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On-Resin Macrocyclization of Peptides Using Vinyl Sulfonamides as a Thiol-Michael “Click” Acceptor

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Abstract

Macrocyclization of linear peptides imparts improved stability to enzymatic degradation and increases potency of function. Many successful macrocyclization of peptides both in solution and on-resin have been achieved but are limited in scope as they lack selectivity, require long reaction times, or necessitate heat. To overcome these drawbacks a robust and facile strategy was developed employing thiol-Michael click chemistry via an *N*-methyl vinyl sulfonamide. We demonstrate its balance of reactivity and high stability through FTIR model kinetic studies, reaching 88% conversion over 30 min, and NMR stability studies, revealing no apparent degradation over an 8 day period in basic conditions. Using a commercially available reagent, 2-chloroethane sulfonyl chloride, the cell adhesion peptide, RGDS, was functionalized and macrocyclized on-resin with a relative efficiency of over 95%. The simplistic nature of this process demonstrates the effectiveness of vinyl sulfonamides as a thiol-Michael click acceptor and its applicability to many other bioconjugation applications.

Graphical Abstract

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Author Contributions

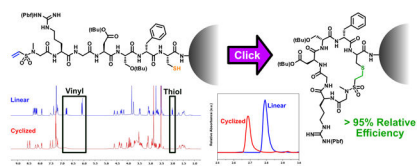
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Supporting Information

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Synthetic procedures for peptide synthesis and functionalization, NMR characterization for peptides and small molecules, and model degradation study

The Authors declare no competing financial interest.



Peptide conjugation is an ever-expanding field that allows for the creation of complex structures¹, molecular tagging², and material functionalization.³ Intramolecular conjugation reactions within a peptide result in a cyclic structure, or macrocycle. Peptide macrocycles are of great interest owing to their enhanced resistance to protease degradation⁴ and improved potency.⁵ A well-known example highlighting cyclic peptide advantages over linear peptides is demonstrated in the simple cell adhesion peptide sequence, RGDS.⁶ Higher stability and efficacy of cyclic peptides have led to further exploration into the synthesis of macrocyclic motifs of various peptide sequences for biological relevance⁷ or self-assembling structures.⁸

Macrocyclic peptides are often synthesized using solid-phase peptide synthesis (SPPS) protocols and cyclizing the product either in solution or on a solid substrate (i.e., on-resin). The solution-phase macrocyclization route implements a conjugation reaction on the fully protected peptide in dilute solution. Examples of solution-phase strategies include native chemical ligation,⁹ imine formation,¹⁰ and palladium-catalyzed conjugation reactions.^{11–12} However, solution-phase strategies largely necessitate an additional deprotection step followed by purification to remove cleaved protecting groups and oligomerized byproducts. The solid-phase macrocyclization route implements a conjugation reaction on the peptides while attached to the resin, resulting in fewer side products and purification steps.¹³ The advantages of on-resin macrocyclization has made it the preferred strategy for synthesizing cyclic peptide structure.

Several on-resin conjugation reactions have been employed for the macrocyclization of peptides, including thiol-ene,¹⁴ azide-alkyne cycloaddition,^{15–16} amide coupling^{17–18}, glacial coupling¹⁹, and ring-closing metathesis.^{20–21} In general, reactions demonstrating click-like characteristics are particularly attractive for the synthesis of cyclic peptides, as they are selective, proceed to full conversion, and produce little to no byproducts.^{22–23} One common click reaction used in peptide conjugation is the copper(I) catalyzed azide-alkyne cycloaddition (Cu-AAC).²⁴ The CuAAC reaction is a bio-orthogonal reaction between an azide and alkyne in the presence of copper(I) to form a triazole product. For example, Ingale et al.¹⁵ demonstrated on-resin macrocyclization of peptides using copper bromide and excess sodium ascorbate; however, long reaction times of 18 h were required to reach sufficient conversions. Another common click reaction used in peptide conjugation is the thiolene reaction, which is the radical mediated addition reaction between a thiol and electron deficient alkene.²⁵ For example, An-seth and coworkers¹⁴ demonstrated a simple thiol-ene conjugation strategy utilizing cysteine as a thiol and a commercially available allyloxycarbonyl (Alloc) protected lysine; however, this approach required multiple additions of photoinitiator and irradiation steps to perform the on-resin macrocyclization.

