Tandem Peptide Cyclization

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A Tandem In Situ Peptide Cyclization through Trifluoroacetic Acid Cleavage**

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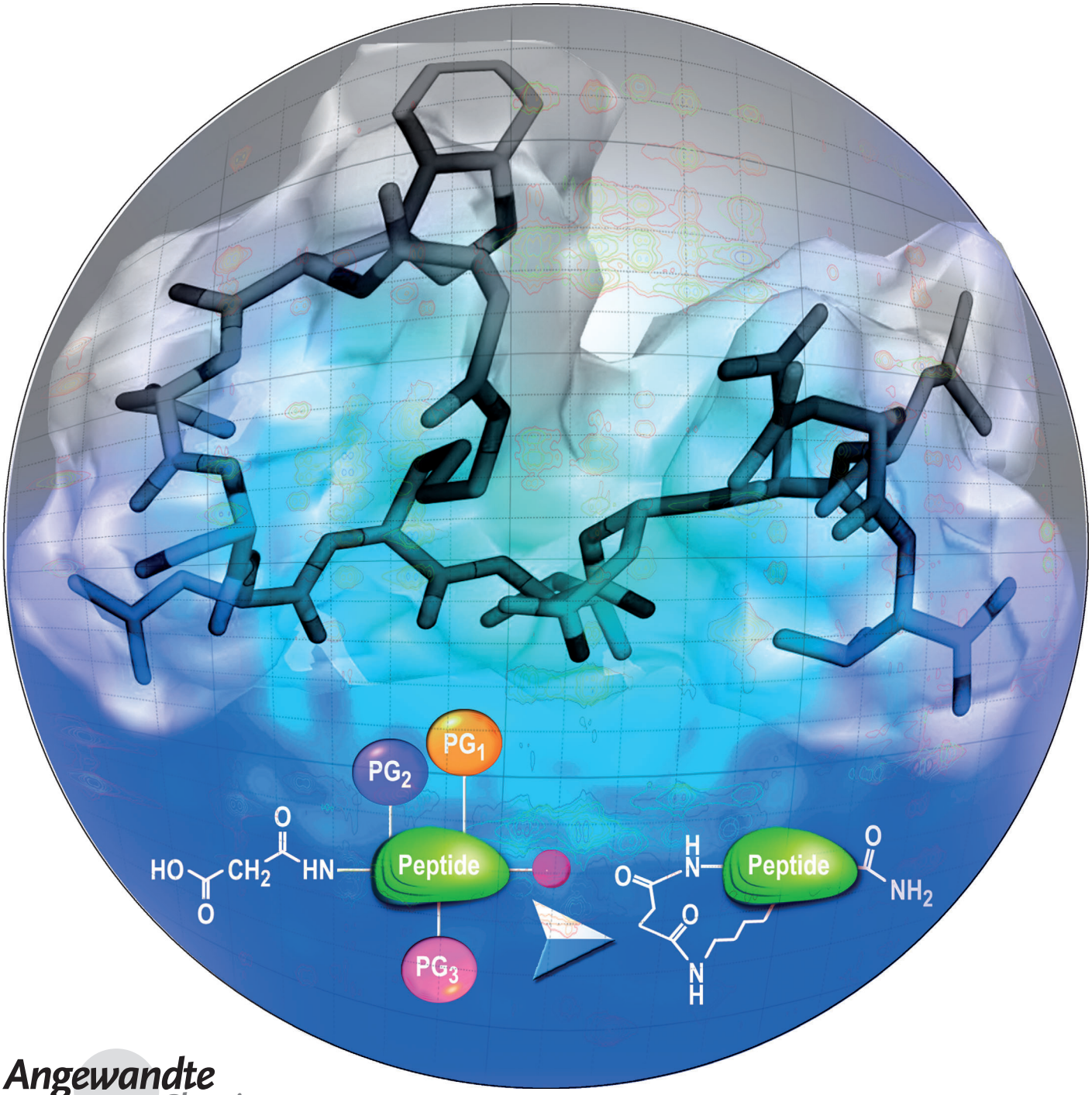

