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Intramolecular Hydrogen Bonding Enables a Zwitterionic Mechanism for Macrocyclic Peptide Formation: Computational Mechanistic Studies of CyClick Chemistry

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Abstract: Macrocyclic peptides have become increasingly important in the pharmaceutical industry. We present a detailed computational investigation of the reaction mechanism of the recently developed "CyClick" chemistry to selectively form imidazolidinone cyclic peptides from linear peptide aldehydes, without using catalysts or directing groups (*Angew. Chem. Int. Ed.* **2019,** *58*, 19073 – 19080). We conducted computational mechanistic to investigate the effects of intramolecular hydrogen bonds (IMHBs) in promoting a kinetically facile zwitterionic mechanism in "CyClick" of pentapeptide aldehyde AFGPA. Our DFT calculations highlighted the importance of IMHB in pre-organization of the resting state, stabilization of the zwitterion intermediate, and the control of the product stereoselectivity. Furthermore, we have also identified that the low ring strain energy promotes the "CyClick" of hexapeptide aldehyde AAGPFA to form a thermodynamically more stable 15+5 imidazolidinone cyclic peptide product. In contrast, large ring strain energy suppresses "CyClick" reactivity of tetra peptide aldehyde AFPA from forming the 9+5 imidazolidinone cyclic peptide product.

Introduction

Since the discovery of insulin, peptide drugs have become an indispensable part of the modern pharmaceutical industry due to their low toxicity and high potency.[1],[2] Among all peptide drug candidates, macrocyclic peptides received increasing attention in recent years.[3] Cyclization of linear peptides reduces their conformational freedom and thus increases their protein binding affinity [4],[5], structural stability [6],[7], and improved cellular penetration [8] compared with their linear counterparts. In the past two decades, the FDA approved 18 cyclic peptide new drugs [9], exemplified by antibiotic *daptomycin* [10],[11], anticancer agent *romidepsin* [12],[13], lupus nephritis *voclosporin* [14], and gastrointestinal disorder drug *linaclotide* [15],[16] (**Figure 1a**). Despite the great potential of cyclic peptides, there exists only a handful of methods for the selective formation of head-to-tail cyclic peptides. [17],[18]. As presented in the review by C. J. White and A. K. Yudin, controlled macrocyclization of all-$_L$ and all-$_D$ peptides are carried out at low concentrations to avoid intermolecular reaction and nearly always requires either a 'internal' conformational element, or an 'external' catalyst [19] or directing group to achieve controlled peptide macrocyclization. For example, previous studies by Ye and co-workers indicated that the use of metal ions promote head-to-tail cyclic product selectivity by bringing the N- and C- termini in close proximity [20]. Alternatively, Yudin and co-workers used Ugi multi-component condensation reaction [21] to prepare a zwitterionic intermediate, which undergoes selective macrocyclization [22] [23] (**Figure 1b**).

In 2019, the Raj group published a novel exclusively intramolecular "CyClick" strategy for peptide macrocyclization [24]. Under mild reaction conditions, the "CyClick" chemistry generates imidazolidinone-cyclic peptides (**3**) from all-$_L$ linear peptide aldehydes (**1**) with high chemoselectivity and stereoselectivity at very high concentrations (**Figure 1c**). CyClick chemistry does not form any intermolecular dimers or oligomers even at relatively high concentrations. Since the "CyClick" chemistry does not use any catalyst, nor a directing group, the reaction mechanism and the origin of high intramolecular selectivity is of great interest. Considering the importance of intramolecular hydrogen bonding in confining the conformational flexibility of macrocyclic peptides [25],[26], we postulated that intramolecular hydrogen bond (IMHB) may be responsible for promoting the "CyClick" intramolecular cyclization by stabilizing the cyclic imine intermediate (**2**) over the linear imine intermediate. Although we found multiple experimental studies recognizing the importance of intramolecular hydrogen bonding in controlling the selectivity of macrocyclization of linear peptides [27],[28],[29],[30],[31], limited computational investigations of the IMHB conformation-directed macrocyclization are available in literature [23],[32], presumably due to the challenging conformational flexibility of peptides [33]. In this work, we present a detailed computational investigation explaining the exclusive intramolecular nature of CyClick chemistry, the origin of high chemo- and stereoselectivity, and the effect of length of linear peptides on "CyClick" reactivity.

