 1H), 3.72 (td, *J* = 6.3, 5.6, 2.8 Hz, 1H), 3.49 (dd, *J* = 17.1, 7.3 Hz, 1H), 3.43 (d, *J* = 6.3 Hz, 2H), 3.25 (d, *J* = 15.9 Hz, 1H), 3.13 (dd, *J* = 14.7, 8.7 Hz, 1H), 3.02 – 2.96 (m, 1H), 2.93 (s, 3H), 2.93 – 2.87 (m, 1H), 2.86 – 2.79 (m, 1H), 2.72 (dd, *J* = 14.5, 6.0 Hz, 1H), 2.66 – 2.62 (m, 1H), 2.60 (s, 3H), 2.47 – 2.44 (m, 1H), 2.20 – 2.03 (m, 1H), 1.96 – 1.82 (m, 2H), 1.82 – 1.74 (m, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 174.4, 173.2, 171.0, 170.3, 169.7, 168.7, 167.4, 155.5, 137.7, 130.2, 129.0, 127.9, 127.6, 125.7, 114.7, 62.7, 58.2, 56.6, 52.8, 50.0, 49.7, 48.7, 47.2, 42.0, 37.3, 36.8, 35.6, 29.8, 28.2, 24.9.

LCMS and HRMS characterization of Table 1, Entries (1-17)

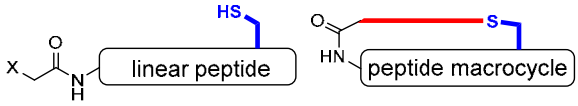


Table 1. Entry 1. Linear: X: Cl; HPLC analysis: t_R = broad peak 5.77-6.14, *Purity* 91%. X: OSO_2Ph-*p*-O-$(CH_2)_3N_3$; HPLC analysis: *tR* = broad peak 10.86-11.75, *Purity* 83.0%. Macrocycle-CR: HPLC analysis: *tR* = 5.16 min, *Purity* 95%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{31}H_{43}O_9N_8S$ 703.2868; found 703.2858.

Table 1. Entry 2. Linear: X: Cl; HPLC analysis: *tR* = broad peak at 5.27-5.78 min, *Purity*96%. X: OSO_2PhO-$(CH_2)_3N_3$; HPLC analysis: *tR* = broad peak 10.25-11.07, *Purity* 82.0%. Macrocycle-CR: HPLC analysis: *tR* = 4.68 min, *Purity* 88.0%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{35}H_{50}O_{11}N_9S$ 804.3345; found 804.3338.

Table 1. Entry 3. Linear: X: Cl; HPLC analysis: *tR* = 5.55 min, *Purity*70%. X: OSO_2PhO-$(CH_2)_3N_3$; HPLC analysis: broad peak *tR* = 9.94-10.72 min, *Purity* 84%. Macrocycle-CR: HPLC analysis: *tR* = 4.84 min, *Purity* 88%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{37}H_{53}O_{12}N_{10}S$ 861.3560; found 861.3550.

Table 1. Entry 4. Linear: X: Cl; HPLC analysis: *tR* = broad peak at 4.32-5.01min, *Purity* 84%. X: OSO_2PhO-$(CH_2)_3N_3$; HPLC analysis: *tR* = broad peak at 8.73-9.34 min, *Purity* 75%. Macrocycle-CR: HPLC analysis: *tR* = 4.49 min, *Purity* 91%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{43}H_{65}O_{13}N_{14}S$ 1017.4571; found 1017.4567.

Table 1. Entry 5. Linear: X: Cl; HPLC analysis: *tR* = broad peak at 6.05-6.40 min, *Purity* 37%. X: OSO_2PhO-$(CH_2)_3N_3$; HPLC analysis: *tR* = 10.83 min, *Purity* 28%. Macrocycle-CR: HPLC analysis: *tR* = 5.71 min, *Purity* 80.0%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{53}H_{76}O_{14}N_{15}S$ 1178.5411; found 1178.5405.

