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Rapid Arene Triazene Chemistry for Macrocyclization

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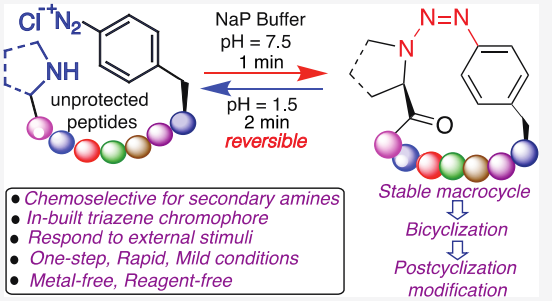
ABSTRACT: Here, we report a novel rapid arene triazene strategy for the macrocyclization of peptides that generates an inbuilt chromophoric triazene moiety at the site of cyclization within a minute. The rapid arene triazene chemistry is chemoselective for secondary amines and *p*-amino phenylalanine. Importantly, the resulting triazene cyclic peptide is highly stable at neutral pH and under harsh conditions but rapidly responds to various external stimuli such as UV radiations and acidic conditions, resulting in the ring opening to generate the linear peptides in an unchanged form, which further cyclizes under neutral pH conditions. This method works with completely unprotected peptides and has been applied for the synthesis of 18- to 66-membered monocycles and bicycles with various amino acid compositions in one pot under neutral pH conditions. Due to the high stability of triazene cyclic peptides, the postcyclization modification was carried out with various functional groups. This rapid, macrocyclization strategy featuring a triazene scaffold, amenable to late-stage diversification and responsive to external stimuli, should find application in various fields of chemical biology, selective drug delivery, and identification of cyclic peptide hits after library screening.



INTRODUCTION

Cyclic peptides are privileged scaffolds as pharmaceuticals and have gained particular attention for inhibiting protein−protein interactions with long interfaces considered to be undruggable by small molecules.[1−3] This is attributable to their medium size, high proteolytic stability, ability to adopt particular secondary structures, and high binding affinity as compared to their linear counterparts because of the lower entropy cost of binding.[4−8] Thus, cyclic peptides merge the high specificity of biomolecules with the high stability of small molecules. This led to a huge surge in the development of new macrocyclization strategies with a particular focus on chemoselectivity, for example, click chemistry,[9] cross-metathesis,[10,11] Pd-catalyzed arylations of thiols and amines,[12,13] dichloroacetone for cyclization,[14] thiol−ene reaction,[15] thio-etherifications,[16−18] oxadiazole synthesis,[19,20] Petasis-boron-Mannich reactions,[21] and recently developed isoindole-bridged cyclic peptides.[22,23] Recently, our group developed an exclusive intramolecular chemoselective cyclization strategy "CyClick" for the synthesis of various cyclic peptides at high concentrations without the formation of any dimers or oligomers.[24] Although there are several methods known for the chemoselective synthesis of stable cyclic peptides, but cyclic peptides that respond to the changes in the external stimulus by the opening and closing of the macrocycle ring are very few including disulfide[25] and iminoboronates.[26] Such cyclization methods are highly desirable for targeted drug delivery and could act as sensors for the local environment in various disease states.

Another key feature required to determine the biological/pharmaceutical activity of cyclic peptides including library screening and binding affinity is the change in the chromophoric property, yet there are only a few methods that generate inbuilt chromophores.[22,23] This property is highly desirable because it obviates the need for the incorporation of a big bulky chromophoric group in the cyclic peptides, which may influence the structure, binding affinity, and solubility of cyclic peptides. In addition, the rapid rate of macrocyclization could lead to the synthesis of cyclic peptide libraries in one pot without any cross-linking byproducts, but chemical methods to achieve this goal are lacking. Consequently, there is a great need to develop new chemoselective macrocyclization methodologies that fulfill all the above criteria and provide a rapid, efficient strategy for easy access to a variety of cyclic peptides that show response to the external stimuli by the opening and closing of the macrocyclic ring. Currently, there are no such methods available that fulfill all the above criteria by a single methodology.

RESULTS AND DISCUSSION

Development of a New Macrocyclization Strategy. Inspired by observation of the facile reaction of the arene

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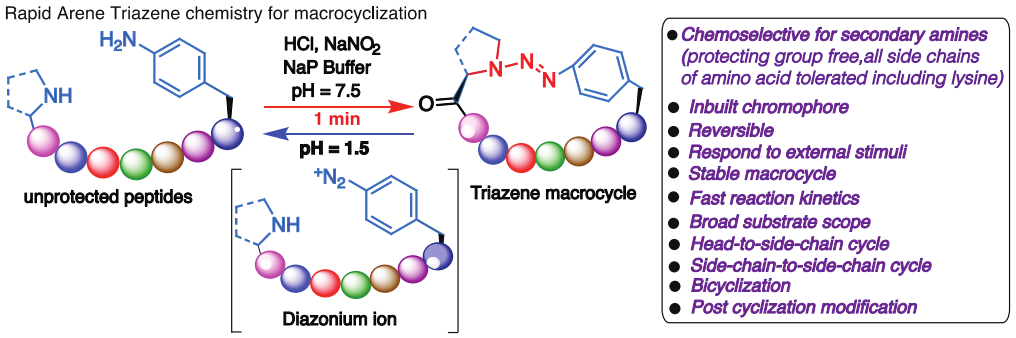

Figure 1. Rapid and chemoselective arene triazene reaction for cyclization of unprotected linear peptides to triazene cycles.

diazonium ion with a secondary amine to form a stable triazene,[27−29] we reasoned that the arene diazonium ion might serve as an attractive starting point, owing to the ease and flexibility of introducing it inside the peptide by the incorporation of commercially available *p*-amino phenylalanine (pAF) in the peptide chain.[30] Arene diazonium ions would then chemoselectively react with secondary amines such as N-terminal proline or monomethyl lysine at pH 7.5 to generate a stable macrocycle with a triazene moiety at the site of cyclization (Figure 1). The triazene moiety generated by secondary amines is a privileged structural motif found in many pharmaceuticals and biologically active compounds as prodrugs with anti-tumor and mutagenic properties[31] such as mitozolomide,[31] dacarbazine,[32−34] and temozolomide.[35,36]