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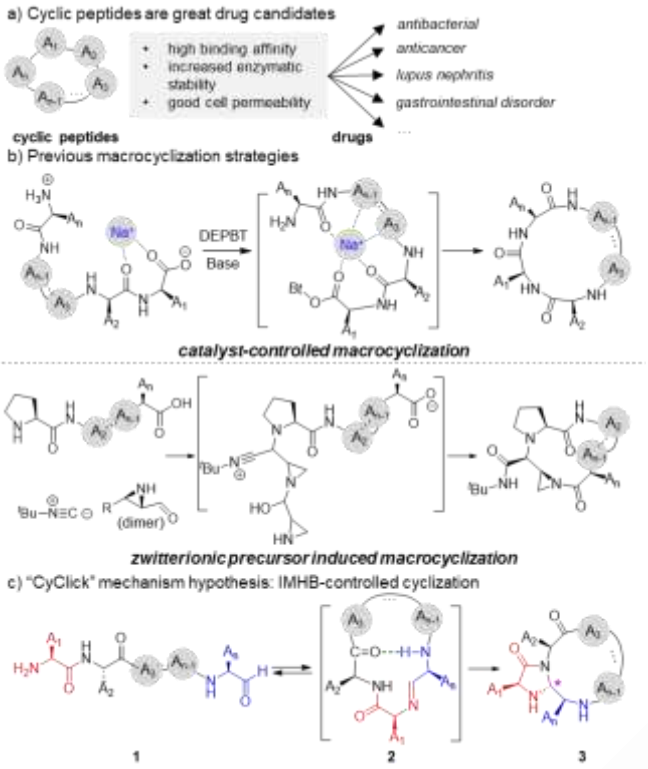
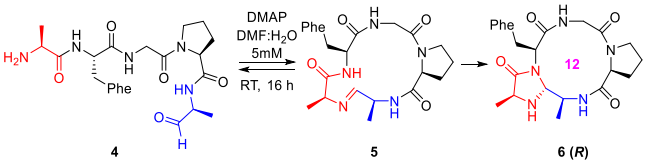


Figure 1. a) Characteristics of cyclic peptides as promising drug candidates; b) previous examples of using metal ion catalysts or zwitterionic precursors for conformational pre-organization to facilitate head-to-tail peptide macrocyclization; c) proposed mechanism, IMHB controlled exclusive intramolecular nature of "CyClick" reaction

Results and Discussion

We conducted detailed computational and experimental investigations to determine the mechanism of the CyClick reaction. We initially investigated experimentally a "CyClick" reaction of a small linear pentapeptide AFGPA **4** that occurs in H_2O:DMF (1:1) solvent at room temperature to generate a 12-membered imidazolidinone cyclic peptide **6 (*R*)** (7%) after 16 h we also observed the formation of cyclic-imine intermediate (5%) that was confirmed by its reduction with sodium cyanoborohydride (**SI Figure S13**). When 7 equivalents of DMAP were added, we noticed a significant increase in the yields of 12-membered cyclic peptide **6 (*R*)** (52%) in 16 h with a high stereoselectivity of >95% *de* (SI Figure S14). Consistent with the work from our previous publication, no intermolecular reactions leading to dimers or oligomers (**Scheme 1**).



Scheme 1. CyClick reaction on linear peptide aldehyde **4** (AFGPA) selectively generates a 12-Membered imidazolidinone cyclic peptide **6 (*R*)**.

Computations on the same reaction are summarized in **Figure 2**, which shows the computed reaction coordinate profile of CyClick reaction of linear peptide aldehyde **4**, AFGPA, to selectively form the 12+5 imidazolidinone cyclic peptide **6 (*R*)**. To save computation time, we used a model substrate **4'**, AAGPA, in which the Phe residue in **4** is replaced by an Ala residue. The formation of a cyclic imine intermediate, **7** is exergonic by 12.1 kcal/mol. Since the formation of cyclic imine intermediate **7** is reversible we assume that the formation of cyclic imine intermediate **7** is not the rate-limiting step. As demonstrated in our conformational analysis, we observed that the cyclic imine intermediate **7** is relatively structurally rigid. We only identified six conformers within the 3.0 kcal/mol window. The second lowest energy conformation of **7** is 2.2 kcal/mol higher in energy compared to the lowest energy conformation shown in **Figure 3**, which is stabilized by a key transannular IMHB (See SI for detailed conformational analysis). The most favorable reaction pathway of the second cyclization reaction of **7** is a zwitterionic pathway. The intramolecular hydrogen transfer transition state **TS1** has a relatively low kinetic barrier of only 17.7 kcal/mol, and formation of the zwitterionic intermediate **8** is endergonic by 16.0 kcal/mol with respect to the resting state **7**. The subsequent collapse of the zwitterion (**TS2-R**) selectively forms the imidazolidinone cyclic peptide product **6' (R)**. Along the zwitterionic pathway, the C–N bond formation step (**TS2-R**) is the rate-limiting transition state, with a total free energy barrier of 20.0 kcal/mol with respect to cyclic imine intermediate **7**. The formation of the product **6' (R)** is exergonic by 3.9 kcal/mol, with respect to **7**. Alternatively, the S-selective stereoisomeric pathway (dashed black pathway) via **TS2-S** requires significantly higher free energy barrier of 36.1 kcal/mol.