An attractive alternative click reaction to the CuAAC and thiol-ene reactions is the thiol-Michael addition.²⁶ In contrast to the radical-mediated thiol-ene reaction, thiol-Michael additions follow an anionic mechanism. Thiol-Michael reactions occur between a thiol and an electron deficient alkene to form a stable thioether bond in the presence of a base or nucleophile catalyst. Thiol-Michael reactions have been exploited in a wide range of biomolecular conjugation reaction, including peptides²⁷, proteins²⁸, and nucleic acids.²⁹ Common thiol-Michael acceptors, such as maleimides and acrylates, exhibit fast kinetics and proceed to full conversion, but present complications when applied to peptide chemistry.^{30–31} Maleimides are known to undergo a ring opening reaction under aqueous conditions, both before and after conjugation,^{32–33} while acrylates are susceptible to hydrolytic cleavage, particularly after thiol addition.³⁴ An alternative water stable thiol-Michael acceptor is the acrylamide functional group; however, they exhibit slow kinetics and incomplete conversions over long time periods.³⁰ Here, we suggest the vinyl sulfonamide as an efficient, water stable thiol-Michael acceptor for peptide macrocyclization. Vinyl sulfonamides have been employed in both bioconjugations³⁵ and polymer networks³⁶ for its high reactivity and hydrolytic stability. Furthermore, simple installation of vinyl sulfonamides on primary or secondary amines is achieved using the commercially available and inexpensive 2-chloroethanesulfonyl chloride.

Solution-phase model reaction systems were performed to compare relative rates of vinyl sulfonamides with several commonly implemented Michael acceptors (Figure 1A), such as maleimide (E1) and acrylate (E2) as well as less reactive hydrolytically stable acrylamides (E3 and E4). The reaction kinetics of thiol Michael conjugations are highly dependent on alkene and thiol structure³⁷. Model mono-vinyl sulfonamide compounds, (E5) and (E6), were synthesized and compared with maleimide, acrylate, and acrylamide functional groups. All thiol-Michael acceptors were conjugated with two commercially available thiols, n-butyl mercaptopropionate (T1) and a cysteine analog (T2). Mercaptopropionic acid is readily conjugated to the *N*-terminus of a peptide or amine side-group on an amino acid through amide coupling, whereas cysteine is a natural amino acid containing a thiol side-group.³⁸

The model maleimide and acrylate compounds proceeded rapidly, while both acrylamides compounds showed minimal conversion after 30 min under the same conditions. A summary of the thiol-Michael kinetics is presented in Figure 1B and Figure 1C. The reaction of phenyl maleimide, E1, with T1 or T2 reacted to full conversion within the short time period of adding and mixing them with 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) and transferring to the FTIR (30 seconds). Maleimide functional groups showed enhanced reactivity for two distinct reasons: alleviation of ring strain and the electron deficiency of the olefin caused by the two adjacent activating carbonyl groups.³⁹ The kinetics of the methyl acrylate, E2, reacted with either the alkyl or cysteine-based thiols proceeded rapidly, reaching conversions of $92 \pm 3\%$ or $91 \pm 1\%$, respectively, at 5 min. In comparison, reactions of either acrylamide functional groups with either type of thiol exhibited sluggish kinetics. The reaction of the secondary acrylamide, E3, with T1 or T2 reached conversions of only $2 \pm 1\%$ or $1.5 \pm 0.3\%$ within 5 min, respectively. Surprisingly, the reaction of the *N*-methyl tertiary acrylamide (denoted henceforth as a tertiary acrylamide), E4, with either T1 or T2 had improved reactivity, reaching $7 \pm 1\%$ within 5 min. We attribute the poor reactivity of both acrylamides to the decrease in electronegativity of the amides as compared with esters.⁴⁰

High electronegative moieties adjacent to olefins result in a more reactive center for thiol-Michael conjugations. Furthermore, the enolate that forms in amides is known to be less stable as compared with ester-based groups.^{41–42} It is believed that an induction effect reduces the stability of the eno-olate species thereby resulting in slower kinetics of the secondary acrylamide in comparison with the tertiary acrylamides.

