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On-bead cyclization in a combinatorial library of 15,625 octapeptides

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Abstract—Combinatorial peptide libraries prepared by split-and-mix synthesis on solid support can be decoded by amino acid analysis (AAA) using the TAGSFREE method, which assigns variable amino acids to 'unique pair' positions. The method was used here to investigate on-bead cyclization in a library of 15,625 octapeptides $X^8X^7X^6X^5X^4$-Lys-X^2-glu(β-Ala-β-Ala-TentaGel Macrobead)-OAllyl, anchored via the side-chain carboxylate of the D-glutamate. Cyclization was carried out by amide bond formation between the free N-terminus and the α-carboxyl group of D-glutamate after selective removal of the Fmoc and allyl protecting groups, and followed using the TNBS test for free amines. Fast-cyclizing sequences often contained a turn element, in particular Ala-(Asp/Thr)-Pro at X^8-X^7-X^6, and phenylalanine at X^2. Slow-cyclizing sequences contained predominantly basic and polar residues, in particular Arg-His-Ser at X^7-X^6-X^5 and threonine at X^8. Fast-cyclizing sequences gave higher preparative yields of cyclic peptides (22–26% purified yields) than slow-cyclizing sequences (6–8%), showing that fast reaction is associated with efficient cyclization. This experiment demonstrates the first use of a TAGSFREE library of cyclic peptides.

1. Introduction

Cyclic peptides represent a privileged class of natural products displaying antiproliferative, anti-microbial, anti-fungal, and immunosuppressive activities.[1–3] Variations of amino acid side chains, stereochemistry, connectivity (α, β, etc.) and length (typically 4–15 amino acids) provide structural diversity enabling specific interactions with various target proteins. Cyclic peptides have lower conformational entropies and more defined conformations compared to their linear counterparts, leading to generally stronger and more selective target recognition and reduced biodegradability by proteases.[4,5]

Cyclic peptides can be prepared by solid-phase peptide synthesis (SPPS) of the linear precursor,[6] followed by cyclization, allowing capitalization on well-established methods and a diversity of available building blocks.[7] However, the production of cyclic peptide libraries by split-and-mix combinatorial synthesis,[8–10] which would facilitate screening for bioactive molecules, is more difficult to implement. Firstly cyclization is strongly sequence dependent, casting doubt on the homogeneity of the products formed. Secondly the identification of library members cannot be accomplished by Edman microsequencing due to the absence of free N-termini (unless a linear peptide is used as encoding tag)[11,12] and requires more complex methods, such as MS-analysis,[13] or the use of bead encoding/decoding with binary tags.[14–16] Such analyses are expensive and not affordable to decode more than a handful of beads per experiment.

We recently reported a general method for designing combinatorial peptide libraries decodable by amino acid analysis (AAA) using an algorithm called TAGSFREE,[17,18] developed in analogy to an earlier method for decoding combinatorial libraries of peptide dendrimers.[19–21] AAA involves total acidic hydrolysis of the peptide, derivatization of the released amino acids with phenyl isothiocyanate, and analytical separation and quantification by HPLC. This analysis is a routine and automated procedure available at low cost from most bioanalytical laboratories, and can be carried out with as little as 50 pmol of product, which is sufficient to detect the amounts present on a typical solid-phase synthesis polymer bead (NovaSyn Tentagel at 0.26 mmol/g: 90 pmol/bead, Rapp TentaGel Macrobeads used in this study at 0.23 mmol/g: 1.5 nmol/bead). The TAGSFREE library design consists in assigning each amino acid

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building block to a so-called 'unique pair' of variable positions, leading to a library in which each amino acid composition as determined by AAA corresponds to only one, sometimes two, or rarely four possible sequences. AAA also delivers a very useful quality control for the material on the bead, with typical fall-out rates of 15–30% due to incomplete sequences or empty beads, such that false positive hits are readily identified. The design limits library size and imposes sequence design because the number of possible 'unique pairs' U and hence the number of possible building blocks is limited by the peptide length N as $U = N(N-1)/2$. Nevertheless, the encoding scheme leads to useful numbers in the perspective of on-bead split-and-mix synthesis, where library size is limited by the number of beads in the synthesis.

Although the method is in principle independent of peptide topology, it was so far only exemplified with linear peptides used as aldolization catalysts[17] or as protease substrates.[18] Herein we report an investigation of the sequence dependence of cyclization efficiency in a library of octapeptides. Fast cyclizing sequences were found to provide significantly higher isolated yields of cyclic peptides compared to slow cyclizing ones. The experiment demonstrates the first application of the TAGSFREE method to cyclic peptides.

2. Results and discussion

We set out to investigate on-bead cyclization efficiency as a function of peptide sequence to test the applicability of TAGSFREE libraries to cyclic peptides. Cyclic peptides are most often synthesized from the linear precursor by cyclization in solution at high dilution, whereby the linear precursor is itself prepared by SPPS,[22–25] or using solution-phase organic synthesis.[26,27] A more direct synthesis is also possible using on-bead cyclization after removal of orthogonal protecting groups.[28–35] In both approaches the cyclization yields are strongly sequence dependent.[22,36] For example the so-called RAFT decapeptide c-(KAKPGKAKPe) has been shown to undergo efficient cyclization on-bead while attached to the support via the side-chain of the D-glutamate residue at position 1.[35,37,38] This high-yielding cyclization is facilitated through preorganization by two turn-inducing sequence elements in the form of a proline-D-glutamate dipeptide at positions 2 and 1, and a proline-glycine dipeptide at positions 6 and 5.