Overall, we identify that the zwitterionic pathway is the most favorable reaction pathway for the intramolecular "CyClick" of 4. The resting state is the cyclic imine intermediate **7**, and the rate-limiting transition state is the second cyclization **TS2**. The computed low free energy barrier and high R-stereoselectivity are consistent with the experimental observations reported in **Scheme 1**.

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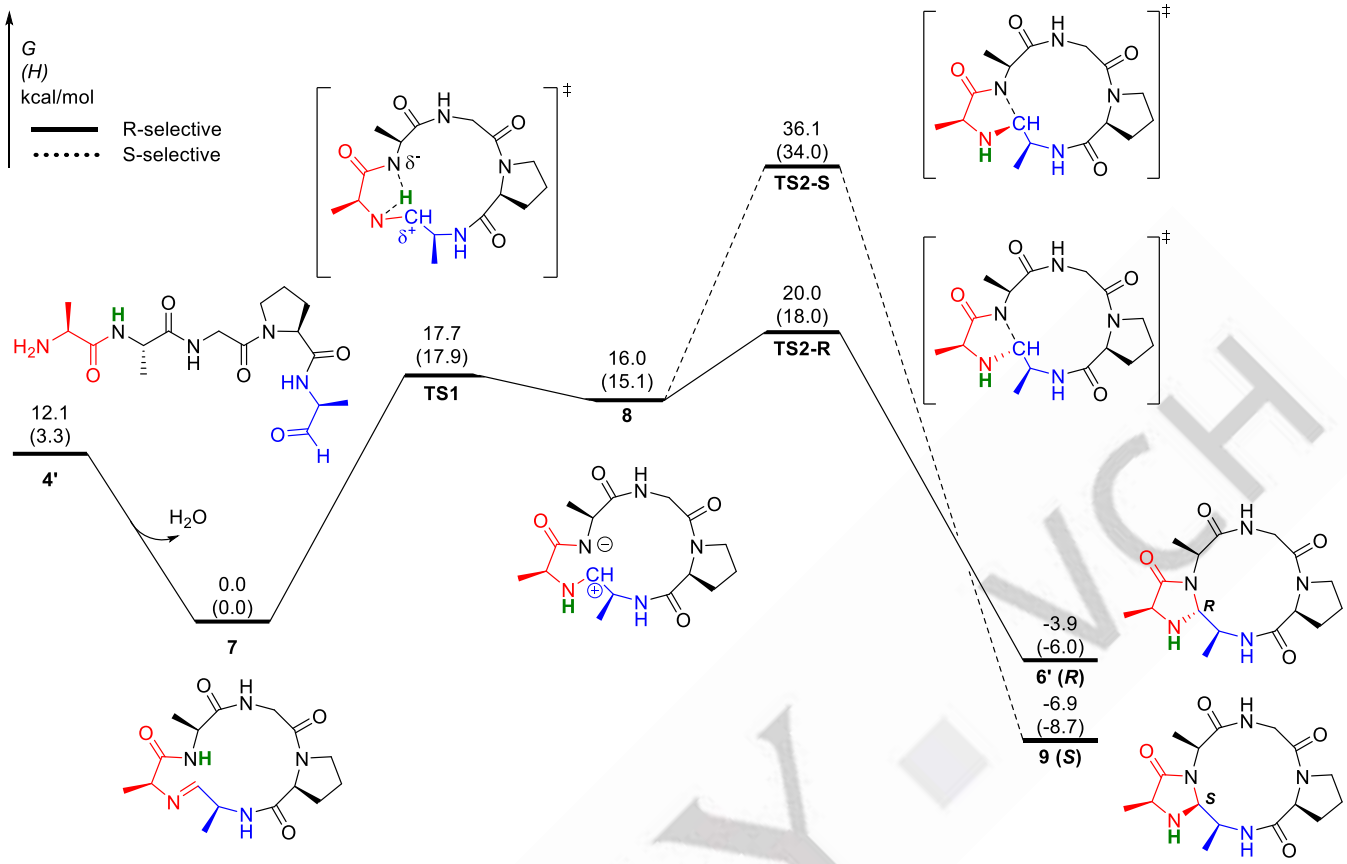

Figure 2. Reaction coordinate profile of intramolecular CyClick reaction of **4'** (AAGPA)