Abstract: *We present a new approach for peptide cyclization during solid phase synthesis under highly acidic conditions. Our approach involves simultaneous in situ deprotection, cyclization and trifluoroacetic acid (TFA) cleavage of the peptide, which is achieved by forming an amide bond between a lysine side chain and a succinic acid linker at the peptide N-terminus. The reaction proceeds via a highly active succinimide intermediate, which was isolated and characterized. The structure of a model cyclic peptide was solved by NMR spectroscopy. Theoretical calculations support the proposed mechanism of cyclization. Our new methodology is applicable for the formation of macrocycles in solid-phase synthesis of peptides and organic molecules.*

Cyclization is one of the most efficient tools for enhancing the stability and selectivity and improving the pharmacological properties of peptide therapeutics.[1] This is achieved by conformational restriction imposed on the peptide upon cyclization.[2] The wide applications of cyclic peptides (CPs)[3] include potential antibiotics, regulation of cellular activities involving neurotransmission,[4] treatment of Alzheimer's and Huntington's diseases,[5] nanomaterials[6] and imaging agents.[7] Several synthetic strategies are used for peptide cyclization.[8] The macrocyclic ring may be formed by an amide,[9a,b] ester,[9c] azo[9d] or disulfide bonds,[9e] triazole linkage,[9f] metal-assisted macrocyclization,[9g] ring closing metathesis,[9h] alkene-based "staples",[9i] electrostatically induced macrocyclization,[9j] use of isocyanide,[9k] multi-component reactions[9l] and more.[9m,n] The above cyclization strategies are performed under basic conditions either on the resin-bound peptide or in solution after cleavage.[9a–c,h,10] However, cyclization under highly acidic conditions has not been reported. The obvious reason is the deactivation of the nucleophilic amine in highly acidic conditions.

Here we describe an easy and efficient new strategy for tandem in situ peptide cyclization under highly acidic conditions during trifluoroacetic acid (TFA) cleavage. The cyclization takes place by forming an amide bond between the amine of a lysine side chain and a succinic acid derivative at the N-terminus of the peptide (Figure 1). We demonstrated our strategy for the model linear peptide LEDGF 361–370(NSLKIDNLDV),[11] which has been shown in our lab to be a potent inhibitor of HIV-1 integrase (IN) and has anti-HIV activity in cells.[11,12] LEDGF 361–370 was selected as a model since cyclization of this peptide by the conventional strategies has already been shown to significantly increase its drug-like properties.[12]

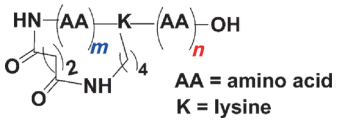

Figure 1. General structure of CPs generated by our new methodology.

To introduce the moiety that reacts with the lysine side chain to form the ring-closing amide bond, various succinic acid derivatives (Table 1 b–d) were treated with the N-terminal free amine of resin-bound LEDGF 361–370 (**4**). The peptide cyclization took place in three successive steps: 1) the formation of an N-terminal succinimide using standard HBTU (*O*-(benzotriazol-1-yl)-tetramethyluronium hexafluorophosphate) coupling in the presence of diisopropyl

Table 1: The formation of CPs (**6**, **20**, **21**, **43**) using different succinic acid derivatives.