Both secondary and tertiary vinyl sulfonamides exhibited comparable kinetics to the acrylate, and much faster kinetics than the secondary and tertiary acrylamides. The reaction of secondary vinyl sulfonamide, E5, with either T1 or T2 reached a conversion of $49 \pm 1\%$ or $21 \pm 1\%$, respectively, over 5 minutes. As observed with secondary and tertiary vinyl acrylamides, the tertiary vinyl sulfonamide demonstrated faster kinetics than the secondary vinyl sulfonamides. The reaction of tertiary vinyl sulfonamide, (E6), reacted more rapidly than E5 within the same timeframe with conversions reaching $76.4 \pm 0.6\%$ and $53.4 \pm 0.7\%$ with T1 and T2, respectively. The increased reactivity of vinyl sulfonamides over vinyl acrylamides and comparable reactivity to acrylates is attributed to two main factors. First, sulfur atoms possess high polarizability leading to a stronger inductive effect for stabilizing the α -carbanion that forms during the thiol-Michael reaction mechanism.⁴³ Second, antibonding orbitals of sulfur atoms create σ -effects that further stabilize the anionic center.^{44–46} The added stability of the α -carbanion results in an increase in the kinetic performance for vinyl sulfonamides in thiol-Michael conjugations. Additionally, it is noteworthy that slower kinetics were experienced across all the alkenes when reacting with the cysteine-based thiol T2. The slower kinetics were an unexpected result based on the apparent thiol pKa (between 6–9). However, the slower kinetics could be attributed to neighboring groups providing steric hinderance or affecting thiol pKa.

Water stability of the thioether product from the thiol reaction with either the secondary and tertiary vinyl sulfonamides was evaluated and compared with the thioether product of an acrylate. Sinha et al.³⁶ showed that tertiary vinyl sulfonamide demonstrated superior stability to acidic and basic environments in polymer networks as compared with networks formed using secondary sulfonamides and acrylates. Using NMR spectroscopy, we confirmed the stability of both secondary- and tertiary-vinyl sulfonamides in both acidic and basic media (Figures S16 to S21). Under basic conditions (in pH 10 borate buffer), the model acrylate underwent rapid hydrolytic cleavage, while both vinyl sulfonamide compounds remained stable over an 8 day period.

Owing to its rapid reaction kinetics and hydrolytic stability, tertiary vinyl sulfonamide was selected to cyclize a ubiquitous cell-adhesion peptide, RGDS; a process which has been shown to both increase its stability to enzymatic degradation and efficacy to promote cell spreading.⁶ The procedure for macrocyclizing the RGDS peptide is summarized in Figure 2. The peptide sequence was preceded by a cysteine residue, bearing a monomethoxy trityl (Mmt) protected thiol. The eventual removal of the Mmt group through well-established protocols provides a thiol chemical handle for the thiol-Michael addition.⁴⁷ A D-phenylalanine residue was coupled after the cysteine and prior to the rest of the sequence, which has been shown to help promote macrocyclization of peptides.^{48–49} To install the tertiary vinyl sulfonamide, a low-cost commercially available Fmoc-protected sarcosine was added at the end of the peptide. The Fmoc protecting group was removed revealing a

secondary amine to which the vinyl sulfonamide was added. To generate the vinyl sulfonamide, a commercially available reagent, 2-chlorosulfonyl chloride, was applied to the resin mixture in the presence of triethylamine (TEA), yielding the Michael-acceptor product. Macrocyclization of the peptide was catalyzed using DBU, a common reagent used in Fmoc SPPS.⁵⁰ Upon macrocyclization, a non-natural sulfonamide linkage is introduced into the backbone of the macrocyclic peptide.