We started our initial investigation on a 10-mer peptide with the sequence PGRGWADGA(pAF) **1a**, where we added pAF by standard Fmoc SPPS[37] (Figure 2a and Supporting Information Figure S1). The pAF generated arene diazonium ions by the addition of 10 mM HCl and sodium nitrite ($NaNO_2$) followed by a change in the pH to 7.5, leading to the rapid formation of the head-to-side chain triazene cyclic product **2a** with N-terminal proline with >99% conversion in 5 min without the formation of any byproducts including dimers or oligomers (Figure 2a, Supporting Information Figure S1). We carried out the reaction with a completely unprotected peptide and did not observe any modification of reactive Asp (D), Arg (R), and Trp (W) side chains. Importantly, coupling reagents, organic solvents, metal catalysts, and harsh conditions (high temperature) were not required in this procedure. The formation of the triazene cyclic peptide **2a** was characterized by high-performance liquid chromatography (HPLC) and high-resolution mass spectrometry (HRMS) (Figure 2a, Supporting Informations Figure S1, Tables S1 and S2). Next, we cyclized 8-mer linear peptide PAMGLAE(pAF) **1b,** and it resulted in the formation of triazene cyclic peptide **2b** with >99% conversion as analyzed by HPLC (Figure 2a, Supporting Information Figure S2, Tables S1 and S2). Because the m/z of the intermediate diazonium ion is equal to m/z of triazene cyclic peptide (Supporting Information Table S2), we further characterized the triazene cyclic peptide **2b** by synthesizing it on a large scale, isolating the pure triazene cyclic peptide **2b** in 58% yield, and carrying out nuclear magnetic resonance (NMR) spectroscopy. We compared the NMR spectra of the linear PAMGLAE(pAF) peptide **1b** with those of cyclic peptide **2b** (Supporting Information Figure S2). The diagnostic NH proton of proline in the linear peptide **1b** observed at δ 2.32−2.24 disappeared in the ^{1}H NMR spectrum of the triazene cyclic **2b** product. The diagnostic alpha protons (2.44−2.33, ppm) and alpha carbon (30.06 ppm) on the proline in the cyclized product **2b** moved upfield as compared to the linear peptide **1b** alpha protons (3.24−3.17, ppm) and alpha carbon (42.31 ppm). Similarly, significant changes were observed in the ^{1}H and ^{13}C NMR of *p*-NH_2-phenylalanine in cyclized **2b** and uncyclized peptide **1b** (Supporting Information Figure S2). To further confirm the formation of the triazene cyclic product, we synthesized another cyclic peptide PGG(pAF), **2c,** in 53% yield and characterized it by HPLC, MS, and NMR studies (Supporting Information Figure S3). The comparison of the NMR of linear peptide PGG(pAF) **1c** with that of cyclized peptide **2c** showed similar changes in the ^{1}H and ^{13}C NMR spectra as in **2b**. Moreover, heteronuclear single-quantum correlation, heteronuclear multiple-bond correlation, and rotating-frame overhauser enhancement spectroscopy (ROESY) NMR experiments further confirmed the formation of the triazene structure in cyclic peptides (Supporting Information Figures S2 and S3). Next, we attempted the macrocyclization with linear peptides containing primary amines such as N-terminal alanine and lysine ATAQS(pAF) **1d** and Ac-KTAQS(pAF) **1e** under various reaction conditions, including longer reaction times. Macrocyclic products of **1d** and **1e** were not observed between the diazonium ion and N-terminal alanine and the side chain of lysine due to the formation of unstable triazenes with primary amines in the absence of stabilizing secondary amine substituents, further confirming the literature reports[38] (Supporting Information Figure S4). Infact, we observed the formation of corresponding peptide arylalcohols **2d** and **2e** as confirmed by the HPLC and MS via path A[38] (Supporting Information Figure S4, Table S1 and S2). We synthesized the arylalcohol product, **2d**, in 35% yield from the linear peptide, ATAQS(pAF) **1d**, on a large scale and characterized it by NMR (Supporting Information Figure S5). We compared the NMR spectra of the linear peptide **1d** with those of the aryl alcohol product, **2d**. NH_2 protons of the N-terminal alanine remained unchanged in both **1d** and **2d**. The significant change in the ^{1}H NMR spectrum for *p*-NH_2-phenylalanine in **2d** with the peak at δ 9.14 for a single proton suggested that NH_2 of the phenyl ring converted into the −OH group. We also observed a significant change in the ^{13}C NMR spectrum of phenyl carbons from δ 137.61 to δ 155.73 (Supporting Information Figure S5). It might be because the electronegative oxygen de-shields the carbon on the phenyl ring, and consequently, the signals moved downfield. To further confirm the structure of alcohol **2d**, we made the **1d** analogue, where *p*-NH_2-phenylalanine was replaced with tyrosine ATAQSY. We compared the NMR of the **1d** analogue ATAQSY with that of **2d**, and the results showed an identical structure (Supporting Information Figure S6). This further confirmed that NH_2 of *p*-