Table 1. Entry 6. Linear: X: Cl; HPLC analysis: *tR* = 6.36 min, *Purity* 51%. X: OSO_2PhO-$(CH_2)_3N_3$; HPLC analysis: *tR* = 10.90 min, *Purity* 50%. Macrocycle-CR: HPLC analysis: *tR* = 5.31 min, *Purity* 78%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{56}H_{81}O_{15}N_{16}S$ 1249.5783; found 1249.5789.

Table 1. Entry 7. Linear: X: Cl; HPLC analysis: *tR* = 6.86 min, *Purity* 29.2%. X: OSO_2PhO-$(CH_2)_3N_3$; HPLC analysis: *tR* = 11.19 min, *Purity* 27%. Macrocycle-CR: HPLC analysis: *tR* = 5.64 min, *Purity* 69.0%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{65}H_{90}O_{17}N_{17}S$ 1412.6416; found 1412.6411.

Table 1. Entry 8. Linear: X: Cl; HPLC analysis: *tR* = 9.16 min, *Purity* 16%. X: OSO_2PhO-$(CH_2)_3N_3$; HPLC analysis: *tR* = 13.04 min, *Purity* 14%. Macrocycle-CR: HPLC analysis: *tR* = 8.76 min, *Purity* 69%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{75}H_{102}O_{18}N_{18}S$ 787.3665; found 787.3664.

Table 1. Entry 9. Linear: X: Cl; HPLC analysis: *tR* = 8.64 min, *Purity* 13%. X: OSO_2PhO-$(CH_2)_3N_3$; HPLC analysis: *tR* = 12.5 min, *Purity* 13%. Macrocycle-CR: HPLC analysis: *tR* = 7.25 min, *Purity* 61%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{80}H_{108}O_{19}N_{19}S$ 1670.7784; found 1670.7770.

Table 1. Entry 10. Linear: X: Cl; HPLC analysis: *tR* = 8.50 min, *Purity* 16%. X: OSO_2PhO-$(CH_2)_3N_3$; HPLC analysis: *tR* = 12.38 min, *Purity* 16%. Macrocycle-CR: HPLC analysis: *tR* = 7.67 min, *Purity* 91%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{79}H_{106}O_{19}N_{19}S_2$ 1688.7348; found 1688.7315.

Table 1. Entry 11. Linear: X: Cl; HPLC analysis: *tR* = 8.60 min, *Purity* 7%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 12.34 min, *Purity* 8%. Macrocycle-CR: HPLC analysis: *tR* = 7.51 min, *Purity* 60%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{85}H_{116}O_{20}N_{20}S$ 884.4192; found 884.4197.

Table 1. Entry 12. Linear: X: Cl; HPLC analysis: *tR* = 7.83 min, *Purity* 8%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 11.69 min, *Purity* 7%. Macrocycle-CR: HPLC analysis: *tR* = 6.55 min, *Purity* 81%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{89}H_{121}O_{23}N_{21}S$ 941.9327; found 941.9334.

Table 1. Entry 13. Linear: X: Cl; HPLC analysis: *tR* = 7.56 min, *Purity* 6.0%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 11.37 min, *Purity* 5%. Macrocycle-CR: HPLC analysis: *tR* = 6.41 min, *Purity* 74%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{91}H_{124}O_{24}N_{22}S$ 970.4434; found 970.4431.

Table 1. Entry 14. Linear: X: Cl; HPLC analysis: *tR* = 7.30 min, *purity* 5.0%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 11.01 min, *Purity* 5%. Macrocycle-CR: HPLC analysis: *tR* = 6.20 min, *Purity* 70%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{95}H_{131}O_{26}N_{23}S$ 1020.9673; found 1020.9668.