Closer evaluation of the DFT optimized geometries of intermediates and transition states along the zwitterionic pathway revealed the vital roles IMHB played in promoting "CyClick" reaction (**Figure 3**). First, in the lowest energy conformation of **4'**, three IMHBs pre-organize the substrate and put the N-terminus in close proximity with the aldehyde C-terminus, which should facilitate the first cyclization reaction to form cyclic imine **7**. Second, a transannular IMHB in **7** pre-organizes the macrocycle backbone into a relative rigid conformation, which persists along the subsequent hydrogen transfer transition state **TS1**, the zwitterionic intermediate **8**, and the rate-limiting **TS2-R**. Finally, a second transannular IMHB between the cationic iminium N–H and the backbone carbonyl group stabilizes the zwitterionic **8** (1.83Å) and the rate-limiting **TS2-R** (2.02Å). Our Hirshfeld charge analysis further validates the zwitterionic nature of intermediate **8** (See SI for details)**.** With stabilization from the two IMHB, formation of the zwitterionic intermediate **8** is only endergonic by 16.0 kcal/mol. In comparison, the acid-base reaction between a model *N*-ethylpropionamide and *N*-ethylpropan-1-imine to form the amide conjugate anion and the iminium cation is endergonic by 23.2 kcal/mol, suggesting that IMHBs stabilizes the zwitterion intermediate **8** by around 7.2 kcal/mol.

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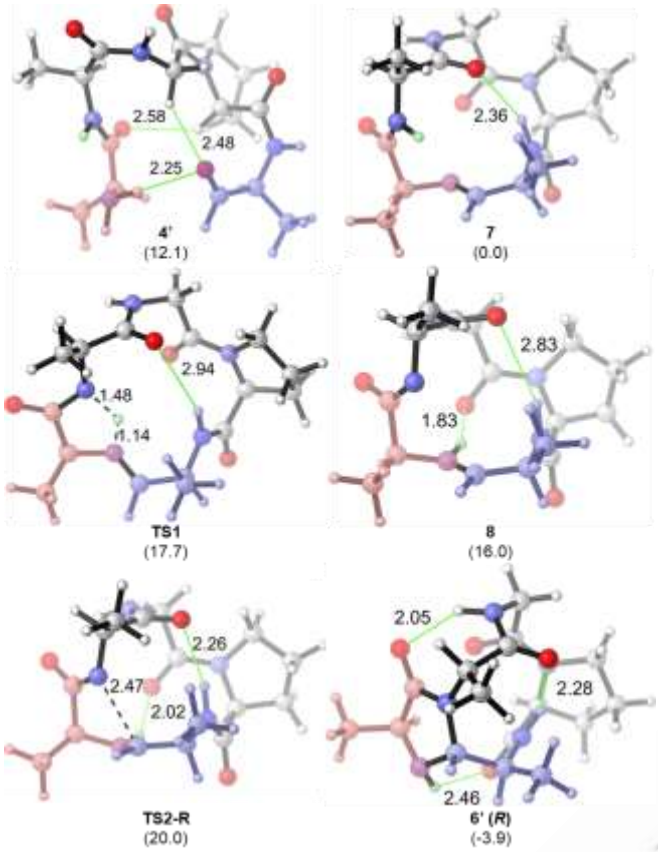

Figure 3. Geometries of intermediates and transition states along the zwitterionic pathway of intramolecular "CyClick" of **4'** (Computed Gibbs free energy ΔG in kcal/mol)

IMHBs also contribute to the origin of stereoselectivity in the intramolecular "CyClick" of **4**'. Our computational study predicts that **TS2-S** has a free energy barrier (37.2 kcal/mol) that is significantly higher than that of **TS2-R** (20.0 kcal/mol) (**Figure 4**). The predicted high R-stereoselectivity is in excellent agreement with experimental observation. Further analysis of the DFT optimized molecular geometries of the stereoselectivity-determining transition states reveal that because of a key transannular IMHB, the low energy **TS2-R** adopts a less distorted conformation as the cyclic imine resting state **7** (E_{dist} = 2.1 kcal/mol), while the **TS2-S** adapts a more distorted macrocycle backbone conformation (E_{dist} = 9.3 kcal/mol). On top of the lower distortion energy, the second transannular IMHB between the cationic iminium N–H and the backbone carbonyl group (2.02Å) further stabilizes **TS2-R**. By contrast, the iminium N–H in **TS2-S** points away from the macrocycle backbone and is not stabilized by IMHB. The iminium cation also adopts a more stable trans conformation in the lower energy **TS2-R**.