Peptides and succinic acid derivative	Cyclic peptide	Obsd. MW	Obsd. r_n	Yield [%]
WNSLKIDNLDV (**4**)				
Succinic acid (**1a**)				66
(Benzyl)succinic acid (**1b**)	*c*(Suc-WNSLK)-			64
(3′-Ascorbic)succinic acid (**1c**)	IDNLDV-$CONH_2$	1396	17.34	60
(6′-Ascorbic)succinic acid (**1d**)	(**6**)			61
Succinic anhydride (**1e**)				65
DGWNSLKIDNLDV (**13**)				
in situ formation from Asp-Gly (**3a**)	*c*(Suc-GWSLK)- IDNLDV-$CONH_2$ (**20**, **21**)	1398	17.33	61
NGWNSLKIDNLDV (**14**)				
in situ formation from Asn-Gly (**3b**)				63
WRRIKRFVSQVIM (**42**)	*c*(Suc-WRRIK)-	1821	16.23	32
Succinic acid (**1a**)	RFVSQVIM- $CONH_2$ (**43**)			

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ethyl amine (DIPEA, basic conditions) in DMF at room temperature for 1 h; 2) the deprotection and cleavage of resin-bound peptide in TFA (acidic conditions) at room temperature for 1.5 h; 3) precipitation of the crude peptide in ether at 0 °C followed by workup in 50 % aqueous acetonitrile for lyophilisation. This led to the spontaneous formation of a cyclic peptide (CP) (**6**) at above 60 % yield under the highly acidic conditions used at step 2 (Table 1). The CP (**6**) had a succinic acid moiety that bridged the N-terminal tryptophan and the lysine side chain (Figure 2). The formation of the CP (**6**) was verified by a negative response towards the conventional color tests such as Kaiser-ninhydrin[13a] and chloranil.[13b] The CP exhibited the correct mass of 1396 Da and had a higher retention time (r_n) in reverse-phase HPLC compared

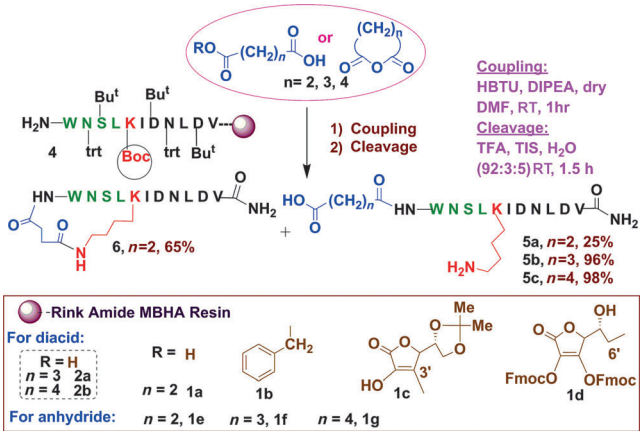

Figure 2. Solid-phase synthesis of CP **6**. CP **6** was formed as a result of cyclization (residues in the ring are shown in green) between a lysine side chain (red) and an N-terminal succinic acid (blue). Cyclization was independent of the nature of the R leaving group (**1a–d** in brown) on the succinic moiety. The degree of cyclization was drastically reduced with the increase in chain length (*n*) of the dicarboxylic acid derivative ($n = 3, 4$).

mechanism and detect the possible common succinimide intermediate, we monitored the cleavage kinetics of the resin-bound succinyl LEDGF peptide (**10**). Up to 10 min at 0 °C, only one product, corresponding to a putative intermediate, was observed. This peak had a retention time of 16.4 min in the analytical reverse-phase HPLC, which was between the retention times of LP **5a** (15.6 min) and CP **6** (17.2 min). After 10 min, the CP (**6**) began to appear along with the intermediate. The intermediate showed a positive response to the Kaiser and chloranil tests, indicating the presence of a free amine. However, its mass remained identical to that of CP **6**, indicating that the intermediate (**11**) must possess a succinimide bound to the N-terminal tryptophan (Figure 3). Once