Inspired by this example, we designed a combinatorial library of octapeptides $X^8X^7X^6X^5X^4$-Lys-X^2-glu(β-Ala-β-Ala-TentaGel Macrobead)-OAllyl, anchored to the solid support via the side-chain carboxyl-group of an α-allyl-protected D-glutamate residue. Application of the TAGSFREE design to the six variable positions by using all 15 available unique pairs resulted in a 15,625-member combinatorial library (Table 1). Proline was placed at the variable pair of positions X^2 and X^6 corresponding to its position in the RAFT peptide for inducing turns. Lysine was held constant at position 3 to ensure good solubility of the deprotected peptides upon purification. The other amino acids were distributed among the remaining pairs to achieve an even distribution of properties along the sequence.

Table 1. TAGSFREE sequence design for octapeptide library[a]

No.	AA	X^8	X^7	X^6	X^5	X^4	X^2
1	Thr	1	1				
2	Leu	1		1			
3	Glu	1			1		
4	Val	1				1	
5	Ala	1					1
6	Arg		1	1			
7	Gly		1		1		
8	Tyr		1			1	
9	Asp		1				1
10	His			1	1		
11	Ile			1		1	
12	Pro			1			1
13	Ser				1	1	
14	Phe				1		1
15	Lys					1	1
	SUM	5	5	5	5	5	5

[a] Fifteen different amino acids are assigned to each of the 15 possible unique pairs for 6 variable positions in library $X^8X^7X^6X^5X^4$-Lys-X^2-glu(β-Ala-β-Ala-TentaGel Macrobead)-OAllyl. The total library size is $5^6 = 15{,}625$ members.

The library was prepared by standard Fmoc-type solid-phase synthesis on a 1.0 g batch of TentaGel Macrobeads® resin (215,000 beads/g, 0.23 mmol/g), ensuring an approximately 13-fold coverage of each sequence in the library. The relatively low loading of these beads per weight unit is known to favor intramolecular reaction during on-bead cyclization.[39,40] The synthesis was initiated by coupling a β-Ala-β-Ala spacer and the α-allyl-Fmoc-D-glutamate, whereby β-Ala also serves as an internal standard in AAA. After removal of the Fmoc group, the resin was split in five equal portions and coupled with each of the five amino acids defined for position X^2. The beads were then deprotected, mixed, and split again for the next coupling round. The procedure was repeated up to attachment of building blocks X^8. The library was then treated with $Pd(PPh_3)_4$ in CH_2Cl_2 in the presence of phenylsilane to effect deallylation of the D-glutamate residue.[35] The last Fmoc-protecting group was finally removed with piperidine to afford the free terminal amine suitable for testing cyclization (Fig. 3).

On-bead cyclization was tested using 90 mg portions of the solid support, which is sufficient to cover approximately 70% of the library (Fig. 1). The cyclization was carried out using PyBop ((Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate) (1.5 equiv) in the presence of Hünig's base (3 equiv) in DMF at room temperature, which are the conditions recommended for this type of cyclization.[35] To determine fast-cyclizing sequences, the reaction was interrupted by washing after 30 min and the beads were stained for free amines using TNBS (trinitrobenzene sulfonic acid), which stains beads dark red if free amines are present.[41] The resin was inspected under the microscope, which showed the presence of approximately 1% colorless beads indicating that cyclization was already com-

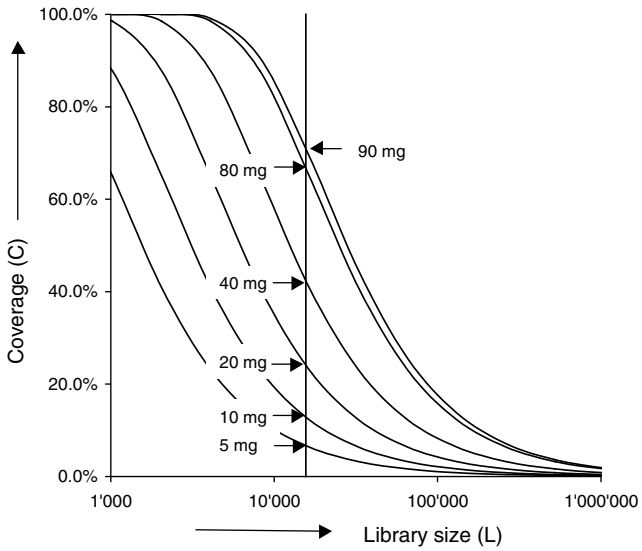

Figure 1. Calculated coverage of sequences as a function of library size L and mass of resin used, calculated for TentaGel Macrobeads at 215,000 beads/g. The vertical line is drawn at $L = 15{,}625$. $C = 1 - ((L-1)/L)^n$, n = number of beads in the mass of resin used.

plete (Fig. 2A). The beads were picked and subjected to AAA. From 29 beads analyzed, 24 returned a readable sequence (Table 2). To identify slow-cyclizing sequences, the experiment was repeated extending the cyclization time to 24 h. In this case, the few remaining deep-red stained beads still containing free amines were picked and subjected to AAA for decoding (Fig. 2B). From 25 beads analyzed, 12 returned a readable sequence (Table 2), the remaining beads having incomplete sequences or being simply empty, reflecting incomplete synthesis in these few beads.

Fast-cyclizing and slow cyclizing sequences were analyzed in terms of the preferred amino acid at each position. For each amino acid and position, the percentage occurrence in fast-cyclizing sequences minus the percentage occurrence in slow-cyclizing sequences was calculated as a measure for cyclization propensity (Table 3, main number). The relative occurrence frequency of each amino acid at each position across all beads sequences was close to 20% as expected from the combinatorial synthesis (Table 3, number in parentheses). Fast cyclizing sequences preferred glycine at position X^7 (+33%), proline at position X^6 (+42%), phenylalanine at position X^2 (+31%), and alanine at position X^8 (+25%). On the other hand slow cyclizing sequences showed a high relative frequency of threonine at position X^8 (−29%), arginine at position X^7 (−42%), and histidine at position X^6 (−38%).