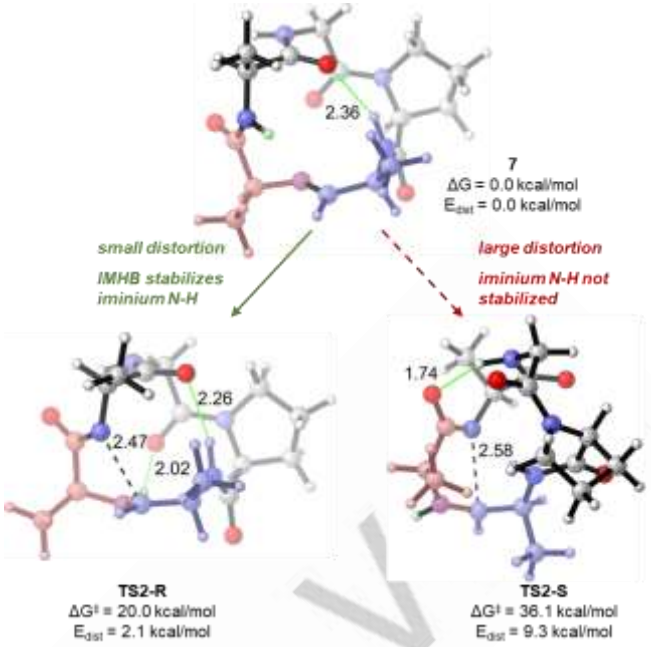

Figure 4. Origin of R-stereoselectivity in intramolecular "CyClick" of **4'**

We also studied the effect of dimethylaminopyridine (DMAP) on the "CyClick" reaction. Experimentally, addition of DMAP increases the yield of **6' (R)** by 45%. The computed reaction coordinate profile (**Figure 5a**) indicates that in presence of the organic base DMAP, the CyClick mechanism is unchanged, but the intramolecular hydrogen atom transfer (**TS3**) to form zwitterionic intermediate **12** is stabilized by a weak van der Waals complex with DMAP (**Figure 5b**). The rate-limiting transition state remains the collapse of the zwitterion (**TS4**). In comparison, the DMAP assisted zwitterionic pathway (dashed red pathway) is less favored. Both zwitterion intermediate **13** and the subsequent **TS5** have higher free energy barriers (ΔG_{13} = 24.9 kcal/mol, $\Delta G^{\ddagger}_{TS5}$ = 29.3 kcal/mol). DMAP does not promote the kinetic barrier of the "CyClick" reaction, but the imidazolidinone cyclic peptide product (**14**) is thermodynamically stabilized (ΔG_{14} = – 6.4 kcal/mol) compared to that without DMAP ($\Delta G_{6'}$ = – 3.9 kcal/mol), and a higher yield is observed in this reversible reaction.

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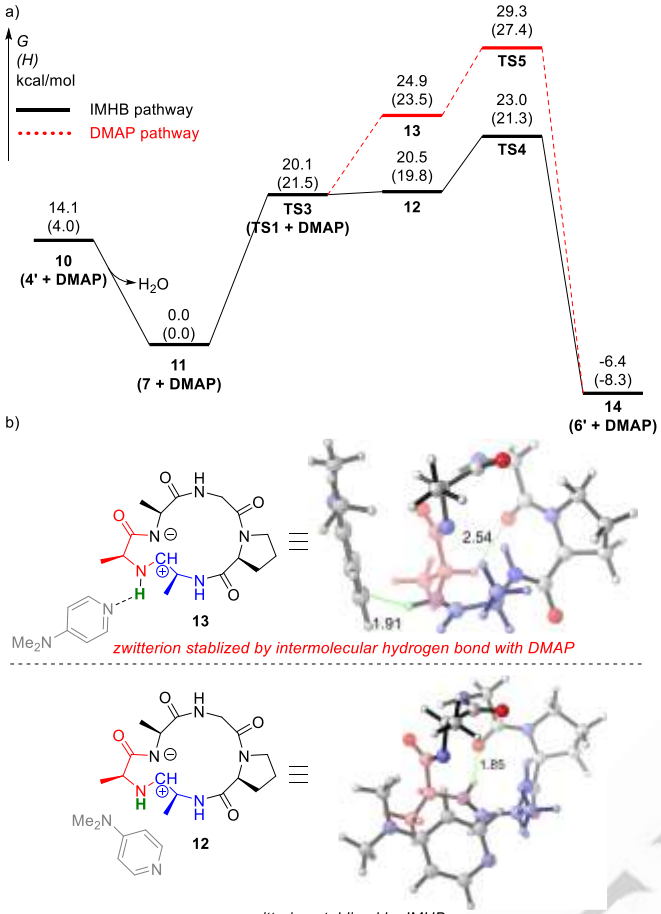

Figure 5. a) Computed reaction coordinate profile of intramolecular CyClick reaction of **4'** (AAGPA) with DMAP additive; b) Effect of DMAP and IMHB in stabilizing the zwitterion intermediate

We have also studied the competing intermolecular CyClick reaction of **4'** (AAGPA) to form linear peptide oligomers (**Figure 6**). To save computational time, we used a model substrate **4''** to model the C-terminus of **4'**. The computed reaction coordinate profile indicates that the formation of the imine intermediate **15** is only slightly exergonic by 2.1 kcal/mol. The hydrogen transfer transition state **TS6** has a free energy barrier of 21.7 kcal/mol with respect to the resting state **15**. The formation of the zwitterionic intermediate **16** via intramolecular hydrogen atom transfer (**TS6**) is endergonic by 17.2 kcal/mol. The subsequent rate-limiting cyclization transition state **TS7** has a relatively higher kinetic barrier of 25.3 kcal/mol, which is 5.3 kcal/mol higher than that of the intramolecular cyclization ($\Delta G^{\ddagger}_{TS2\text{-}R}$ = 20.0 kcal/mol).