The macrocyclization of the peptide is achieved on-resin by reacting the thiol of the cysteine with the tertiary vinyl sulfonamide end group. First, the resin was washed followed by the selective deprotection of the Mmt protected cysteine, using a dilute acid solution of 3% trifluoroacetic acid (TFA)/5% triethylsilane (TES) in dichloromethane (DCM). The peptide resin was then neutralized using a dilute TEA solution and was collected. One third of the resin was subjected to cleavage from the resin using a 95% TFA cleavage cocktail. This peptide was collected, precipitated, and purified by HPLC to produce the linear peptide with an overall yield of 10.0 mg or 14.2% based on the initial resin's molarity. The other two-thirds of the resin was subjected to the macrocyclization procedure. A catalytic amount of DBU in dimethylformamide (DMF) was added and mixed for one hour. The resin was then washed multiple times with DMF and DCM and was subjected to the resin cleavage cocktail, precipitated, and purified by reversed-phase high-performance liquid chromatography (RP-HPLC). The final product was lyophilized resulting in 19.4 mg or 13.8% yield of fully macrocyclized peptide. The structure of the fully cleaved macrocyclized peptide is presented in Figure 3A. Based on the final yield of the peptide the overall relative efficiency of the macrocyclization was >95%. Such a high efficiency of macrocyclization demonstrates the effectiveness of using vinyl sulfonamides as a thiol-Michael acceptor for peptide macrocyclizations. All attempts at cyclizing the peptide using a tertiary acrylamide were unsuccessful at macrocyclizing on-resin further demonstrating the superior reactivity of vinyl sulfonamides over acrylamides. As expected, attempts at performing solution-phase macrocyclizations under highly dilute conditions utilizing the same vinyl sulfonamide chemistry resulted in minimal macrocycle product.¹³

¹H NMR was performed to verify the disappearance of the vinyl sulfonamide protons, denoted by 'a' and 'b' in Figure 3B. As expected after undergoing cyclization on-resin, the vinyl proton shifts ($\delta = 6.07, 6.78$ ppm) disappeared from the cyclized peptide spectrum. The thiol peak ($\delta = 2.00$), denoted as 'c,' also disappeared post cyclization. Furthermore, the shifts in the ¹H spectra post-cyclization further signifying a structural change to the peptide has occurred. For example, ¹H chemical shifts in the amide region ($\delta = 7.25$ to 9.00 ppm) are broader prior to macrocyclization. As linear peptides adopt a random coil conformation, amide peaks form a broad distribution of conformations demonstrated by the clustering of the protons between 8.00 and 8.25 ppm. Once a peptide forms a macrocycle, the discrete conformations are observed from the spreading of the amide protons as they adopt distinct orientations.

Correlation between protons using 2D NMR techniques further confirmed the structure of the cyclic peptide. ¹H-¹³C HSQC and HMBC of the cyclic peptide were overlaid to compare the single and multiple bond correlations between ¹H and ¹³C peaks, shown in Figure 3C. With these correlations, protons were assigned to the resulting thioether product and cysteine

residue. Upon macrocyclization, the thioether carbon (C3; $\delta = 34.08$; 2.73/2.92 ppm) was used to determine the correlation with the cysteine α -proton (C1; $\delta = 52.69$; 4.39 ppm), as denoted by the black arrows. These correlations confirm that the thiol-Michael reaction has occurred. Furthermore, COSY, NOSEY, and TOCSY NMR data were collected on both the linear and macrocyclized peptides to confirm these findings (Figure S28 to S43).

On-resin microcleavages were performed on the linear and macrocyclic peptides for further confirmation of macrocyclization. A shift was observed when analyzing the macrocyclized peptide which eluted at 2.69 min, while the linear peptide eluted at 2.79 min. Upon macrocyclization, the structural changes in the peptide results in a faster elution time on the UPLC column. The R groups of the amino acid residues are forced outward allowing for more direct interactions of the polar R groups with the hydrophobic C18 column packing resulting in a faster elution time. A shift in retention time was again observed during purification of the linear and macrocyclized peptide using RPHPLC (Figures S2 and S3).