Analysis in terms of amino acid pairs in the sequences showed that 298 of the 375 possible pairs (80%) were observed in at least one in the 39 attributed sequences. However, only 70 of these 298 pairs occurred in both fast-cyclizing and slow-cyclizing sequences, and only two of these pairs occurred more than once in both sets (Gly-Lys and Ser-Val at X^5-X^4), illustrating the sequence differences between the two sets. The fast-cyclizing sequences had frequently Asp-Pro or Gly-Leu at X^7-X^6 (each 5 occurences, none in slow cyclizing sequences) and Pro-Gly at X^6-X^5 (6 occurrences, none in slow cyclizing sequences). The tripeptide Ala-(Asp/Thr)-Pro at X^8-X^7-X^6 occurred 7 times in fast cyclizing sequences and not at all in slow cyclizing sequences. This sequence element clearly combines the turn-inducing proline residue at X^6 with alanine at X^8, which is the amino acid that mostly favors cyclization at that position. On the other hand the slow cyclizing sequences showed frequently Arg-His at X^7-X^6 (3 occurrences, none in fast cyclizing sequences) and His-Ser at X^6-X^5 (4 occurrences, none in fast cyclizing sequences), including three occurrences of the tripeptide Arg-His-Ser at X^7-X^6-X^5, one of which (**S9**) also includes the least cyclizing amino acid threonine at position X^8. The unreactivity of this sequence might be caused by bulky protecting groups combined with the absence of a turn-inducing element.

Four fast-cyclizing sequences (**F9**, **F13**, **F15**, and **F18**) and two slow-cyclizing sequences (**S11** and **S12**) were synthesized as individual cyclic peptides to test the influence of the sequence preferences observed in the combinatorial experiment on the preparative yields. The syntheses were performed on 250 mg batches of TentaGel Macrobeads RAM® (215,000 beads/g, 0.23 mmol/g, TFA-labile Rink linker). In each case 50 mg of the resin were cleaved directly to provide the reference linear peptide. The remaining resin was subjected to on-bead

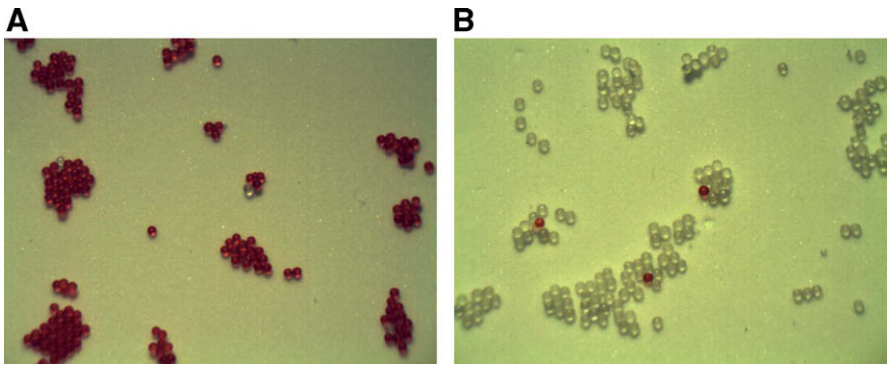

Figure 2. On-bead cyclization of octapeptide library. The beads were subjected to cyclization conditions (PyBop, Hünig's base, DMF, RT), washed, and stained for free amines using TNBS (trinitrobenzene sulfonic acid). (A) Beads containing fast-cyclizing octapeptides are identified as few colorless beads after 30 min of reaction. (B) Beads containing slow-cyclizing octapeptides are identified as few red-stained beads after 24 h of reaction.