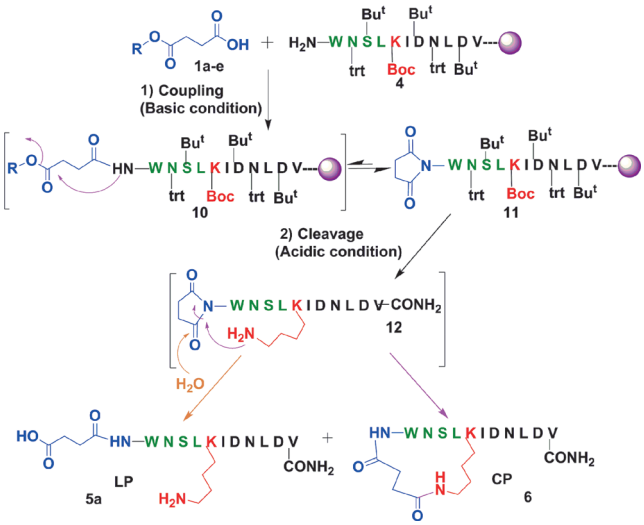

Figure 3. Proposed mechanism for in situ cyclization via a succinimide intermediate (SI). A resin-bound succinyl peptide was formed before cleavage, followed by the intramolecular attack of the amine group of the lysine that took place during the cleavage. R represents a leaving group.

to the parent linear peptide (LP, **5a**). Cyclization was also achieved without the presence of leaving groups such as with succinic acid (**1a**) and succinic anhydride (**1e**). The presence of the lysine was crucial for the formation of the CP. No cyclization was obtained when the lysine in LEDGF 361–370 (**7**) was acetylated or when the lysine was replaced by ornithine (Figure S1 in the Supporting Information). Rather, in this case we isolated LP **8** in which the N-terminal succinic moiety was converted into succinimide. This raised the question whether this succinimide is the common intermediate for all the above cyclization reactions involving linear peptide **4** and the succinic acid derivatives **1a–e**.

The cyclization reaction comprises three distinct steps that occur during the final stages of peptide synthesis: 1) HBTU-mediated coupling to enable the formation of the succinimide intermediate (SI) in the resin-bound peptide; 2) TFA-mediated cleavage and deprotection of the side chain protecting groups, including the Boc (tert-butoxycarbonyl) on the lysine, which enables step 3; 3) in-situ cyclization mediated by a nucleophilic attack of the deprotected lysine side chain on the succinimide. To gain insight into the reaction

the cyclic peptide was formed, it was very stable. Exposing CP **6** to 60 % aqueous acetonitrile (containing 0.1 % TFA) for several days did not result in ring opening. This rules out the possibility that the cyclization may take place under the weakly acidic HPLC conditions. To further prove this point, we synthesized diastereomeric CPs (**20**, **21**) from pre-made[14] succinimidyl-LEDGF 361–370 (**19**), which was prepared by modifying its N-terminus with either aspartic acid-glycine (**17**) or asparagine-glycine residues (**18**) (Figure 4). These sequences are known to form succinimide spontaneously in solid-phase peptide synthesis (SPPS).[14] A similar intramolecular cyclization (**23**) reaction was observed when a pre-synthesized succinimide was present in the middle of the LEDGF peptide (**22**, Table S2). When the succinic acid (**1a**) was replaced by glutaric acid (**2a**) or adipic acid (**2b**) or their monocarboxylic derivatives, no cyclization was observed. This means that the formation of six- or seven-membered N-terminal ring was less favored than the five-membered succinimide (**25**, **27**, Table S2). To further test these parameters, we applied our strategy to another model peptide, LEDGF 401–413 (WRRIKRFVSQVIM, **42**). This peptide is