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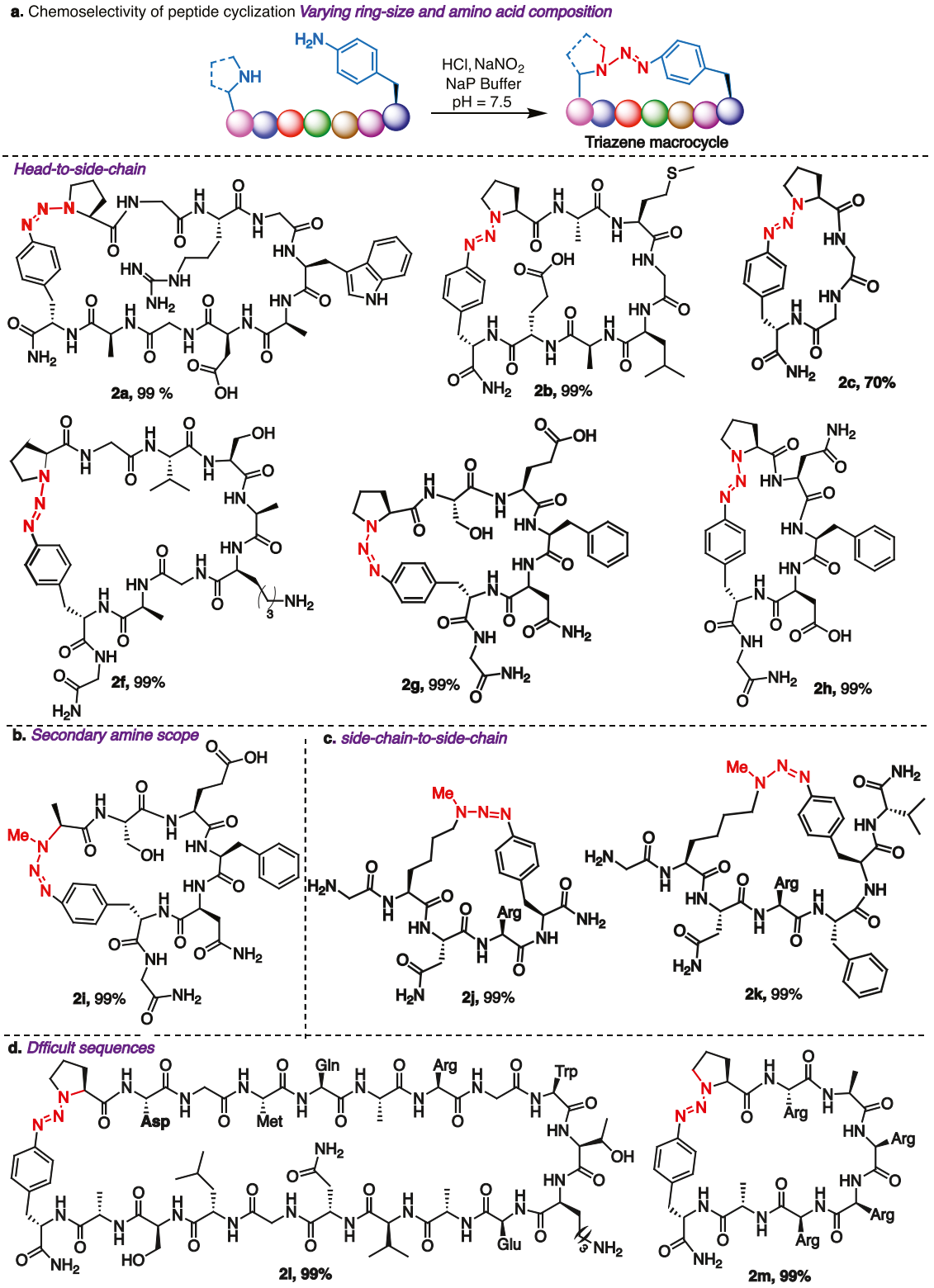

Figure 2. Substrate scope of arene triazene chemistry. (a) High HPLC conversions of cyclic peptides (18- to 66-membered) with various amino acid residues and lengths of peptide chains. (b) Secondary amine scope with N-methylated alanine. (c) Side-chain-to-side-chain macrocyclization of cyclic peptides and secondary amine scope with the *N*-methyl lysine side chain. (d) Cyclization of difficult sequences including 20 amino acid-long peptides and multiple arginines containing peptides with high conversions to triazene cyclic peptides **2l** and **2m**.

NH_2-phenylalanine converted to tyrosine (−OH) due to the degradation of the triazene cyclic product obtained between primary amine and diazonium ions via path A in the mechanistic pathway (Supporting Information Figure S4).[38]

Next, we carried out the macrocyclization reaction with a peptide PGVSAKGA(pAF)G **1f** containing both lysine and N-terminal proline under the reaction conditions. We observed the formation of triazene macrocyclic product **2f** with proline