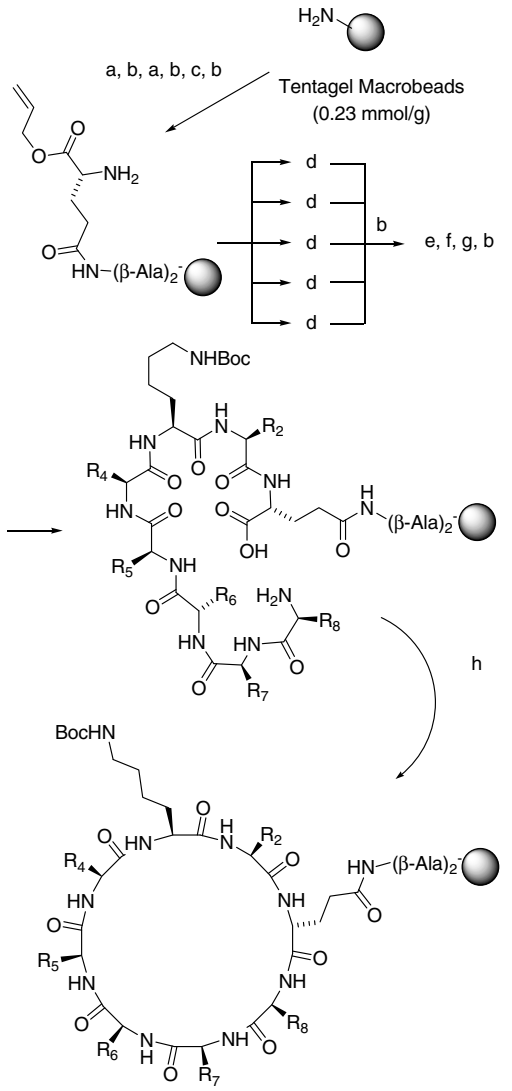

Figure 3. Solid phase synthesis of the octapeptide library and on-bead cyclization. Conditions: (a) Fmoc-β-AlaOH 3 equiv, PyBop 3 equiv, (*i*-Pr)$_2$EtN 6 equiv in DMF; (b) 20% piperidine in DMF; (c) Fmoc-α-allyl-D-glutamate 3 equiv, PyBop 3 equiv, (*i*-Pr)$_2$EtN 6 equiv in DMF; (d) 5 different FmocAAOH as given for position X^2, Table 1, conditions as (a); (e) Fmoc(Boc)LysOH, conditions as (a); (f) repeat split and (d), (b) five times with FmocAAOH as given for positions X^4 to X^8, Table 1, conditions as (a); (g) 0.25 equiv $Pd(PPh_3)_4$, 21 equiv $PhSiH_3$ in CH_2Cl_2 2 × 20 min, (h) PyBop 1.5 equiv, (*i*-Pr)$_2$EtN 3 equiv in DMF, 30 min. to 24 h, RT.

Table 2. Fast-cyclizing and slow-cyclizing octapeptides from library $X^8X^7X^6X^5X^4$-Lys-X^2-glu

	X^8	X^7	X^6	X^5	X^4	X^2
F1	Glu	Tyr	Arg	Glu	Lys	Ala
F2	Val	Asp	Pro	Gly	Lys	Ala
F3	Ala	Asp	Pro	Gly	Lys	Asp
F4	Glu	Thr	Arg	Ser	Val	Asp
F5	Ala	Asp	Pro	Gly	Lys	Lys
F6[a]	Ala	Asp	Pro	Phe	Ser	Lys
F7	Ala	Gly	Leu	His	Tyr	Lys
F8	Ala	Thr	Pro	Phe	Ser	Lys
F9	Val	Gly	Leu	His	Ile	Phe
F10	Leu	Gly	Pro	His	Ser	Phe
F11	Leu	Tyr	Arg	Phe	Ile	Phe
F12	Leu	Arg	Ile	Phe	Tyr	Phe
F13	Ala	Thr	Pro	Ser	Lys	Phe
F14	Ala	Gly	Leu	Gly	Lys	Phe
F15	Ala	Thr	Pro	Gly	Tyr	Phe
F16	Glu	Gly	Leu	Gly	Val	Phe
F17	Thr	Gly	Leu	Gly	Val	Phe
F18	Ala	Asp	Pro	Gly	Tyr	Pro
F19	Leu	Gly	His	Phe	Ser	Pro
F20	Val	Arg	Pro	Gly	Tyr	Pro
F21	Leu	Thr	Leu	Phe	Tyr	Pro
F22	Glu	Gly	His	Glu	Val	Pro
F23	Glu	Gly	Pro	Ser	Val	Pro
F24	Ala	Tyr	Leu	His	Ile	Pro
S1	Ala	Arg	His	Ser	Ile	Ala
S2	Ala	Arg	Ile	His	Ser	Ala
S3	Glu	Arg	His	Ser	Val	Asp
S4	Leu	Tyr	His	Ser	Val	Lys
S5	Leu	Thr	Ile	His	Ile	Lys
S6[b]	Thr	Gly	Arg	His	Lys	Ala
S7	Thr	Tyr	Arg	His	Tyr	Lys
S8[c]	Thr	Asp	Arg	Gly	Lys	Pro
S9	Thr	Arg	His	Ser	Val	Phe
S10	Val	Thr	Arg	Phe	Ser	Pro
S11	Val	Arg	His	Glu	Ser	Asp
S12	Val	Tyr	Leu	His	Ser	Lys

Fast-cyclizing (F) and slow-cyclizing (S) sequences were identified as described in Figure 2. Amino acid sequences were deduced from AAA data using the TAGSFREE program. Only three beads gave double sequences upon decoding.

[a] Alternative sequence for **F6**: Ala-Asp-Pro-Ser-Lys-Lys-Phe-glu.

[b] Alternative sequence for **S6**: Thr-Arg-His-Gly-Lys-Lys-Ala-glu.

[c] Alternative sequence for **S8**: Thr-Arg-Pro-Gly-Lys-Lys-Asp-glu.

cyclization. For fast-cyclizing sequences cyclization was completed within 30 min of reaction, while slow-cyclizing sequences required at least 24 h reaction time for completion. This reactivity was comparable to that observed in the library screening, suggesting that substitution of the Rink linker for the β-Ala-β-Ala spacer did not influence cyclization significantly. Analytical HPLC of the crude product after cleavage showed that the fast cyclizing sequences contained predominantly the expected cyclic peptides, which were isolated in good yields by preparative HPLC purification. By contrast, the crude products of the slow cyclizing sequences showed mainly side-products and non-cyclized material. Nevertheless the cyclic peptides were also isolated in pure form by preparative HPLC in these cases, although in only modest yields (Table 4).

The preparative experiments confirmed the sequence trends observed in the combinatorial experiment. Thus, fast-cyclizing sequences clearly gave higher cyclization yields than the slow-cyclizing sequences. The differences in yield of cyclic peptide were due to cyclization efficiencies and not due to the efficiency of linear peptide synthesis, as evidenced by the comparable yields obtained for the linear peptides in both series.