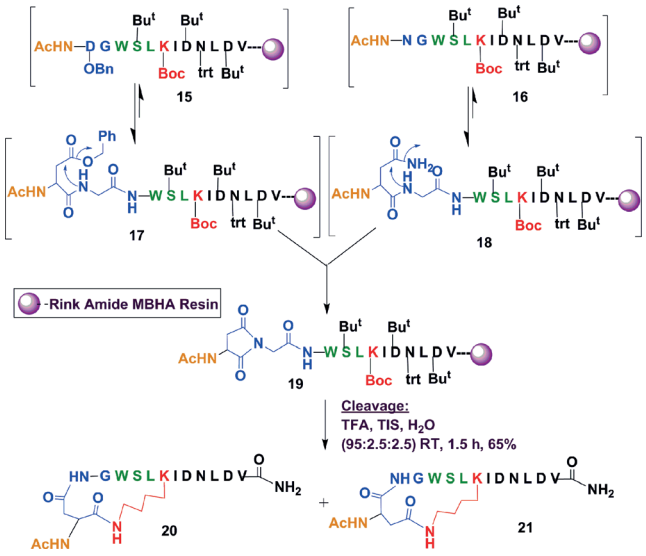

Figure 4. Evidence for in situ cyclization via a succinimide intermediate. Succinimide was formed in situ from Asn or β-benzyl Asp followed by Gly, leading to the formation of the same cyclic peptides as with the succinic acid derivatives.

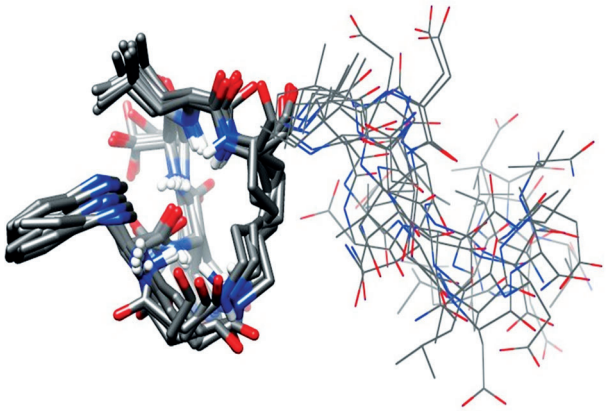

Figure 5. NMR structure of the cyclic peptide. Low-energy NMR-derived solution structure ensemble of CP **6** in dimethyl sulfoxide (DMSO).

also derived from the interaction interface between LEDGF/p75 and IN and was shown to inhibit IN.[11] Cyclization took place also in this case with similar parameters (CP **43**), although at lower yield (Table 1).

To further prove the cyclization, we determined the solution structure of CP **6** using 2D-NMR spectroscopy.[15] Compared to LP **5a**, the ^{1}H NMR backbone α-hydrogen and amide hydrogen signals in the fingerprint region (8.3–9.7 ppm) showed an increase in the range of amide chemical shift values by more than 0.5 ppm (Figure S2). This suggests that CP **6** indeed adopted a cyclic structure. All the ^{1}H NMR spectra of CP **6** derived from various reactions were identical, reflecting a single isomer in solution. In the SI, the succinic methylenes were observed at 2.3 and 2.6 ppm, as opposed to 3.2 and 3.4 in CP **6**, showing a significant difference in chemical shift. The NOESY spectrum showed an interaction between the tryptophan NH at 9.2 ppm and the α-hydrogens of the succinic moiety at 3.2 ppm. The lysine NH interacted with proximate α-hydrogens of the succinic moiety at 8.8 and 3.4 ppm (Figure S3 and Table S1). An interaction of both the asparagine NH and the serine NH with one of the succinic hydrogens was also observed. All these interactions indicate that the lysine side chain is bound to the peptide N-terminus by cyclization via the succinic bridge. The hydrophobic side chain residues also provided additional stabilization by forming a cyclic core group "$N_{methylene}$KL" that reduces the peptide flexibility.

The NMR-derived conformational ensemble of CP **6** was solved in [D_6]DMSO (Figure 5). 32 of 50 initial structures had no violations of NOE-derived constraints or canonical geometry. The backbone RMSD value (Figure S4) of the cycle (succinate-WNSLK) of the 32 non-violated conformers was 0.93 Å, while a low-energy 20-member ensemble gave 0.81 Å. The tail of the molecule (IDNLDV) was disordered. The cyclic core residues (suc-WNS) showed potential intramolecular hydrogen bonds between the succinic carbonyls and the Asn-side chain, and HN-O between Ser and Trp. The tryptophan and leucine side chains formed a hydrophobic patch on one face of the ring.