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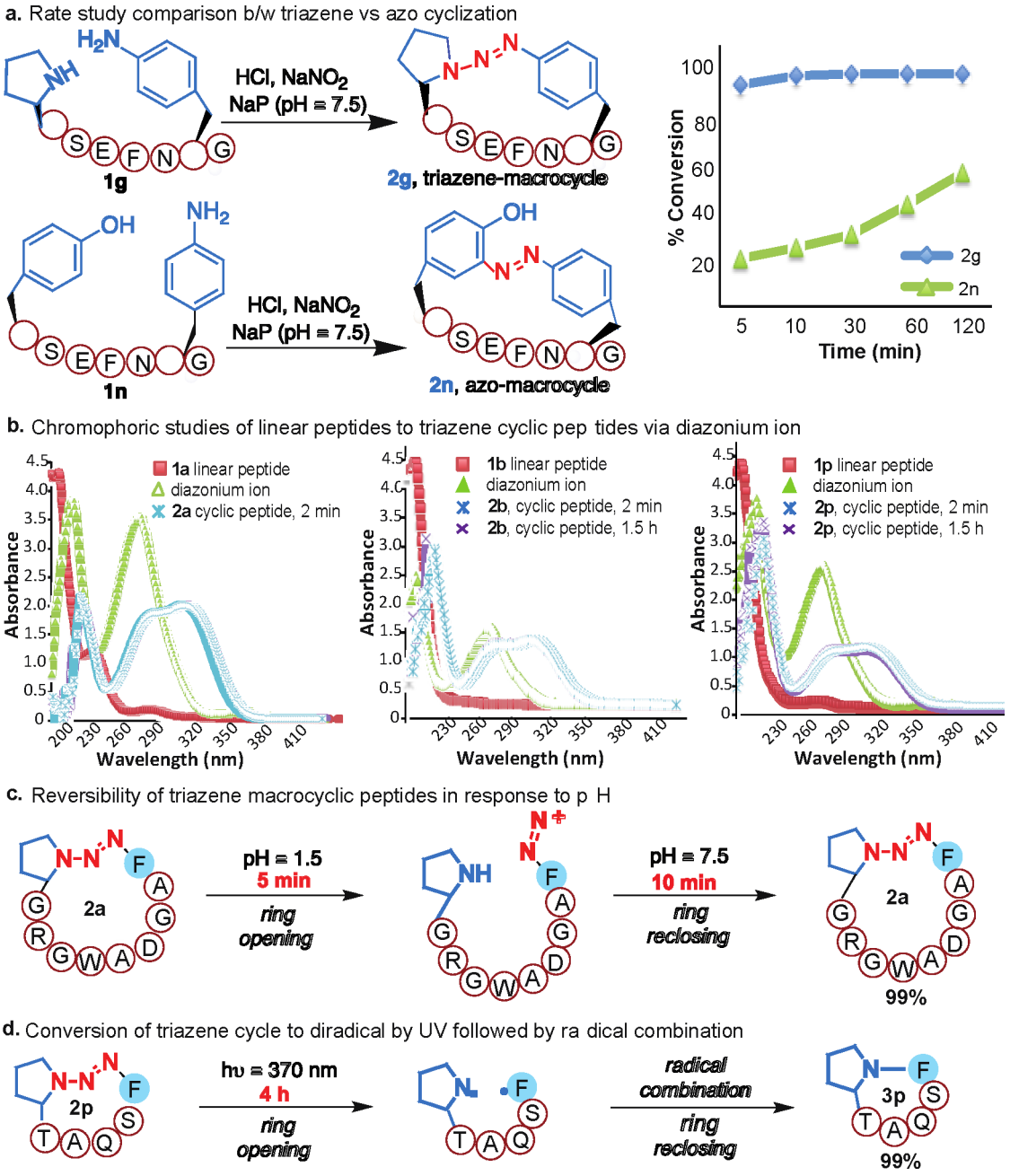

Figure 3. (a) Rate study comparison of the arene triazene macrocyclization of proline with well-known azo cyclization with tyrosine. The reaction with **1g** showed higher reactivity as compared to azo macrocyclization with peptide **1n**. Reaction conditions: unprotected peptide (3 mM) in 10 mM HCl and $NaNO_2$ (1.5 equiv, 4.5 mM) were added and stirred for 10 min on ice to generate diazonium ions followed by the addition of sodium phosphate buffer (NaP, pH 8.5) to make the pH of the resulting mixture 7.5 and incubated from 5 min to 2 h at room temperature. The reaction was performed in triplicate, and data are average of three experiments. (b) Spectrophotometric analysis of arene triazene cyclization using UV. Triazene cyclic peptides **2a, 2b,** and **2p** exhibited characteristic peaks at 289 and 313 nm wavelength, and corresponding diazonium ions showed maxima at 273 nm. Linear counterparts **1a, 1b,** and **1p** did not show any absorbance in this range. (c) Triazene macrocycle **2a** showed sensitivity with change in the pH resulting in linearization of the cyclic peptide at low pH (1.5) and formation of the cyclic peptide **2a** again at physiological pH 7.5. (d) Triazene macrocycle **2p** showed sensitivity to UV radiations at 370 nm to generate biradicals followed by their combination to generate stable new macrocycle **3p** between proline and the aromatic ring of pAF.

in >99% conversion (44% yield) as analyzed by HPLC, HRMS, and NMR (Figure 2, Supporting Information Figure S7, Table S1 and S2). The diagnostic NH proton of proline of **1f** was observed at δ 2.32−2.27 but disappeared in the ^{1}H NMR spectrum of cyclized product **2f**. In ^{13}C NMR, the diagnostic alpha carbon (29.2 ppm) on the proline in the cyclized product **2f** moved upfield as compared to the linear peptide **1f** alpha carbon (46.15 ppm) (Supporting Information Figure S7). There were no significant changes observed in the δ values for lysine side chain in the NMR of cyclic peptide **2f**, confirming that proline is the site of cyclization. Together, these results confirm that the arene triazene cyclization reaction is highly selective for secondary amines over the primary amines.

Scope of Arene Triazene Chemistry. Having established the optimal conditions, we sought to demonstrate the

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versatility of this chemistry with different amino acids and ring sizes. As outlined in Figure 2, unprotected linear peptides PGRGWADGA(pAF) **1a**, PAMGLAE(pAF) **1b**, PGG(pAF) **1c**, PGVSAKGA(pAF)G **1f**, PSEFN(pAF)G **1g,** and PNFE-(pAF)G **1h**, bearing reactive amino acids such as Trp, Arg, Lys, Asp, Asn, Glu, Ser, and Met and different chain lengths with variable-spacing 3−8 amino acid residues, cyclized efficiently and provided the corresponding triazene macrocycles (**2a-2c and 2f-2h**) of different rings between pAF and N-terminal proline with complete conversions as judged by LC−MS analyses of the crude reaction mixtures (Figure 2 and Supporting Information Figure S8, Tables S1 and S2). Most importantly, we did not observe side reactions with any reactive amino acids on unprotected peptides and none of the peptide sequences contained turn inducers, thus suggesting that turn inducers are not required for the highly efficient macrocyclization and demonstrating high reactivity and chemoselectivity of the arene triazene reaction. Notably, we did not observe the formation of any linear or cyclic oligomers.