In principle dimer or polymer formation would also lead to decoloration in the TNBS test used for selection of the library since free amino termini are also consumed by these side-reactions. Nevertheless, the high cyclization yields and the presence of turn-inducing sequence

Table 3. Cyclization propensities of each amino acid[a]

No.	AA	X^8	X^7	X^6	X^5	X^4	X^2
1	Thr	−29% (14%)	+4% (19%)				
2	Leu	+4% (19%)		+21% (22%)			
3	Glu	+13% (17%)			0% (8%)		
4	Val	−13% (17%)				−4% (22%)	
5	Ala	+25% (33%)					−17% (14%)
6	Arg		−42% (22%)	−13% (17%)			
7	Gly		+33% (26%)		+25% (29%)		
8	Tyr		−13% (17%)			+17% (19%)	
9	Asp		+17% (15%)				−13% (13%)
10	His			−38% (21%)	−21% (24%)		
11	Ile			−13% (8%)		−4% (14%)	
12	Pro			+42% (32%)			+17% (24%)
13	Ser				−19% (21%)	−19% (21%)	
14	Phe				+15% (18%)		+31% (29%)
15	Lys					+10% (24%)	−19% (21%)

[a] Data given: N% (P%). The cyclization propensity N% is defined as N% = F% − S% where F%=percentage occurrence of the amino acid at the given position in the fast-cyclizing sequences; S%=percentage occurrence of the amino acid at the given position in the slow-cyclizing sequences. The relative occurrence P% is the observed frequency of the amino acid at the given position across all beads sequenced. For sequences **F6**, **S6**, and **S8**, each of the two possible sequences was assigned equal weight for counting.

Table 4. Preparative synthesis of selected cyclic octapeptides and their linear analogues

No.	Sequence[a]	Analytical HPLC cyclic/linear/n.i.[b]	Crude yield[c]	Yield after prep-HPLC[d]	MS calcd	MS obsd[e]
F9	c(VGLHIKFq)	72%/—/28%	33 mg (56%)	11 mg (26%)	923.6	923.5
F13	c(ATPSKKFq)	71%/—/29%	24 mg (42%)	9 mg (22%)	888.5	888.4
F15	c(ATPGYKFq)	77%/1%/22%	37 mg (69%)	10 mg (23%)	893.5	893.4
F18	c(ADPGYKPq)	33%/—/67%	41 mg (34%)	11 mg (28%)[f]	857.4	857.4
S11	c(VRHESKDq)	35%/13%/52%	31 mg (24%)	2.7 mg (6%)	980.5	980.4
S12	c(VYLHSKKq)	25%/14%/61%	28 mg (15%)	3.6 mg (8%)	984.6	984.6
F9L	VGLHIKFq	—/78%/—	3.8 mg (27%)	—	941.6	941.6
F13L	ATPSKKFq	—/76%/—	4.2 mg (30%)	—	906.5	906.4
F15L	ATPGYKFq	—/80%/—	4.8 mg (37%)	—	911.5	911.4
F18L	ADPGYKPq	—/98%/—	4.6 mg (45%)	—	875.4	875.4
S11L	VRHESKDq	—/93%/—	5.6 mg (46%)	—	998.5	998.2
S12L	VYLHSKKq	—/96%/—	4.7 mg (39%)	—	1002.6	1002.6

[a] D-Glutamine (q) is obtained from TFA-cleavage of the side-chain anchor to the rink-amide resin.
[b] HPLC of crude products. n.i. denotes non-identified side-products. Cyclic dimers are often formed in on-bead peptide cyclization.
[c] The yield given is calculated from the crude weight taking the percentage product in the analytical HPLC data into account.
[d] Weight and yield of pure product.
[e] Cyclic monomer and cyclic dimer can be differentiated by MS in the isotopic distribution at the $[M+H]^+$ peak. MS-spectra enlargements are provided in Supporting information.
[f] The absence of cyclic dimer lead to a higher number of pure fraction during the prep. HPLC and hence a higher isolated yield.

elements in fast cyclizing sequences clearly show that faster cyclization is indeed achieved by favoring intramolecular cyclization, and not by polymerization. Therefore, rapid on-bead cyclization would appear as a valid criterion for any peptide library to yield cyclic peptides for testing. On the other hand, slow-cyclizing libraries probably mostly contain failed cyclization products and should be avoided as sources for cyclic peptides. Although it was not investigated here, slow cyclization probably also leads to partial racemization of the D-glutamate.

3. Conclusion

The experiments above demonstrate the first application of TAGSFREE library design by 'unique pairs' to cyclic peptides, which allows decoding of the beads by amino acid analysis (AAA). A 15,625-membered split-and-mix combinatorial library of octapeptides linked to the solid support via the side-chain carboxyl group of an α-allyl-D-glutamate residue was prepared on TentaGel Macrobeads and tested for fast-cyclizing and slow-cyclizing sequences by peptide bond formation between the N-terminus at position X^8 and the deprotected α-carboxyl group of the D-glutamate residue at position 1, using the TNBS test for free amines as reaction indicator.

Fast-cyclizing sequences contained predominantly turn-inducing elements, in particular the tripeptide Ala-(Asp/Thr)-Pro at X^8-X^7-X^6. By contrast, slow-cyclizing sequences contained basic and polar amino acids, in particular in the tripeptide Arg-His-Ser at X^7-X^6-X^5, as well as threonine at position X^8. Preparative syntheses of selected sequences and HPLC purification gave much higher isolated yields of cyclic peptides for the fast-cyclizing sequences (22–28%) than for the slow-cyclizing

sequences (6–8%), confirming that fast reaction as indicated by decoloration in the TNBS test is associated with efficient cyclization. This suggests that combinatorial peptide libraries can be tested for rapid cyclization as an indication of their suitability as a source for cyclic peptides.