Table 1. Entry 15. Linear: X: Cl; HPLC analysis: *tR* = 7.00 min, *Purity* 5.0%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 10.69 min, *Purity* 4%. Macrocycle-CR: HPLC analysis: *tR* = 5.97 min, *Purity* 75%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{99}H_{136}O_{29}N_{24}S$ 1078.4807; found 1078.4802.

Table 1. Entry 16. Linear: X: Cl; HPLC analysis: *tR* = 7.44 min, *Purity* 5%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 10.98 min, *Purity* 5%. Macrocycle-CR: HPLC analysis: *tR* = 6.43 min, *Purity* 75%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{108}H_{145}O_{31}N_{25}S$ 1160.0124; found 1160.0138.

Table 1. Entry 17. Linear: X: Cl; HPLC analysis: *tR* = 8.05 min, *Purity* 4%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 11.49 min, *Purity* 4%. Macrocycle-CR: HPLC analysis: *tR* = 6.88 min, *Purity* 70%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{112}H_{152}O_{32}N_{26}S$ 1202.5388; found 1202.5376.

LCMS and HRMS characterization of Table 2, Entries (1-12)

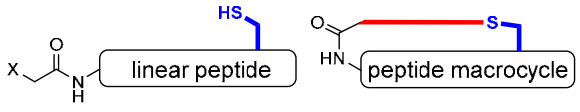


Table 2. Entry 1. Linear: X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 10.95 min, *Purity* 72%. Macrocycle-CR: HPLC analysis: *tR* = 4.78 min, *Purity* 93%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{29}H_{42}O_9N_7S$ 664.2759; found 664.2764.

Table 2. Entry 2. Linear: X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 11.42 min, *Purity* 62%. Macrocycle-CR: HPLC analysis: *tR* = 5.71 min, *Purity* 98%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{38}H_{51}O_{11}N_8S$ 827.3393; found 827.3395.

Table 2. Entry 3. Linear: X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 14.06 min, *Purity* 64%. Macrocycle-CR: HPLC analysis: *tR* = 8.35 min, *Purity* 92%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{48}H_{62}O_{12}N_9S$ 988.4233; found 988.4235.

Table 2. Entry 4. Linear: X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 13.21 min, *Purity* 62%. Macrocycle-CR: HPLC analysis: *tR* = 7.45 min, *Purity* 97%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{51}H_{67}O_{13}N_{10}S$ 1059.4604; found 1059.4603.

Table 2. Entry 5. Linear: X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 12.74 min, *Purity* 60%. Macrocycle-CR: HPLC analysis: *tR* = 6.18 min, *Purity* 96%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{55}H_{74}O_{15}N_{11}S$ 1160.5081; found 1160.5073.

Table 2. Entry 6. Linear: X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 14.93 min, *Purity* 12%. Macrocycle-CR: HPLC analysis: *tR* = 9.48 min, *Purity* 96%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{65}H_{85}O_{16}N_{12}S$ 1321.5922; found 1321.5957.

Table 2. Entry 7. Linear: X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 14.40 min, *Purity* 31%. Macrocycle-CR: HPLC analysis: *tR* = 8.52 min, *Purity* 92.0%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{70}H_{92}O_{17}N_{13}S$ 1418.6449; found 1418.6483.

Table 2. Entry 8. Linear: X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 15.92 min, *Purity* 12%. Macrocyclic-CR: HPLC analysis: *tR* = 10.85 min, *Purity* 96%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{81}H_{102}O_{18}N_{15}S$ 1604.7242; found 1604.7273.

Table 2. Entry 9. Linear: X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 13.86 min, *Purity* 18%. Macrocycle-CR: HPLC analysis: *tR* = 8.86 min, *Purity* 98%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{87}H_{115}O_{19}N_{19}S$ 880.9163; found 880.9176.

Table 2. Entry 10. Linear: X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 13.64 min, *Purity* 8%. Macrocycle-CR: HPLC analysis: *tR* = 8.35 min, *Purity*97%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{90}H_{120}O_{20}N_{20}S$ 916.4349; found 916.4362.