This computed reaction coordinate profile predicts that the intermolecular CyClick reaction is considerably slower than the intramolecular CyClick reaction, which is in good agreement with the experimentally observed exclusive intramolecular chemoselectivity. Previous experimental analysis from the Raj group [24] showed the formation of intramolecular cyclic product occurs even at very high concentrations of peptide (100 mM). Analysis of the DFT optimized molecular geometries revealed that although IMHB exists in both the resting state **15** and the rate-limiting **TS7**, the cationic iminium N–H in **TS7** points away from any hydrogen bond acceptors. Moreover, the peptide backbone of **TS7** is slightly more distorted (E_{dist} = 3.1 kcal/mol), than the pre-organized macrocyclic peptide backbone in the intramolecular **TS2-R** (E_{dist} = 2.1 kcal/mol).

Overall, we conclude that IMHBs are essential in the "CyClick" cyclization of **4'** to a) pre-organize the linear substrate and promote the first cyclization to form the cyclic imine intermediate; b) stabilize cationic iminium N–H in the zwitterionic intermediate and the rate-limiting second cyclization transition state; c) promotes R-stereoselectivity and intramolecular chemoselectivity.

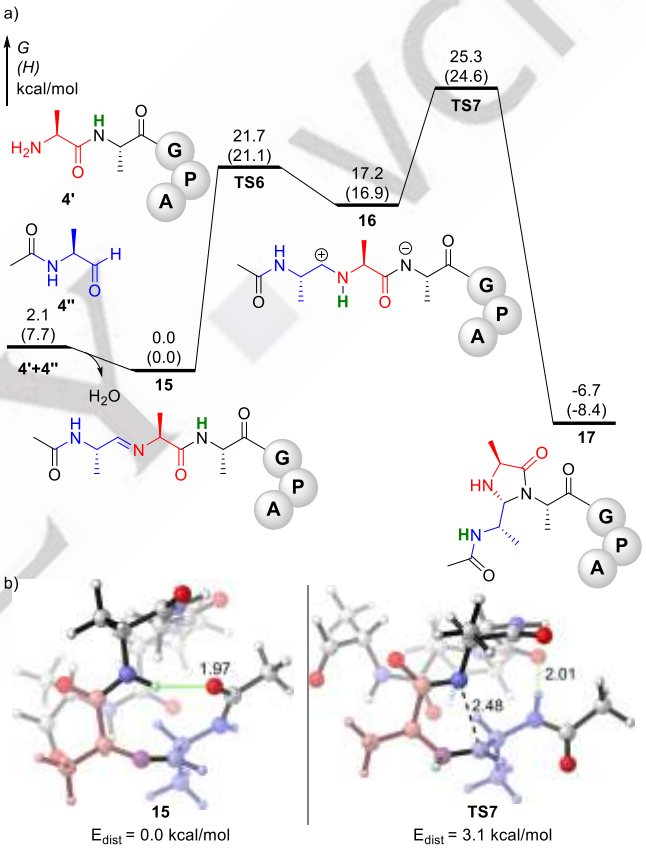

Figure 6. a) Computed reaction coordinate profile of intermolecular "CyClick" of **4'**; b) DFT optimized geometries of resting state **15** and rate-limiting transition state **TS7**

With a good understanding of the CyClick reaction mechanism of the 12+5 substrate, we subsequently studied the effect of the length of linear peptide aldehyde on the CyClick reaction experimentally and computationally. We also computed the reaction coordinate profile of the "CyClick" reaction of **18'** (AAGPAA) and **21'** (AAPA) [34], which form the 15+5 and 9+5 imidazolidinone cyclic peptides **20'** and **23'**, respectively.

Experimentally, the cyclization of linear hexa-peptide aldehyde, **18** (AAGPFA), which is the linear peptide aldehyde substrate with one additional Ala residue compared with **4.** In H_2O:DMF (1:1) and 7