4. Experimental

4.1. General

Reagents were purchased in the highest quality available from Fluka, Sigma, Bachem, or Novabiochem. TentaGel Macrobeads were purchased from Rapp Polymere GmbH. The following commercially available amino acids were used in synthesis: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Thr(^{t}Bu)-OH, Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Val-OH. Fmoc-D-glu-1-allyl ester was prepared as previously described by P. Dumy and co.[35] All solvents used in reactions were bought in p.a. quality or distilled and dried prior to use. Solvents for washings were distilled from technical quality. Chromatographic purifications (flash) were performed with Silica Gel 60 from Fluka (0.04–0.063 nm; (230–400) mesh ASTM). Preparative RP-HPLC (flow rate 80 mL min^{-1}) was performed with a Waters Delta Prep 4000 system with a Waters Prepak Cartridge (500 g) as column and Waters 486 Tunable Absorbance Detector. Analytical RP-HPLC (flow rate 1.5 mL min^{-1}) was performed on Waters 600E systems with a Waters Atlantis (4.6 × 100 mm, dC18, 5 lm) column, UV detection with Waters 996 photodiode array detector at 214 nm. Eluents for all systems were: A: water and 0.1% TFA and C: Acetonitrile/H_20 with TFA (90:10:0.1).

Mass spectra were obtained by electron spray ionization (ES-MS) on a Micromass Autospec Q (Waters/Micromass) instrument in the positive mode and provided by the mass spectrometry service of the Department of Chemistry and Biochemistry, University of Bern.

4.2. Library synthesis

The on-bead cyclic-octapeptides library was synthesized on TentaGel Macrobeads NH_2resin (1.0 g, loading: 0.23 mmol/g) using the split-and-mix procedure. The resin was acylated with 3 equiv of *N*-α-Fmoc amino acid in the presence of PyBOP (3 equiv, 380 mg, 0.69 mmol) and *N*,*N*′-diisopropylethylamine ((*i*-Pr)$_2$EtN) (6 equiv, 230 μL, 1.38 mmol) in DMF. After 2 × 60 min the resin was washed (3× 5 mL each) with DMF, CH_2Cl_2, and MeOH and controlled with the TNBS (trinitrobenzenesulfonic acid) test followed by acetylation. Fmoc protecting groups were removed by treatment with piperidine 20% in DMF (3 mL, 3 × 10 min) and then washed with DMF, CH_2Cl_2, and MeOH.

Mix and split was carried out after α-amine deprotection. The resin was suspended in DMF–CH_2Cl_2(2:1, v/v), mixed via nitrogen bubbling for 15 min and distributed in five equal portions for the next coupling. After the last coupling step, the resin bearing the library of octapeptides (1.0 g, 0.23 mmol) was dried under vacuum for 1 h, then swollen in a glass reactor fitted with a sintered glass frit with dry CH_2Cl_2 (10 mL, 2 × 15 min) and dry DMF (10 mL, 1 × 15 min). The resin was treated with $PhSiH_3$ (617 μL, 5 mmol) in dry CH_2Cl_2 (10 mL) for 5 min. $Pd(PPh_3)_4$ (69 mg, 0.06 mmol) was then added and the resin was stirred under argon gas for 20 min. The reagents were removed by filtration, the resin washed with CH_2Cl_2 (10 mL, 4 × 1 min) and DMF (6 mL, 3 × 1 min) and the procedure was repeated once. The resin was finally washed with CH_2Cl_2 (10 mL, 2 × 1 min), dioxane–water (9:1, 10 mL, 2 × 1 min), (*i*-Pr)$_2$EtN 5% in DMF (6 mL, 3 × 1 min) and DMF (10 mL, 2 × 1 min). Finally, the last Fmoc protecting group was removed as described above.

4.3. Screening for fast and slow cyclizing sequences

On-bead cyclization was tested in 90 mg portions of the solid support (~19,350 beads, 0.0207 mmol). PyBoP (1.5 eq, 18 mg, 0.031 mmol) and (*i*-Pr)$_2$EtN (3 equiv, 10 μL, 0.062 mmol) were added to the resin in DMF (4 mL) and the mixture was stirred at room temperature for 30 min. The reagents were removed by washing with NMP (5 mL, 2 × 1 min), MeOH (5 mL, 1 × 1 min), and CH_2Cl_2 (4 mL, 2 × 1 min). TNBS test was performed by swelling the resin in 200 μL of TNBS solution 10% in DMF and 200 μL of 10% (*i*-Pr)$_2$EtN in DMF for 5 min. The swelling mixture was removed by filtration, the beads suspension washed with CH_2Cl_2 and then plated out onto a silica gel plate. The beads were observed under a microscope and colorless beads were picked as fast cyclizing sequences and subjected to AAA for decoding. For detecting slow cyclizing sequences the experiment was repeated and the resin was allowed to stir for 24 h in presence of the condensating reagents. Test with trinitrobenzenesulfonic acid was performed as described above and deep red stained beads were picked for sequence determination.

4.4. Bead analysis

Single-peptide-containing resin beads were hydrolyzed with aqueous HCl (6 M) at 110 °C for 22 h, and their amino acid composition was determined quantitatively by HPLC after derivatization with phenyl isothiocyanate (PITC). The sequences were then determined by TAGSFREE decoding.