Table 2. Entry 11. Linear: X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 13.83min, *Purity* 11%. Macrocycle-CR: HPLC analysis: *tR* = 8.90 min, *Purity* 93%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{99}H_{129}O_{22}N_{21}S$ 997.9665; found 997.9686.

Table 2. Entry 12. Linear: X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 13.22 min, *Purity* 8%. Macrocycle-CR: HPLC analysis: *tR* = 8.28 min, *Purity* 95%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{103}H_{135}O_{24}N_{23}S$ 1054.9880; found 1054.9891.

LCMS and HRMS characterization of Table 3, Entries (1-15)

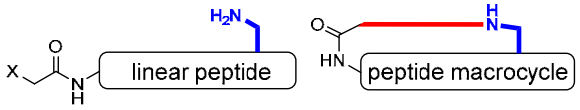


Table 3. Entry 1. Linear: X: Cl; HPLC analysis: *tR* = 4.22 min, *Purity* 82%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 7.98 min, *Purity* 72%. Macrocycle-CR: HPLC analysis: *tR* = 4.57 min, *Purity* 96%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{34}H_{45}O_8N_8$ 693.3355; found 693.3340.

Table 3. Entry 2. Linear: X: Cl; HPLC analysis: *tR* = 7.16 min, *Purity* 51%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 12.22 min, *Purity* 63%. Macrocycle-CR: HPLC analysis: *tR* = 6.64 min, *Purity* 93%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{44}H_{56}O_9N_9$ 854.4196; found 854.4174.

Table 3. Entry 3. Linear: X: Cl; HPLC analysis: *tR* = 8.48 min, *Purity* 5%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 13.53 min, *Purity* 4%. Macrocycle-CR: HPLC analysis: *tR* = 8.67 min, *Purity* 100%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{54}H_{72}O_{12}N_{11}$ 1066.5356; found 1066.5352.

Table 3. Entry 4. Linear: X: Cl; HPLC analysis: *tR* = 7.74 min, *Purity* 8%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 12.88 min, *Purity* 5%. Macrocycle-CR: HPLC analysis: *tR* = 8.65 min, *Purity* 100%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{56}H_{75}O_{13}N_{12}$ 1123.5571; found 1123.5555.

Table 3. Entry 5. Linear: X: Cl; HPLC analysis: *tR* = 6.48 min, *Purity* 7%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 11.13 min, *Purity* 5%. Macrocycle-CR: HPLC analysis: *tR* = 8.18 min, *Purity* 86%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{59}H_{80}O_{15}N_{13}$ 1210.5891; found 1210.5886.

Table 3. Entry 6. Linear: X: Cl; HPLC analysis: *tR* = 8.04 min, *Purity* 6%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 12.67 min, *Purity* 6%. Macrocycle-CR: HPLC analysis: *tR* = 8.82 min, *Purity* 76%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{64}H_{89}O_{16}N_{14}$ 1309.6575; found 1309.6572.

Table 3. Entry 7. Linear: X: Cl; HPLC analysis: *tR* = 6.73 min, *Purity* 8%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 10.78 min, *Purity* 8%. Macrocycle-CR: HPLC analysis: *tR* = 6.87 min, *Purity* 55%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{72}H_{101}O_{20}N_{16}$ 1509.7373; found 1509.7362.

Table 3. Entry 8. Linear: X: Cl; HPLC analysis: *tR* = 11.11 min, *Purity* 16%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 14.79 min, *Purity* 16%. Macrocycle-CR: HPLC analysis: *tR* = 11.12 min, *Purity* 68%. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{88}H_{115}O_{20}N_{18}$1743.8530; found 1743.8495.

Table 3. Entry 9. Linear: X: Cl; HPLC analysis: *tR* = 6.62 min, *Purity* 23%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 10.66 min, *Purity* 18%. Macrocycle-CR: HPLC analysis: *tR* = 6.99 min, *Purity* 59%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{79}H_{108}O_{19}N_{20}$ 820.4044; found 820.4029.