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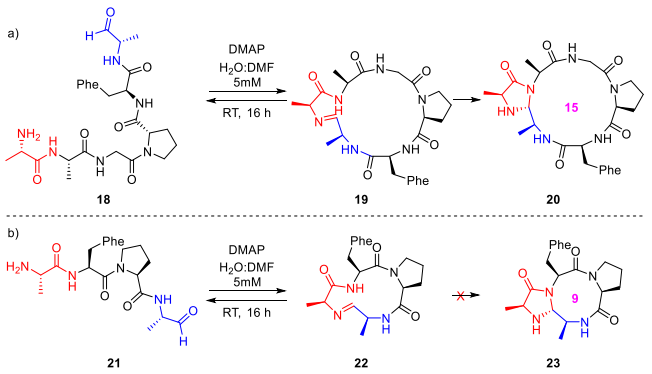
equivalents of DMAP at room temperature, we observed the 15-membered cyclic peptide product with 45% conversion (**Scheme 2a, SI Figure S15**). Alternatively, the tetrapeptide **21** (AFPA), which is the linear peptide aldehyde substrate with one less glycine compared with **4**, did not lead to the formation of any 9-membered macrocycle in 16 h (**Scheme 2a, SI Figure S16**).



Scheme 2. CyClick reaction on linear peptide aldehyde a) **18** (AAGPFA) selectively generates a 15-membered imidazolidinone cyclic peptide **20**; b) **21** (AFPA) does not generate 9-membered imidazolidinone cyclic peptide **23**

The "CyClick" of **18'** (AAGPAA) along the zwitterionic pathway (**Figure 7**) is similar to reaction mechanism presented in earlier sections. The formation of the cyclic imine intermediate **24** is exergonic by 12.4 kcal/mol. The subsequent hydrogen transfer transition state ($\Delta G^{\ddagger}_{TS5}$ = 18.3 kcal/mol) leads to the formation of the zwitterionic intermediate **25**. The rate-limiting transition state is the second cyclization **TS9-R** with a free energy barrier of 20.6 kcal/mol. The formation of the product 15+5 imidazolidinone cyclic peptide **20'** is exergonic by 9.5 kcal/mol, with respect to cyclic imine intermediate. The S-selective **TS9-S** has a kinetic barrier of 25.2 kcal/mol. Overall, we predict that the CyClick reaction of **18'** will readily take place at room temperature to selectively form **20'**, which agrees with the experimental observations reported in **Scheme 2**.

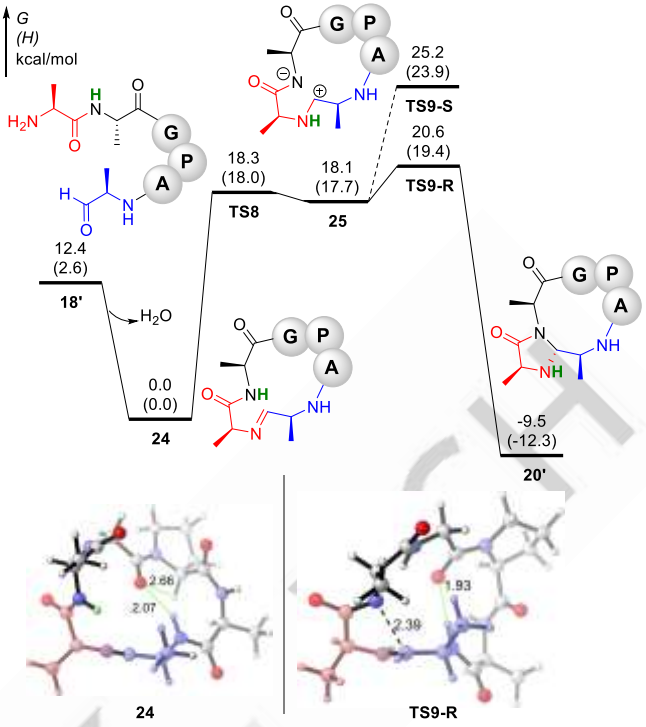


Figure 7. Reaction coordinate profile of CyClick reaction of **18'** (AAGPAA)

We also modeled the hypothetical "CyClick" of **21'** (AAPA), which is the linear peptide aldehyde substrate with one less glycine compared with **4**' (**Figure 8**). The computed reaction coordinate profile suggests that the rate-limiting transition state for CyClick of **21'** is the hydrogen transfer transition state **TS10**. Consistent with the experimental observation, the computed free energy barrier ($\Delta G^{\ddagger}_{TS10}$ = 33.5 kcal/mol) is very high so that the reaction should not take place at room temperature. The formation of the 9+5 imidazolidinone cyclic peptide **23'** is exergonic by only 1.9 kcal/mol.