Next, we explored the reactivity of the arene triazene reaction toward different secondary amines. We first introduced *N*-methylated alanine at the N-terminus A(me)-SEFN(pAF)G **1i** and observed high conversion to the head-to-side chain triazene-cyclic product **2i** within 10 min under the reaction conditions (Figure 2 and Supporting Information S8, Tables S1 and S2). Next, we carried out macrocyclization with unprotected peptides bearing a free primary N-terminus and monomethyl lysine GK(me)NR(pAF) **1j** and GK(me)NRF-(pAF)V **1k**, and full conversion to the triazene-cyclized products **2j−2k** was observed under the optimized conditions as analyzed by HPLC and MS (Figure 2 and Supporting Information S8, Tables S1 and S2). Notably, this reaction does not differentiate between cyclic secondary amines (proline) and non-cyclic secondary amines (*N*-methyl amine) and the side chain amino group and the N-terminal amine and is thus capable of producing both head-to-side chain and side chain-to-side chain triazene cyclic peptides. Orthogonal protecting groups are not needed with any reactive amino acids for diazonium ions, including the free N-terminus or side chains of lysine and tyrosine, which is in contrast to other inbuilt-chromophore macrocyclization strategies.[22,23]

We explored the scope of our approach for the cyclization of difficult sequences including a short peptide with only 4 amino acids, a long peptide with 20 amino acids, and a peptide with multiple Arg residues.[39] With four-mer peptide PGG(pAF) **1c**, we observed 70% conversion to the cyclic product **2c** and rest 30% was the cyclic dimer **3c** (Figure 2 and Supporting Information S8, Tables S1 and S2). With both a long peptide PDGMQARGWTKEAVNGLSA(pAF) **1l** and a multiarginine-containing peptide PRARRRA(pAF) **1m**, we observed full conversion (>99%) to the triazene cyclic products **2l−2m** without the formation of any dimers or oligomers (Figure 2 and Supporting Information S8, Tables S1 and S2). Thus, the chemoselective arene triazene cyclization strategy provides a simple way to cyclize unprotected native peptides, which are otherwise difficult to cyclize in a rapid manner.

Rate of Arene Triazene Cyclization. To evaluate the rate of the arene triazene reaction, we started our initial investigation by carrying out a macrocyclization of a peptide PSEFN(pAF)G **1g** (3 mM in 10 mM HCl) in the presence of $NaNO_2$ (1.5 equiv of 4.5 mM) in phosphate buffer (100 mM and pH 8.5). The overall pH of the solution was 7.5, and reaction was stirred at room temperature. We analyzed the reaction progress by taking samples at regular intervals of time followed by analysis using HPLC and MS. The cyclization of the peptide **1g** was found to reach near-completion **2g** with >99% conversion in less than 5 min (Figure 3a, Supporting Information S9). The cyclization of the peptide **Y**SEFN(pAF)-G **1n** containing tyrosine leading to the formation of azo cyclic product **2n** with pAF showed only 20% conversion after 10 min and 50% conversion in 2 h under the similar reaction conditions (Figure 3a, Supporting Information S9). The HPLC and MS data clearly showed the formation of azo cyclic product **2n** (50% conv.) after 2 h with retention time R_t = 21.2 min and diazonium ions with R_t = 16.02 min in the HPLC (Figure 3a, Supporting Information S9). We observed the formation of the azo cyclic product with Tyr in high conversion at elevated pH only (pH 9), corroborating previous reports.[40−45] The MS of the peak at 16 min showed the formation of the (M-N2) peak, which is characteristic of the diazonium ions (Supporting Information Figure S9). We were not able to isolate/identify the diazonium ion in the reaction with proline because the reaction was very fast and rapidly formed traizene cyclic product **2g** within minutes (Figure 3a). To further characterize the diazonium-ion intermediate, we made a peptide AcATAQS(pAF) **1o** with arene amine for diazotization but without any free secondary or primary amine. We subjected the peptide AcATAQS(pAF) **1o** to the cyclization conditions by first converting it to the diazonium adduct **2o** using $NaNO_2$ followed by changing the pH to 7.5. Because there was no reactive group to trap the diazonium ion, we characterized the formation of AcATAQS(pAF) diazonium ion **2o** by HPLC and MS (Supporting Information S10). We observed the *m*/*z* of the diazonium ion (M) and M-N2 peaks in MS spectra, confirming the formation of diazonium adduct **2o** at 8 min in HPLC. To further confirm the formation of diazonium ion **2o**, we carried out the reaction with piperidine and KI and generated triazene **3o** and iodinated product **4o**, respectively, as analyzed by HPLC and MS (Supporting Information Figure S10). To further characterize the formation of diazonium ions, we carried out the NMR of the diazonium-ion tetrafluoroborate salt synthesized from small molecule Fmoc-Phe(4-NH_2)-OH on a large scale (Supporting Information Figure S11). NMR studies and comparison with commercially available *p*-methoxyphenyl diazonium salt clearly showed the formation of the diazonium adduct under our reaction conditions (Supporting Information Figure S11).

Chromophoric Properties of Triazene Cyclic Peptides. Because this approach generates a chromophoric triazene moiety, we set to explore photophysical properties of triazene cyclic peptides and related diazonium ions and linear peptides by using a UV spectrophotometer. The UV study on triazene cyclic peptide PGG(pAF) **2c** showed characteristic absorbance for triazene at 289 and 313 nm wavelengths. In contrast, linear counterpart **1c** did not show any absorbance in this region (Figure 3 Supporting Information S12). Because triazene **2c** and linear peptide **1c** showed distinct absorbance in UV and the rate of triazene formation is very high, we monitored the course of reaction on three different linear peptides PGRGWADGA(pAF) **1a**, PAMGLAE(pAF) **1b**, and PTAQS(pAF) **1p** by directly carrying out the cyclization reaction in UV cuvettes (Figure 3b, Supporting Information S13). All the three peptides showed a peak at 273 nm, characteristic of diazonium ions, immediately after the addition of $NaNO_2$ under acidic conditions. Next, we changed the pH of the solution to 7.5,