Table 3. Entry 10. Linear: X: Cl; HPLC analysis: *tR* = 10.53 min, *Purity* 5%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 14.34 min, *Purity* 4%. Macrocycle-CR: HPLC analysis: *tR* = 11.38 min, *Purity* 76%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{86}H_{115}O_{20}N_{17}$ 852.9247; found 852.9246.

Table 3. Entry 11. Linear: X: Cl; HPLC analysis: *tR* = 8.14 min, *Purity* 9%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 12.16 min, *Purity* 7%. Macrocycle-CR: HPLC analysis: *tR* = 8.07 min, *Purity* 100%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{82}H_{111}O_{18}N_{19}$ 824.9172; found 824.9160.

Table 3. Entry 12. Linear: X: Cl; HPLC analysis: *tR* = 8.83 min, *Purity* 1%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 14.63 min, *Purity* 0.7%. Macrocycle-CR: HPLC analysis: *tR* = 11.17 min, *Purity* 100%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{91}H_{122}O_{21}N_{18}$ 901.4515; found 901.4510.

Table 3. Entry 13. Linear: X: Cl; HPLC analysis: *tR* = 10.42 min, *Purity* 1%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 14.29 min, *Purity* 0.8%. Macrocycle-CR: HPLC analysis: *tR* = 10.47 min, *Purity* 100%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{94}H_{127}O_{22}N_{19}$ 936.9696; found 936.9703.

Table 3. Entry 14. Linear: X: Cl; HPLC analysis: *tR* = 11.80 min, *Purity* 1%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 13.76 min, *Purity* 0.7%. Macrocyclic-CR: HPLC analysis: *tR* = 11.43 min, *Purity* 100%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{100}H_{138}O_{23}N_{20}$ 993.5116; found 993.5117.

Table 3. Entry 15. Linear: X: Cl; HPLC analysis: *tR* = 11.93 min, *Purity* 1%. X: $OSO_2PhO\text{-}(CH_2)_3N_3$; HPLC analysis: *tR* = 13.71 min, *Purity* 0.9%. Macrocyclic-CR: HPLC analysis: *tR* = 11.49 min, *Purity* 100%. HRMS (ESI-TOF) m/z: $[M+2H]^{2+}$ Calcd for $C_{109}H_{147}O_{25}N_{21}$ 1075.0433; found 1075.0437.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website. The document includes Method optimization, HPLC chromatographs of all linear and macrocyclic peptides, synthesis of Cap **A-C** and resin **A-C**, Copies of ^{1}H-NMR, ^{13}C-NMR spectra of intermediates, reagents, and representative macrocyclic peptides, MS/MS characterization of macrocyclic side products and full automation program using SYMPHONY X peptide synthesizer.

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REFERENCES

(1) Yudin, A. K. *Chem. Sci.* **2015**, *6*, 30-49.

(2) Verdine, G. L.; Walensky, L. D. *Clin. Cancer Res.* **2007**, *13*, 7264-7270.

(3) Kotz, J. *SciBx,* **2012**, *5(45)*.

(4) Chatterjee, J.; Gilon, C.; Hoffman, A.; Kessler, H. *Accounts Chem. Res.* **2008**, *41*, 1331-1342.

(5) Rezai, T.; Bock, J. E.; Zhou, M. V.; Kalyanaraman, C.; Lokey, R. S.; Jacobson, M. P. *J. Am. Chem. Soc.* **2006**, *128*, 14073-14080.

(6) Biron, E.; Chatterjee, J.; Ovadia, O.; Langenegger, D.; Brueggen, J.; Hoyer, D.; Schmid, H. A.; Jelinek, R.; Gilon, C.; Hoffman, A.; Kessler, H. *Angew. Chem.-Int. Edit.* **2008**, *47*, 2595-2599.