A facile protocol for macrocyclization of peptides using tertiary vinyl sulfonamides was established and shown to be compatible with standard Fmoc-based SPPS protecting groups and methods. This functional group is easily generated using any secondary amine handle utilizing a low-cost commercially available reagent, 2-chlorosulfonyl chloride. Additionally, it was shown to possess comparable reactivity to acrylates and was considerably more reactive than acrylamides. This functional handle is stable in harsh basic environments unlike acrylates. The higher stability of tertiary vinyl sulfonamides suggests that it can be applied in applications where the conjugation bond stability is desired. Due to the wide availability, low-cost, high selectivity, and increased stability of the tertiary vinyl sulfonamide, we expect the scope of this thiol-Michael click reaction to expand beyond peptide macrocyclization and broaden the toolbox of reaction handles for thiol-Michael conjugations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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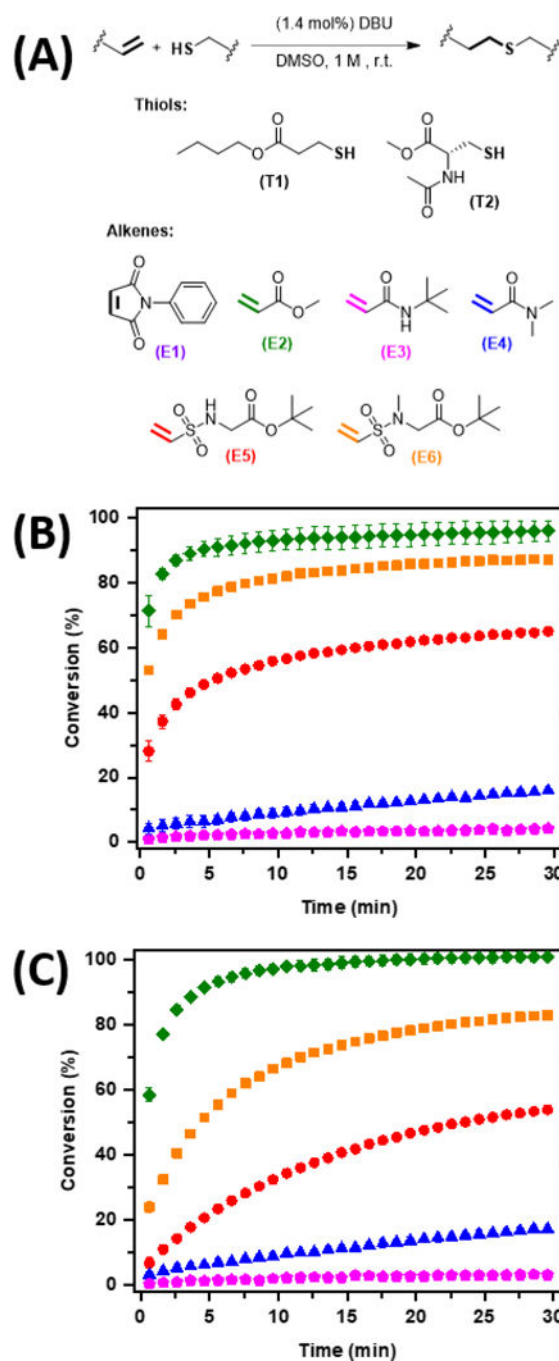
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**Figure 1.**

Reaction kinetics of various thiols and electron poor alkenes. A) The general thiol-Michael reaction scheme, and the model thiols and alkenes used in this study. B) Reaction kinetics were monitored at room temperature over 30 minutes using FTIR spectroscopy by tracking the disappearance of the thiol-peak between 2484 and 2545 cm^{-1} . 1 eq of the thiol, T1, was reacted with 1 eq of each of the alkenes, E2 (green, filled diamond), E3 (magenta, filled pentagon), E4 (blue, filled triangle), E5 (red, filled circle), and E6 (orange, filled square) in the presence of DBU (1.4 mol%) in DMSO. (C) Similarly, 1 eq of the thiol, T2, was reacted

with 1 eq of each alkene, E2 (green, filled diamond), E3 (magenta, filled pentagon), E4 (blue, filled triangle), E5 (red, filled circle), and E6 (orange, filled square). E1 is not shown as it reached full conversion with both T1 and T2 within the first 30 s of the experiment.