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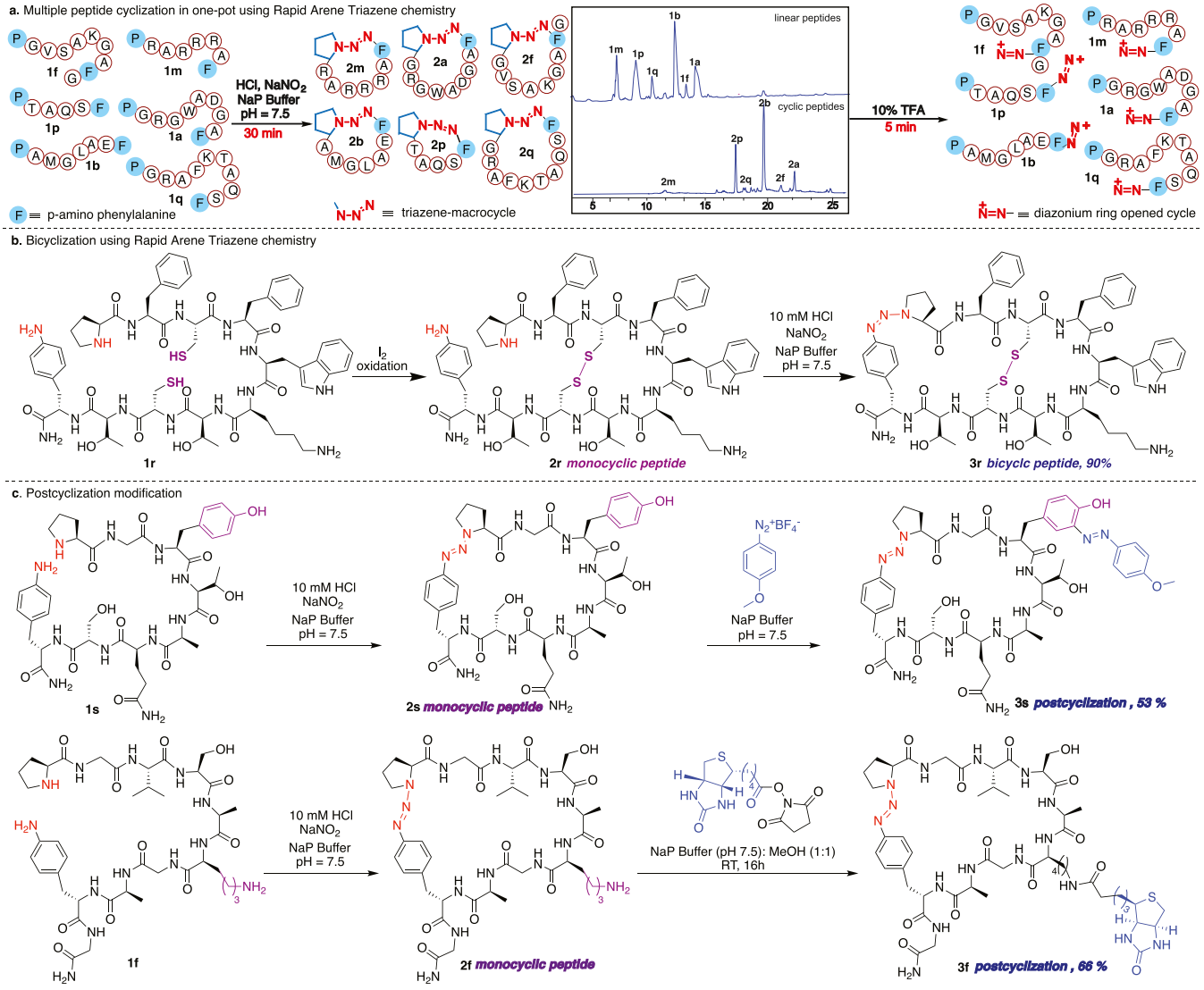

Figure 4. (a) Rapid arene triazene-mediated cyclization of multiple linear peptides **1a**−**1b**, **1f**, **1m**, **1p**, and **1q** in one pot to generate corresponding cyclic peptides **2a**−**2b**, **2f**, **2m**, **2p**, and **2q** without any cross-linking or dimerization followed by ring opening under acidic conditions for sequencing by LC−MS/MS. (b) Bicyclization of linear peptide **1r** via disulfide formation **2r** followed by rapid arene triazene cyclization generated bicyclic peptide **3r**. (c) Postcyclization modification of a triazene cyclic peptide **2s** by modification of tyrosine via 4-methoxy phenyldiazonium ion (4-MDz) and generated **3s** and postcyclization modification of a triazene cyclic peptide **2f** by modification of lysine via biotin-NHS ester and generated **3f**. Both postcyclization modification reactions were performed in one pot directly from linear peptides **1s** and **1f** without isolation of triazene cyclic peptides **2s** and **2f**.

and within 30 s, the new peaks at 289 and 313 nm were observed, characteristic of the triazene cyclic products for all the three cyclic peptides **2a**−**2b** and **2p** (Figure 3b, Supporting Information S13). Excitingly, we observed the full conversion to the triazene cyclic products within a minute with all the three peptides independent of the size and nature of the amino acids. We further confirmed the complete conversion to the triazene cyclic products **2a**−**2b** and **2p** by carrying out the HPLC and MS on the resulting solutions.

Inspired by these UV studies, we sought to determine the fate of the cyclization reaction of peptide **1b** at room temperature within 1 min and observed the full conversion to triazene cyclic product **2b** (>99%) as analyzed by HPLC and MS (Supporting Information Figure S14). We also injected the crude reaction mixture directly in the MS system after 1 min and observed the m/z of only cyclic peptide **2b** without any unreacted starting linear peptide **1b** or diazonium intermediate. All these studies showed that arene triazene cyclization chemistry is rapid and generate chromophores in cyclic peptides. Next, we determined the extinction coefficient of triazene cyclic peptides **2a** and **2c** (58,500−59,900) by recording UV at different concentrations of the triazene cyclic products **2a** and **2c** followed by using linear regression (Supporting Information Figure S15).