(7) White, T. R.; Renzelman, C. M.; Rand, A. C.; Rezai, T.; McEwen, C. M.; Gelev, V. M.; Turner, R. A.; Linington, R. G.; Leung, S. S. F.; Kalgutkar, A. S.; Bauman, J. N.; Zhang, Y. Z.; Liras, S.; Price, D. A.; Mathiowetz, A. M.; Jacobson, M. P.; Lokey, R. S. *Nat. Chem. Biol.* **2011**, *7*, 810-817.

(8) Wang, C. K.; Northfield, S. E.; Colless, B.; Chaousis, S.; Hamernig, I.; Lohman, R. J.; Nielsen, D. S.; Schroeder, C. I.; Liras, S.; Price, D. A.; Fairlie, D. P.; Craik, D. J. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 17504-17509.

(9) Borel, J. F.; Feurer, C.; Gubler, H. U.; Stahelin, H. *Agents Actions* **1976**, *6*, 468-475.

(10) Schreiber, S. L.; Crabtree, G. R. *Immunol. Today* **1992**, *13*, 136-142.

(11) Blackwell, H. E.; Grubbs, R. H. *Angew. Chem.-Int. Edit.* **1998**, *37*, 3281-3284.

(12) Wilson, R. M.; Stockdill, J. L.; Wu, X. Y.; Li, X. C.; Vadola, P. A.; Park, P. K.; Wang, P.; Danishefsky, S. J. *Angew. Chem.-Int. Edit.* **2012**, *51*, 2834-2848.

(13) Verdine, G. L.; Hilinski, G. J.: Stapled Peptides for Intracellular Drug Targets. In *Methods in Enzymolog Protein Engineering for Therapeutics, Vol 203, Pt B*; Wittrup, K. D., Verdine, G. L., Eds.; Methods in Enzymology; Elsevier Academic Press Inc: San Diego, 2012; Vol. 503; pp 3-33.

(14) Frost, J. R.; Scully, C. C. G.; Yudin, A. K. *Nat. Chem.* **2016**, *8*, 1105-1111.

(15) Malins, L. R.; deGruyter, J. N.; Robbins, K. J.; Scola, P. M.; Eastgate, M. D.; Ghadiri, M. R.; Baran, P. S. *J. Am. Chem. Soc.* **2017**, *139*, 5233-5241.

(16) Bashiruddin, N. K.; Suga, H. *Curr. Opin. Chem. Biol.* **2015**, *24*, 131-138.

(17) Ito, K.; Passioura, T.; Suga, H. *Molecules.* **2013**, *18*, 3502-3528.

(18) Kotz, J. *SciBx.* **2012**, *5*.

(19) Osapay, G.; Prokai, L.; Kim, H. S.; Medzihradszky, K. F.; Coy, D. H.; Liapakis, G.; Reisine, T.; Melacini, G.; Zhu, Q.; Wang, S. H. H.; Mattern, R. H.; Goodman, M. *J. Med. Chem.* **1997**, *40*, 2241-2251.

(20) Roberts, K. D.; Lambert, J. N.; Ede, N. J.; Bray, A. M. *Tetrahedron Lett.* **1998**, *39*, 8357-8360.

(21) Robey, F. A.; Fields, R. L. *Anal. Biochem.* **1989**, *177*, 373-377.

(22) Yamagishi, Y.; Shoji, I.; Miyagawa, S.; Kawakami, T.; Katoh, T.; Goto, Y.; Suga, H. *Chem. Biol.* **2011**, *18*, 1562-1570.

(23) Hayashi, Y.; Morimoto, J.; Suga, H. *ACS. Chem. Biol.* **2012**, *7*, 607-613.

(24) Iwasaki, K.; Goto, Y.; Katoh, T.; Yamashita, T.; Kaneko, S.; Suga, H. *J. Mol. Evol.* **2015**, *81*, 210-217.