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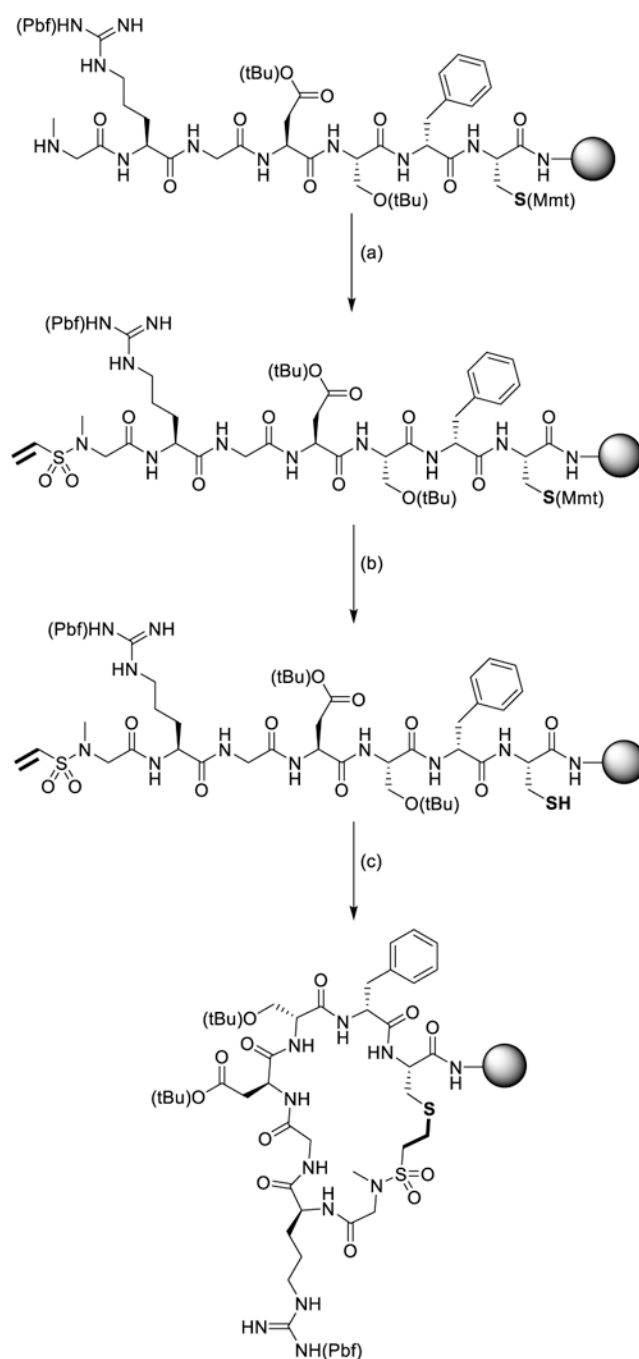


Figure 2.

On-resin macrocyclization of an RGDS peptide. The following procedure was used to functionalize and macrocyclize the peptide: (a) (4 eq) 2-chloroethanesulfonyl chloride, (8 eq) TEA, r.t., for 2 h, 2 times, (b) 3% TFA/5% TES in DCM, r.t., 30 s, 15 times, and (c) 10 mol% DBU in DMF, r.t., 1 h.