Reversibility of Triazene Cyclic Peptides: Response to External Stimuli. Based on our recent study with diazonium ions for labeling monomethyl lysine,[27] we hypothesized that triazene cyclic peptides will respond to a change in the pH by the protonation of the triazene. We hypothesized that at low pH, cyclic traizene will undergo protonation followed by the opening of the triazene ring to unchanged starting linear peptide with diazonium ions and N-terminal proline. To test our hypothesis, by taking **2a** as an example, at pH 7.4, the M + 1 adduct of the cyclic peptide **2a** was clearly seen in the HPLC

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(retention time, R_t = 26.379) and its mass spectrometric analysis (Supporting Information S16). By lowering the pH to 1.5, we observed a huge change in the retention time in the HPLC (R_t = 22.829) within 5 min and the mass of the new peak corresponded to the linear peptide M−N_2 adduct. It is common to observe −N_2 adducts of the diazonium salts in the mass spectrometer. To further confirm that it is the linear diazonium ion and not the proto-diazotization product that leads to the generation of phenylalanine at the site of pAF in the peptide, we carried out two different experiments. First, we changed the pH to 7.5 and again observed the formation of the cyclic triazene peptide **2a** within 5 min as analyzed by both HPLC retention time (R_t = 25.81) and mass spectrometry, which clearly showed the M + 1 adduct of the cyclic peptide **2a** (Figure 3c, Supporting Information S16). We also synthesized a linear peptide PGRGWADGAF with Phe(F) at the site of pAF, and HPLC analysis showed a peak at a completely different retention time (R_t = 21.36), confirming the lack of the formation of the proto-diazotization product (Supporting Information S16). Both the above experiments confirmed the unique feature of the triazene cyclic peptides responding to the change in the pH, leading to the opening and closing of the ring by varying the pH conditions, which is in contrast to the conventional methods of macrocyclization. This unique property of triazene cyclic peptides could be utilized for the sequencing of hit cyclic peptides obtained after library screening. In addition to pH, the triazene cyclic peptide **2p** showed response to UV radiation at 370 nm, and the cyclic triazene ring opened within 4 h to generate a diradical intermediate, which further combined to form a new cyclic peptide **3p** (35% yield) with a C−N bond between the proline and phenyl ring of p-NH_2-Phe at the site of cyclization (Figure 3d, Supporting Information S17). We have confirmed the structure of cyclic peptide **3p** by MS and NMR studies and compared it with linear peptide **1p** (Supporting Information Figure S17). The NH proton of proline in **1p** was observed at δ 2.36−2.31 as a multiplet but disappeared in the ^{1}H NMR spectrum of product **3p**. The diagnostic alpha protons (4.02−3.96, ppm) and alpha carbon (54.12 ppm) on the proline in the cyclized product **3p** shifted more downfield as compared to linear peptide **1p** alpha protons (3.26−3.22 and 3.19 ppm) and alpha carbon (46.32 ppm), indicating more deshielding (Supporting Information Figure S17). This is a characteristic shift in contrast to triazene cyclic product **2p,** where alpha proton and carbon of proline are more shielded and an upfield shift was observed as shown in all the NMR spectra of the cyclic triazene peptides. Another characteristic shift of **3p** is observed in ROESY spectra, indicating the interaction of the alpha proton of proline with protons of the phenyl ring of pAF. Such interaction was not observed in triazene cyclic products because these protons were not near the aromatic ring, further suggesting the formation of a C−N bond between the proline and phenyl ring of p−NH_2−Phe (Supporting Information Figure S17).

Cyclization in One Pot and Sequencing of Triazene Cyclic Peptides. Owing to its chemoselectivity yet robust reactivity toward secondary amines at pH 7.5, we moved to determine the application of arene triazene peptide cyclization under optimized reaction conditions. A higher reaction rate would lead to a lower probability of the intermolecular reaction at low concentrations, and a variety of cyclic peptides can be synthesized in one pot from the corresponding linear peptides. To analyze the compatibility of our approach for cyclization in one pot, we incubated six linear peptides **1a**−**1b**, **1f**, **1m**, **1p**, and **1q** in one pot and subjected them to optimized reaction conditions (Figure 4a, Supporting Information Figure S18; conc. 3 mM of each peptide). We successfully cyclized all the six linear peptides in one pot with full conversion to the triazene cyclization products **2a**−**2b**, **2f**, **2m**, **2p**, and **2q** and did not observe any intermolecular cross-linking products as analyzed by HPLC and MS (Figure 4a, Supporting Information Figure S18). To demonstrate the application of ring opening for the sequencing of HIT cyclic peptides after screening, we opened the library of six cyclic peptides to linear counterparts by using 10% TFA in buffer and observed full ring opening as analyzed by LC−MS (Figure 4a, Supporting Information Figure S18). Next, we randomly picked three linearized peptides **1b**, **1p**, and **1q** from this mixture and determined their sequence by LC−MS/MS (Supporting Information Figure S18).

Postcyclization Modification of Triazene Cyclic Peptides. The stability of cyclic peptides is a major concern for pharmaceutical applications. To evaluate the stability of a triazene cyclic peptide, we incubated cyclic peptide **2b** in aqueous solution, a water: ACN mixture. The triazene cyclic peptide **2b** remained unchanged up to 24 h, and no decomposition was observed (Supporting Information Figure S19). Solid triazene cyclic peptide **2b** was stable for at least 2 months when stored at 20 °C in the dark. We also exposed triazene cyclic peptide **2b** to sodium dithionite (5 equiv), and the cyclic peptide **2b** was stable for 2 h without any degradation, which is in contrast to diazo compounds formed between the Tyr side chain and diazonium ions, which underwent complete degradation in 5 min. We have also shown the orthogonality of our approach by exposing triazene cyclic peptide **2a** to piperidine used in Fmoc-SPPS, and no degradation of **2a** for 12 h was observed as analyzed by HPLC and MS (Supporting Information Figure S20).[27]

To evaluate the potential of triazene cyclic peptides for biological applications, we examined the proteolytic stability of a cyclic peptide in comparison with its linear counterpart. Linear peptide PGRAFKTAQS(pAF) **1q** and corresponding triazene cyclic adduct **2q** were incubated with trypsin, which hydrolyzed peptide bonds at the C-terminal side of lysine and arginine. Results showed that in the presence of trypsin, triazene cyclic peptide **2q** remained completely intact for up to 2 h, whereas its linear counterpart **1q** degraded completely, as determined by HPLC and MS analysis (Supporting Information Figure S21).