4.5. Synthesis of single sequences

Reactions were carried out in the PSW 1100 multiple peptide synthesizer (Chemspeed Technologies), equipped with one solid phase block (up to 16 reactors). In each reactor, 250 mg of TentaGel Macrobeads RAM® (0.0575 mmol) of resin were swollen with CH_2Cl_2 and the synthesis was done with *N*-α-Fmoc protected amino acids (3 equiv, 0.17 mmol), 3 equivalents of

PyBOP and 6 equiv of $(i\text{-Pr})_2$EtN, as previously described. Allyl deprotection, N-terminal Fmoc deprotection, and condensation were performed manually in 5 mL syringes (Braun Injekt) following the same procedure than for the library synthesis.

4.5.1. Synthesis of linear sequences. After final Fmoc deprotection and washing of the resin, a 50 mg sample containing the fully deprotected linear counterpart was taken from each reaction vessel and cleaved using TFA-H_2O-TIS (triisopropylsilane) as a (95:2.5:2.5, v/v) solution for 5 h (1 mL/100 mg of resin). The peptide was precipitated in methyl tert-butyl ether and analyzed by analytical HPLC with no further purification.

4.5.2. Synthesis of fast and slow cyclizing sequences. The remaining 200 mg of resin was subjected to cyclization under the same conditions described above. Fast cyclizing showed a negative TNBS test after 30 min. Slow cyclizing sequences still showed the presence of free amines after 24 h. PyBop and $(i\text{-Pr})_2$EtN were removed by filtration and the resin was washed with DMF (3×5 mL) and CH_2Cl_2 (3×5 mL). The peptide was cleaved with TFA as described and precipitated in diisopropyl ether then dissolved in water–acetonitrile mixture. All the peptides were purified by preparative RP-HPLC.

Cyclic peptide **F9** c(VGLHIKFq) from TentaGel Macrobeads RAM (200 mg, 0.046 mmol) was obtained as colorless foamy solid after preparative HPLC purification (11 mg, 26%); Anal. RP-HPLC (99% A, 1% C to 20% A, 80% C in 25 min, $\lambda = 214$ nm): $t_R = 17.71$ min; ESI MS(+): Calcd for $C_{42}H_{63}N_{12}O_{10}$ ($[M+H]^+$): *m/z*: 923.56 $[M+H]^+$; found 923.5. ^{1}H NMR (300 MHz, D_20) δ 8.67 (s, 1 H, CH–Ar His), 7,24-7,37 (m, 6H, CH–Ar His and CH–Ar Phe), 4.54-4,79 (m, 2H), 3.98–4.35 (m, 6H), 3.74–3.78 (ss, 1H), 3.27–3.42 (m, 2H), 2.96–3.18 (m, 4H), 1.06–2.12 (m, 17H), 0.71–0.91 (m, 18H, CH_3 Ile, CH_3 Val, CH_3 Leu).

Linear peptide **F9L** VGLHIKFq from TentaGel Macrobeads RAM (50 mg, 0.012 mmol) was obtained as colorless foamy solid after methyl tert-butyl ether precipitation (3.8 mg, 27%); Anal. RP-HPLC (99% A, 1% C to 20% A, 80% C in 25 min, $\lambda = 214$ nm): $t_R = 15.50$ min; ESI MS(+): Calcd for $C_{42}H_{65}N_{12}O_{11}$ ($[M+H]^+$): *m/z*: 941.55 $[M+H]^+$; found 941.6.

Cyclic peptide **F13** c(ATPSKKFq) from TentaGel Macrobeads RAM (200 mg, 0.046 mmol) was obtained as colorless foamy solid after preparative HPLC purification (9 mg, 22%); Anal. RP-HPLC (99% A, 1% C to 20% A, 80% C in 25 min, $\lambda = 214$ nm): $t_R = 13.38$ min; ESI MS(+): Calcd for $C_{41}H_{66}N_{11}O_{11}$ ($[M+H]^+$): *m/z*: 888.49 $[M+H]^+$; found 888.4. ^{1}H NMR (300 MHz, D_20) δ 7.22–7.38 (m, 5H, CH–Ar Phe), 2.94–4.56 (m, 18H), 1.15–2.30 (m, 27H).

Linear peptide **F13L** ATPSKKFq from TentaGel Macrobeads RAM (50 mg, 0.012 mmol) was obtained as colorless foamy solid after methyl tert-butyl ether precipitation (4.2 mg, 30%); Anal. RP-HPLC (99% A, 1% C to 20% A, 80% C in 25 min, $\lambda = 214$ nm): $t_R = 11.87$ min; ESI MS(+): Calcd for $C_{41}H_{68}N_{11}O_{12}$ ($[M+H]^+$): *m/z*: 906.51 $[M+H]^+$; found 906.4.

Cyclic peptide **F15** c(ATPGYKFq) from TentaGel Macrobeads RAM (200 mg, 0.046 mmol) was obtained as colorless foamy solid after preparative HPLC purification (9.5 mg, 23%); Anal. RP-HPLC (99% A, 1% C to 20% A, 80% C in 25 min, $\lambda = 214$ nm): $t_R = 13.28$ min; ESI MS(+): Calcd for $C_{43}H_{61}N_{10}O_{11}$ ($[M+H]^+$): *m/z*: 893.45 $[M+H]^+$; found 893.4. ^{1}H NMR (300 MHz, D_20) δ 7.21–7.35 (m, 5H, CH–Ar Phe), 7.04–7.07 (d, 2H, CH–Ar Tyr), 6.77–6.79 (d, 2H, CH–Ar Tyr), 3.84–4.65 (m, 8H, CH alpha), 3.67 (s, 2H), 2.83–3.20 (m, 8H), 1.54–2.31 (m, 10H), 1.48–1.50 (d, 2H), 1.23–1.26 (d, 2H), 1.19 (s, 6H, CH_3 Ala, CH_3 Thr).