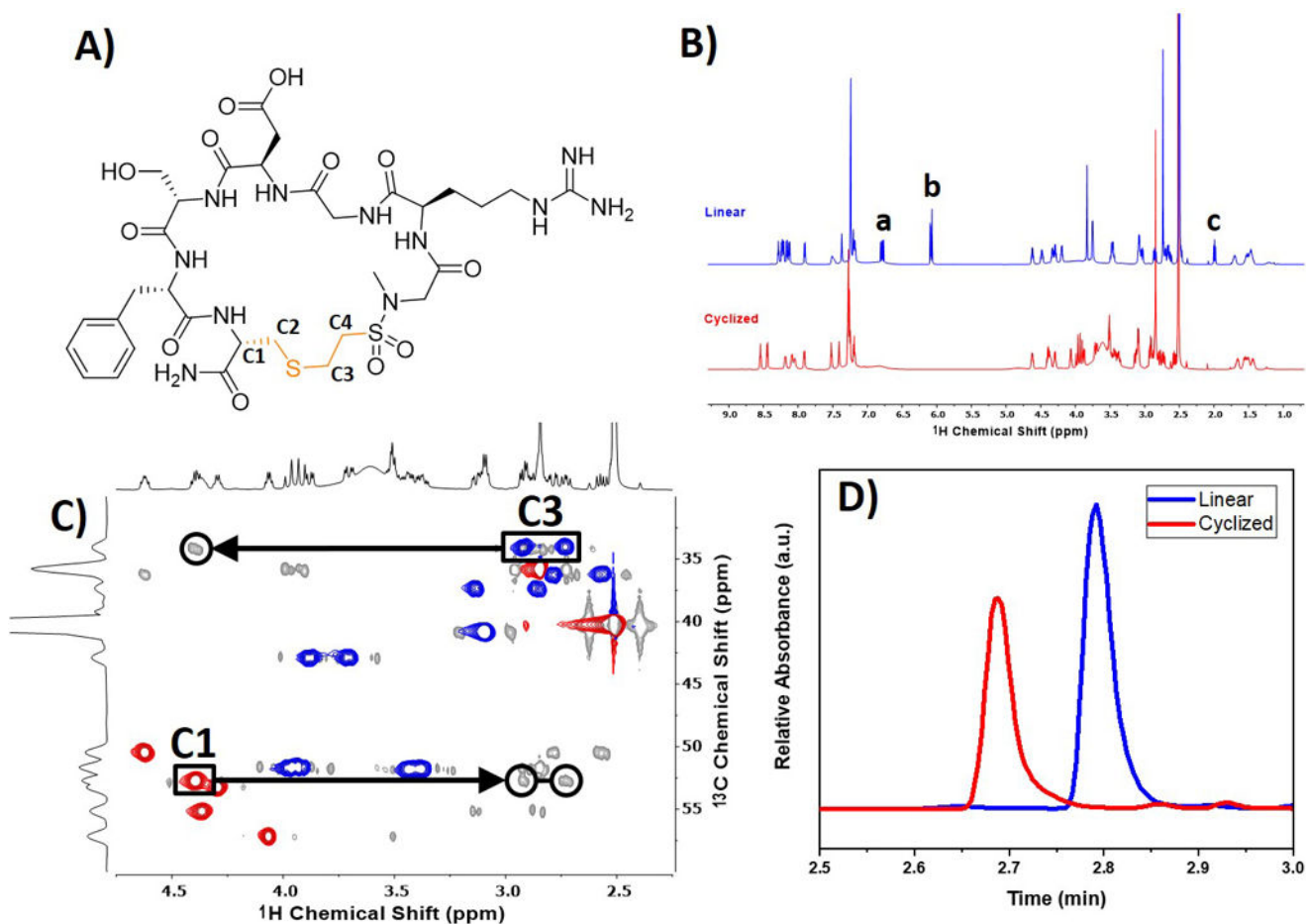


Figure 3.

Characterization of macrocyclic RGDS peptide. (A) Structure of the macrocyclized peptide cleaved from the resin with important carbons for confirming macrocyclization numbered C1 through C4. The proton NMR (B) shows the linear peptide in blue and the cyclic peptide in red. Alkenyl protons, denoted by 'a' and 'b,' disappear after macrocyclization. Additionally, the thiol peak 'c' disappears after performing the macrocyclization. An overlay of ^1H - ^{13}C HSQC (red/blue) and HMBC (grey) is shown in (C) with black arrows highlighting the correlation between the newly formed thioether (C1) and cysteine residue (C3) for the macrocyclized peptide. For the edited ^1H - ^{13}C HSQC, the blue and red peaks correspond to CH₂ or CH/CH₃ carbons, respectively. The ESI-UPLC trace shown in (D) of the microcleaved linear and macrocyclized peptides shows a shift in the elution time of the two peptide structures.