To validate the proposed cyclization mechanism via SI, we carried out theoretical analyses of the corresponding reaction mechanisms. For simplicity, we choose the LK-based benzyl succinate (R′, Figures S5 and S6) as our starting system that mimics the actual experiment. We systematically performed a conformational search to determine the lowest energy conformers for reactants, intermediates and products using force field and ab initio methods (see Supporting Information). The energy profiles were determined by a density functional theory based hybrid B3LYP[16a,b] method with a 6-31+g** basis set[16c] using Gaussian 09 program suit.[16d] Zero point and thermal energies were included to calculate the activation barriers at room temperature.

The overall reaction proceeded in two steps: First, the formation of SI and second, the succinimide ring opening to form the CP′ and LP′. In the first step, the neutral form of the leucine in the LK dipeptide (R′) is in equilibrium with its anionic form (Rn in Figure S5) under basic conditions. In the neutral form, the lowest energy pathway for the SI′ (Figs S5 and S6) formation proceeded by a two-step mechanism. In the first step, an O-protonated tetrahedral cyclic intermediate (TDi1) was formed due to proton transfer from the N-terminal leucine to the carbonyl oxygen of succinate via a four-membered transition state (TS1a). Then the benzyl group was removed from TDi1 via another four-membered TS (TS1b) by a concerted proton transfer mechanism. The calculated energy barriers for these two consecutive steps were 46.7 kcal mol^{-1} (TS1a) and 36.7 kcal mol^{-1} (TS1b). The overall SI′ formation reaction is endothermic by 6.9 kcal mol^{-1}. On the other hand, the reaction barrier in the anionic form, where the negatively charged N-terminal leucine (Rn in Figure S5) attacks the sp^2 hybridized carbonyl carbon of the succinate, was found to be very low (10.2 kcal mol^{-1}, TS1n). In Figure 6 A we show only the essential part of the optimized structure of TS1n in which the corresponding reaction coordinate is where the negatively charged N1 attacks C2.

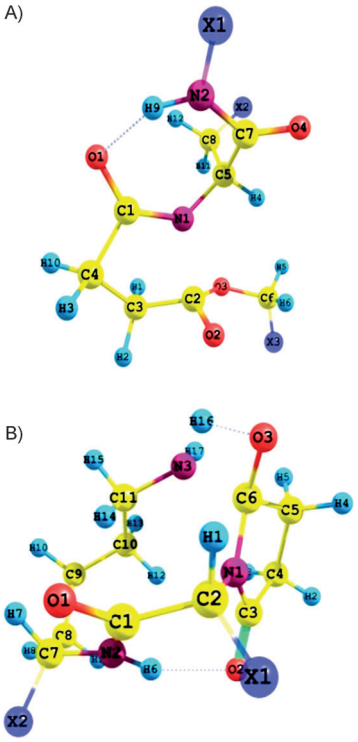

Figure 6. A) The essential part of the optimized TS for the formation of the five-membered ring; B) The essential part of the optimized TS for the first step of the CP′ formation via a four-membered ring (N3-H16-O3-C6). X1, X2, etc., are dummy atoms representing the rest of the molecule.

This pathway was further calculated in solution phase using the integral equation formalism variant polarisable continuum model (IEFPCM)[17] and DMF as solvent. The calculated reaction barrier was 11.0 kcal mol^{-1}, similar to a gas-phase barrier, showing that the anionic pathway is the most favorable process for forming the five-membered SI′.

To assess the cyclization step, we considered SI′ as a reactant from the first step. The lowest energy pathway for the cyclization followed a two-step mechanism under neutral and acidic conditions. In the first step, a tetrahedral intermediate (TDi2) was formed due to proton transfer from the N-terminus lysine (N13 in Figure 6B) to the carbonyl oxygen (O3 in Figure 6B) of SI′ via a four-membered (N3-H16-O3-C6 in Figure 6B) TS (TS2a). In the second step, SI′ broke to form CP′ via another four-membered TS (TS2b) through a concerted proton transfer mechanism (Figure S5). The energy barriers for these two mechanisms in neutral conditions were 46.4 and 42.2 kcal mol^{-1}, respectively. The overall cyclization is endothermic by 4.0 kcal mol^{-1}.