Linear peptide **F15L** ATPGYKFq from TentaGel Macrobeads RAM (50 mg, 0.012 mmol) was obtained as colorless foamy solid after methyl tert-butyl ether precipitation (4.8 mg, 37%); Anal. RP-HPLC (99% A, 1% C to 20% A, 80% C in 25 min, $\lambda = 214$ nm): $t_R = 12.11$ min; ESI MS(+): Calcd for $C_{43}H_{63}N_{10}O_{12}$ ($[M+H]^+$): *m/z*: 911.46 $[M+H]^+$; found 911.4.

Cyclic peptide **F18** c(ADPGYKPq) from TentaGel Macrobeads RAM (200 mg, 0.046 mmol) was obtained as colorless foamy solid after preparative HPLC purification (9 mg, 23%); Anal. RP-HPLC (99% A, 1% C to 20% A, 80% C in 25 min, $\lambda = 214$ nm): $t_R = 12.59$ min; ESI MS(+): Calcd for $C_{39}H_{57}N_{10}O_{12}$ ($[M+H]^+$): *m/z*: 857.42 $[M+H]^+$; found 857.4. ^{1}H NMR (300 MHz, D_20) δ 7.14–7.22 (m, 2 H, CH–Ar Tyr), 6.84–6.91 (m, 2H, CH–Ar Tyr), 3.39–5.20 (m, 12H), 2.70–3.12 (m, 6H), 1.56–2.42 (m, 17H), 1.03–1.45 (m, 5H).

Linear peptide **F18L** ADPGYKPq from TentaGel Macrobeads RAM (50 mg, 0.012 mmol) was obtained as colorless foamy solid after methyl tert-butyl ether precipitation (4.6 mg, 45%); Anal. RP-HPLC (99% A, 1% C to 20% A, 80% C in 25 min, $\lambda = 214$ nm): $t_R = 8.94$ min; ESI MS(+): Calcd for $C_{39}H_{59}N_{10}O_{13}$ ($[M+H]^+$): *m/z*: 875.43 $[M+H]^+$; found 875.4.

Cyclic peptide **S11** c(VRHESKDq) from TentaGel Macrobeads RAM (200 mg, 0.046 mmol) was obtained as colorless foamy solid after preparative HPLC purification (2.7 mg, 6%); Anal. RP-HPLC (99% A, 1% C to 20% A, 80% C in 25 min, $\lambda = 214$ nm): $t_R = 11.61$ min; ESI MS(+): Calcd for $C_{40}H_{66}N_{15}O_{14}$ ($[M+H]^+$): *m/z*: 980.49 $[M+H]^+$; found 980.4. ^{1}H NMR (300 MHz, D_20) δ 8.62 (s, 1H, CH–Ar His), 7.27 (s, 1H, CH–Ar His), 3.84–4.34 (m, 8H), 2.82–3.51 (m, 8H), 1.39–2.40 (m, 21H), 0.91–0.95 (dd, 6H, CH_3 Val).

Linear peptide **S11L** VRHESKDq from TentaGel Macrobeads RAM (50 mg, 0.012 mmol) was obtained as colorless foamy solid after methyl tert-butyl ether precipitation (5.6 mg, 46%); Anal. RP-HPLC (99% A, 1% C to 20% A, 80% C in 25 min, $\lambda = 214$ nm): $t_R = 10.78$ min; ESI MS(+): Calcd for $C_{40}H_{68}N_{15}O_{15}$ ($[M+H]^+$): *m/z*: 998.5 $[M+H]^+$; found 998.2.

Cyclic peptide **S12** c(VYLHSKKq) from TentaGel Macrobeads RAM (200 mg, 0.046 mmol), was obtained as colorless foamy solid after preparative HPLC purification (3.6 mg, 8%); Anal. RP-HPLC (99% A, 1% C to 20% A, 80% C in 25 min, $\lambda = 214$ nm): $t_R = 14.27$ min; ESI MS(+): Calcd for $C_{46}H_{74}N_{13}O_{11}$ ($[M+H]^+$): *m/z*: 984.57 $[M+H]^+$; found 984.6. ^1H NMR (300 MHz, D_2O) δ 8.62 (s, 1H, CH–Ar His), 7.20 (s, 1H, CH–Ar His), 7.12–7.15 (d, 2H, CH–Ar Tyr), 6.82–6.85 (d, 2H, CH–Ar Tyr), 4.50–4.55 (m, 2H), 4.17–4.31 (m, 5H), 4.39–4.00 (m, 2H), 3.67–3.72 (m, 1H), 2.81–3.33 (m, 10H), 2.33–2.56 (m, 2H), 2.02–2,21 (m, 3H), 1.84–1.92 (m, 2H), 1.23–1.72 (m, 10H), 1.13 (m, 1H), 0.81–0.92 (dd, 6H, CH_3 Val), 0.72 (s, 3H, CH_3 Leu), 0.59 (s, 3H, CH_3 Leu).

Linear peptide **S12L** VYLHSKKq from TentaGel Macrobeads RAM (50 mg, 0.012 mmol), was obtained as colorless foamy solid after methyl tert-butyl ether precipitation (4.7 mg, 41%); Anal. RP-HPLC (99% A, 1% C to 20% A, 80% C in 25 min, $\lambda = 214$ nm): $t_R = 12.83$ min; ESI MS(+): Calcd for $C_{46}H_{76}N_{13}O_{12}$ ($[M+H]^+$): *m/z*: 1002.58 $[M+H]^+$; found 1002.6.

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Supplementary data

Compound characterization (MS, ^1H NMR) and amino acid analyses of resin beads form on bead assay. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.01.045.

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