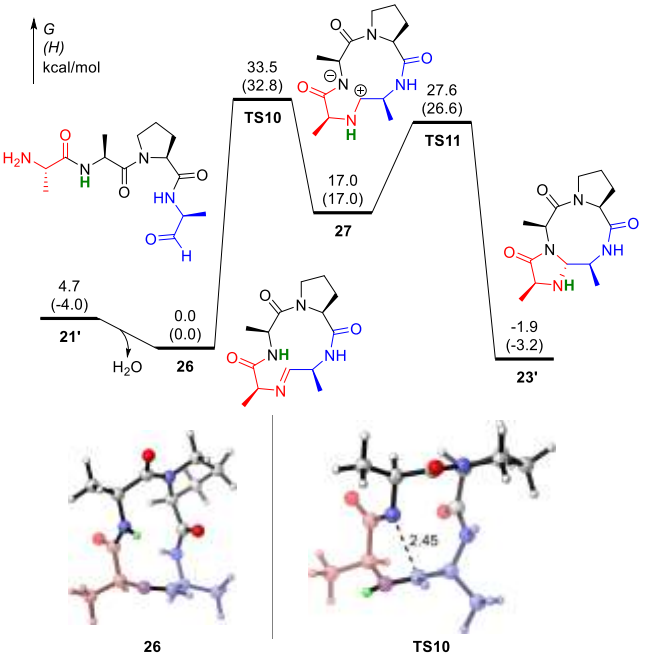


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Figure 8. Reaction coordinate profile of CyClick reaction of **21'** (AAPA)

The ring sizes of imidazolidinone-peptide bicyclic products have a significant effect on their thermodynamic stability. The computed heat of reaction to form the imidazolidinone-peptide product decreases as the ring size increases (**Figure 9**). The **20'** (n =3) is the most stable product ($\Delta H_{20'}$= -12.3 kcal/mol), while the **23'** (n =1) is the lease stable ($\Delta H_{23'}$ = -3.2 kcal/mol), with respect to their corresponding cyclic imine resting states. Calculation of the ring strain with a model homodesmotic reaction (Q_{strain}) [35] revealed that **23'** is thermodynamically less stable than **6'-R** and **20'** because of its larger ring strain energy (Q_{strain}, n=1 = 14.3 kcal/mol).

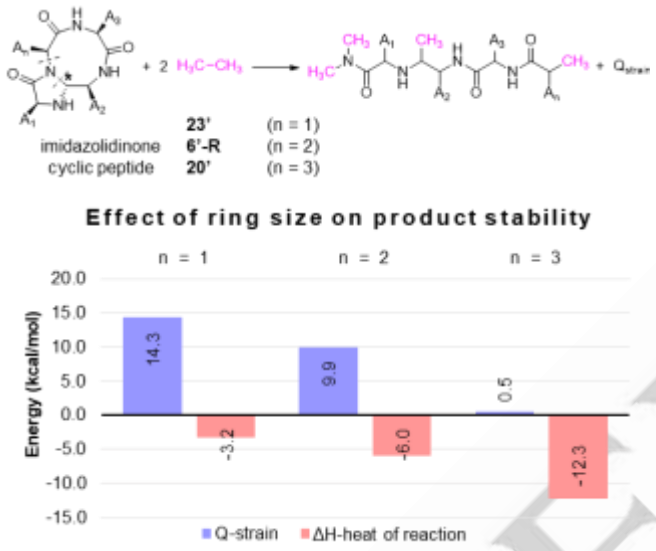

Figure 9. Effect of ring size on the stability of the product imidazolidinone-cyclic peptide

Conclusion

We have presented a detailed mechanistic investigation on the "CyClick" reaction. DFT calculations identify that the "CyClick" mechanism involves a reversible formation of the cyclic imine resting state, followed by rate-limiting cyclization transition state to form the imidazolidinone cyclic product. Along the favored zwitterionic pathway, intramolecular hydrogen bonding (IMHB) is shown to play an essential role in promoting the reactivity, intramolecular chemoselectivity, and stereoselectivity in "CyClick" reactions to form cyclic peptides. Two transannular IMHBs stabilize the key zwitterionic intermediate and the rate-determining cyclization transition state, consequently promoting the "CyClick" reactivity. The IMHB between the iminium N–H and backbone carbonyl also controls the intramolecular chemoselectivity and R-stereoselectivity. Our calculations show that longer linear peptide aldehydes form thermodynamically more stable imidazolidinone-cyclic peptide due to favorable ring strain in the final product.

Acknowledgements

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Keywords: Reaction Mechanism; DFT Calculation; Macrocyclic peptide, Stereoselectivity

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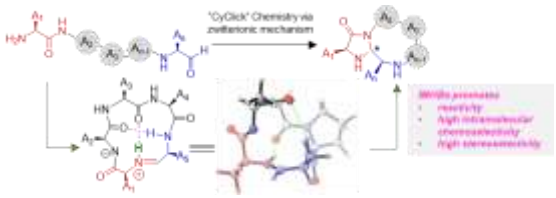

This work presents a detailed computational investigation on reaction mechanisms of the macrocyclization of linear peptide aldehydes to selectively form imidazolidinone cyclic peptides. Our work demonstrates that intramolecular hydrogen bonds (IMHBs) act as transient internal factors to control stereoselectivity by promoting a kinetically facile zwitterionic mechanism.