To check the influence of acid and solvent, we explicitly considered the presence of a TFA molecule in the vicinity of one of the carbonyl oxygens of the succinimide ring and also as a solvent using IEFPCM similar to the experimental conditions. The energy barriers for TS2a and TS2b fell to 41.3 and 39.2 kcal mol^{-1}, respectively. Note that the B3LYP functional underestimates barrier heights by an average of 4.4 kcal mol^{-1}.[18] This cyclization step was always in competition with an intermolecular attack by water to form LP′. Here again we found that a two-step processes was the lowest energy pathway with energy barriers of 36.5 and 42.1 kcal mol^{-1} respectively in neutral conditions. Our experimental analysis for this peptide slightly favoured LP, as in the theoretical results.

We performed systematic structure–activity relationship (SAR) studies of the effects of the peptide sequence, hydrophobicity and resin type on the reaction as detailed in the Supporting Information (Figure S7, Table S2). The lysine residue should be in the middle of the sequence and not directly attached to the resin and should be separated from the succinic acid by at least one residue. The cyclization was neither influenced by the external nucleophiles nor by altering the sequence and stereochemistry of the peptide.

The ability of CPs **6**, **20** and **21** to inhibit IN was tested by an ELISA based assay.[11,12] At a concentration of 40 μM, CPs **20** and **21** inhibited IN activity much better than the parent LEDGF (361–370) peptide (Figure 7A). CP **6** at the same concentration did not have any effect. The additional glycine residue and acetamide groups in CPs **20** and **21** were apparently essential for obtaining the conformation required for inhibition. The stability of CPs **6** and **20** was tested by incubation with trypsin monitored using reversed phase HPLC.[11,12] CPs **6** and **20** showed high resistance towards trypsin digestion compared to the linear parent peptide (Figure 7B). We conclude that cyclization improved the activity and stability of the LEDGF 361–370 peptide.

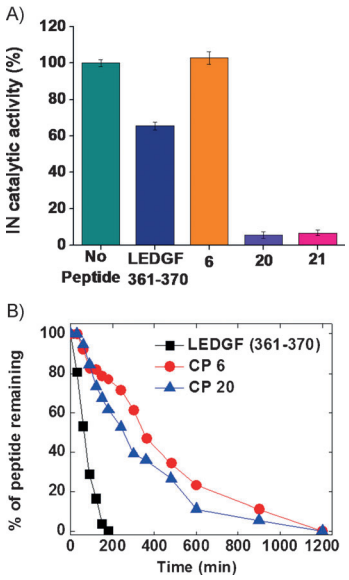

Figure 7. IN inhibitory activity and stability towards tryptic degradation of the CPs. A) IN inhibitory activity of CPs **6**, **20** and **21**; B) stability of CPs **6** and **20** towards tryptic degradation compared to the parent linear LEDGF (361-370) peptide.

In summary, we have developed a new tandem strategy for in situ side chain to N-terminus peptide cyclization under highly acidic cleavage conditions. The main advantage of our approach is the spontaneous in situ formation of cyclic peptides via concurrent deprotection, cyclization and cleavage. The N-terminal succinic acid derivative was used to form

a succinimide intermediate that allowed a conformationally favorable intramolecular attack by the lysine side chain. Kinetic, structural, and theoretical studies supported this mechanism. "Classic" cyclization of peptides under basic conditions may lead to the undesired side reaction of racemization due to the prolonged exposure to base. The racemization leads to the formation of inactive diastereomers that are usually difficult to separate from the desired cyclic peptide. Racemization is negligible under acidic conditions, giving an extra advantage to the cyclization presented herein. The cyclization mechanism presented here could have wide applications for different peptide sequences containing lysine at relevant positions. Our method paves the way for easy and efficient synthesis of cyclic peptides and peptidomimetics.

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