(25) Kodan, A.; Yamaguchi, T.; Nakatsu, T.; Sakiyama, K.; Hipolito, C. J.; Fujioka, A.; Hirokane, R.; Ikeguchi, K.; Watanabe, B.; Hiratake, J.; Kimura, Y.; Suga, H.; Ueda, K.; Kato, H. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 4049-4054.
(26) Morimoto, J.; Hayashi, Y.; Suga, H. *Angew. Chem.-Int. Edit.* **2012**, *51*, 3423-3427.
(27) Yamagata, K.; Goto, Y.; Nishimasu, H.; Morimoto, J.; Ishitani, R.; Dohmae, N.; Takeda, N.; Nagai, R.; Komuro, I.; Suga, H.; Nureki, O. *Structure* **2014**, *22*, 345-352.
(28) Tanaka, Y.; Hipolito, C. J.; Maturana, A. D.; Ito, K.; Kuroda, T.; Higuchi, T.; Katoh, T.; Kato, H. E.; Hattori, M.; Kumazaki, K.; Tsukazaki, T.; Ishitani, R.; Suga, H.; Nureki, O. *Nature* **2013**, *496*, 247-251.
(29) http://contents.xj-storage.jp/xcontents/45870/153f6866/eb01/4010/b742/326cb2a242cf/20161226144241324s.pdf.
(30) Teixido, M.; Albericio, F.; Giralt, E. *J. Pept. Res.* **2005**, *65*, 153-166.
(31) Urban, J.; Vaisar, T.; Shen, R.; Lee, M. S. *Int. J. Pept. Protein Res.* **1996**, *47*, 182-189.
(32) Nessen, M. A.; Kramer, G.; Back, J.; Baskin, J. M.; Smeenk, L. E. J.; de Koning, L. J.; van Maarseveen, J. H.; de Jong, L.; Bertozzi, C. R.; Hiemstra, H.; de Koster, C. G. *J. Proteome Res.* **2009**, *8*, 3702-3711.
(33) Aucagne, V.; Valverde, I. E.; Marceau, P.; Galibert, M.; Dendane, N.; Delmas, A. F. *Angew. Chem.-Int. Edit.* **2012**, *51*, 11320-11324.
(34) Zhang, M. C.; Pokharel, D.; Fang, S. Y. *Org. Lett.* **2014**, *16*, 1290-1293.
(35) Gaulier, C.; Hospital, A.; Legeret, B.; Delmas, A. F.; Aucagne, V.; Cisnetti, F.; Gautier, A. *Chem. Commun.* **2012**, *48*, 4005-4007.
(36) Saito, F.; Noda, H.; Bode, J. W. *ACS. Chem. Biol.* **2015**, *10*, 1026-1033.
(37) The coupling condition for installation of the third generation azide sulfonyl group onto the N-terminus of the protected peptide was optimized and is summarized in Table S1 of the Supporting Information.
(38) Riener, C. K.; Kada, G.; Gruber, H. J. *Anal. Bioanal. Chem.* **2002**, *373*, 266-276.
(39) As the relative recovery is a ratio of yields, it is possible to be over 100% if the macrocyclization reaction is more efficient with the arylsulfonyl leaving group relative to chloroacetate.
(40) Ambaye, N. D.; Lim, R. C. C.; Clayton, D. J.; Gunzburg, M. J.; Price, J. T.; Pero, S. C.; Krag, D. N.; Wilce, M. C. J.; Aguilar, M. I.; Perlmutter, P.; Wilce, J. A. *Biopolymers.* **2011**, *96*, 181-188.
(41) Oddo, A.; Munzker, L.; Hansen, P. R. *Org. Lett.* **2015**, *17*, 2502-2505.
(42) McGeary, R. R.; Bennett, A. J.; Tran, Q. B.; Prins, J.; Ross, B. P. *Tetrahedron* **2009**, *65*, 3990-3997.
(43) Bijukumar, G.; Maloyesh, B.; Sampat, S. S.; Bhirud, S. B.; Rajendra, A. *Synth. Commun.* **2008**, *38*, 1718-1724.