These results demonstrated that the triazene moiety generated during cyclization significantly improved the stability of cyclic peptides against both proteolysis and degradation over a range of other harsh conditions. Together, these results demonstrate the applicability of the rapid arene triazene chemistry in generating potentially bioactive cyclic peptidomimetics as molecular tools to study biological systems.

Encouraged by the stability results of triazene cyclic peptides, we planned to utilize this strategy for the synthesis of bicyclic peptides and late-stage derivatization of the triazene cyclic peptides. For bicyclization, an analogue of octreotide with two cysteine residues was synthesized PFCFWKTCT-(pAF) **1r**, followed by the cyclization under oxidative conditions to form a monocyclic peptide with a disulfide bond **2r** (Figure 4b, Supporting Information Figure S22). Arene triazene cyclization of the disulfide cyclic peptide **2r** gave bicyclic product **3r** with >90% conversion as analyzed by

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LCMS (Figure 4b, Supporting Information S22). Next, we carried out the late-stage derivatization of triazene cyclic peptides using different chemistries. Linear peptides with tyrosine PGYTAQS(pAF) **1s** and lysine PGVSAKGA(pFA)G **1f** were synthesized, followed by the cyclization using arene triazene chemistry to generate triazene cyclic peptides **2s** and **2f**. The tyrosine side chain of **2s** was modified by the addition of electron-rich 4-MDz, leading to the formation of a diazo-complex **3s** with a Tyr side chain as analyzed by LCMS (53% conv., Figure 4c, Supporting Information Figure S23). We observed lower conversion 53% of the late-stage modification product due to the inefficiency of tyrosine labeling with an electron-donating arene diazonium ion 4-methoxy phenyl diazonium ion (4MDz); 28% of triazene cyclic peptide **2s** remained unmodified (Supporting Information Figure S23). Notably, we performed the arene triazene cyclization and postcyclization modification in one pot by sequentially adding 4MDz into the reaction mixture. For detailed analysis, we carried out the cyclization of peptide PGYTAQS(pAF) **1s** containing both Pro and Tyr and generated 81% triazene cyclized product **2s** (49% yield) and 19% azo cyclic product **2s′** under the reaction conditions as analyzed by HPLC, MS, and NMR of the major adduct **2s** (Supporting Information S24).

We further modified the lysine side chain of cyclic peptide **2f** by the addition of biotin NHS-ester, forming a stable amide bond **3f** with 66% conversion as analyzed by LCMS (Figure 4c, Supporting Information S25). It is worth noting that arene triazene cyclization and postcyclization modification were performed in one pot by sequentially adding NHS-ester into the reaction mixture (Supporting Information Figure S25). This late-stage modification further diversifies the structural complexity of the cyclic peptides, which could be utilized for structure−activity relationship studies, leading to the discovery of high-affinity binders.

CONCLUSIONS

In summary, we have developed the arene triazene reaction for the rapid cyclization of peptides, generating an inbuilt chromophore as analyzed by UV. This method is highly chemoselective for reactions between secondary amines and pAF and leads to the efficient synthesis of triazene cyclic peptides without the formation of any undesired side products due to linear and cyclic dimerization or oligomerization. The potency of the arene triazene reaction is well demonstrated by the broad substrate scope encompassing a variety of peptides with different amino acid compositions including difficult sequences containing all L-amino acids without any turn inducers, various secondary amines, and different chain lengths including peptides with 4 and 20 amino acids and a peptide with multiple arginine residues as shown in Figure 2. The arene triazene cyclization reaction exhibits high reaction kinetics and efficiently macrocyclizes peptides within minutes with almost complete conversion irrespective of the distance between two reactive groups and generates both head-to-side chain and side chain-to-side chain macrocyclized products. More interestingly, the resulting cyclic peptides are highly stable under neutral pH conditions and other harsh conditions required for Fmoc-SPPS; thus, the cyclic peptides from rapid arene triazene cyclization were further modified with NHS-esters and diazonium ions to incorporate new functional groups. This approach was also utilized for making bicyclic peptides by using completely unprotected peptides using orthogonal chemistries. Excitingly, the triazene cyclic peptides can be readily reversed to unchanged linear peptides under low-pH conditions and thus utilized for the sequencing of the cyclic peptides after ring opening. Interestingly, ring opening of triazene cyclic peptides at a particular wavelength generated a new type of cyclic peptides obtained by diradical combination. We will explore this peptide cyclization in more details in future. We anticipate that the quick reversal of the arene triazene cycle in response to pH will make the strategy useful for a wide range of applications in the field of chemical biology and for sequencing of cyclic peptide binders. Very few cross-linking methods afford response to external stimuli and reverse macrocyclization. Considering the simple setup of the arene triazene macrocyclization reaction, fast kinetics, high yields, high chemoselectivity, ability to work with completely unprotected peptides to synthesize both mono- and bi-cycles, formation of highly stable cyclic peptides, reversibility in response to external stimuli, inbuilt-chromophore triazene at the linkage, and late-stage diversification with multiple functional groups, we anticipate that this method will become a highly useful tool for the synthesis of peptide conjugates and branched peptides for both chemical biology studies and drug discovery.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c00464.

Detailed experimental procedures and spectra for all cyclic peptides (PDF)

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Notes

The authors declare no competing financial interest.

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