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An unexpected stereochemical effect of thio-substituted Asp in native chemical ligation

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ABSTRACT

This study presents an unexpected finding that the *cis* isomer of β-thio-Asp exhibits higher ligation activity than the *trans* isomer. This discovery sheds light on the intricate nature of native chemical ligation and highlights the importance of factors beyond the steric effects of the side chain in modulating ligation activity.

Native chemical ligation (NCL) is a highly selective chemical reaction that enables the formation of native peptide bond structures [1]. Since its invention in 1994, NCL has emerged as the predominant method for assembling peptide fragments in protein chemical synthesis [2,3]. The original NCL approach involved the reaction of a peptide containing a C-terminal thioester with a peptide containing an N-terminal cysteine in a mild aqueous solution. The proposed mechanism entails a reversible transthioesterification reaction followed by an intramolecular S-N acyl transfer, resulting in the formation of the desired peptide bond (Scheme 1A) [4,5]. Despite its utility, the limited abundance of Cys in natural proteins (approximately 1.8%, primarily located at the C-terminus) significantly restricts the applicability of Cys-based NCL [6,7]. Consequently, in numerous cases, the introduction of Cys mutations or other amino acid substitutions becomes necessary to facilitate synthesis [8,9]. However, concerns arise regarding the potential impact of such mutations on the properties and functionalities of the synthesized proteins.

To address the aforementioned concerns, significant efforts have been dedicated to expanding the applicability of NCL beyond cysteine by exploring modifications of other naturally occurring amino acids [10–15]. One common strategy involves introducing a thiol group at the β- or γ-position of these amino acids to facilitate NCL reactions. Subsequently, the thio-amino acids can be restored to their original structures through desulfurization upon completion of the reaction (Scheme 1B) [16–18]. Considering that most natural amino acids possess side chains, the introduction of a thiol group generates two stereoisomers: The *trans* isomer, where the side chain group and P2 are positioned on opposite sides of the S-N acyl transfer plane, and the *cis* isomer, where they are positioned on the same side (Scheme 1C). As the artificially introduced chiral center can be removed through desulfurization after NCL, it does not affect the synthesized protein [19,20]. However, for achieving more efficient chemical protein synthesis, selecting between *cis* and *trans* stereoisomers remains necessary when utilizing non-Cys-based NCL, as the introduced stereocenter can influence ligation activity [21–23]. For instance, Danishefsky and colleagues made a noteworthy discovery indicating that the *trans* isomers of β-thio-Leu [21] and γ-thio-Pro [22] exhibit significantly higher reaction activity compared to their *cis* counterparts (Scheme 1C). These observations led to the assumption that *trans* isomers may offer certain advantages in NCL reactions, potentially attributed to reduced steric hindrance and favorable transition state conformation, as depicted in Scheme 1C. However, due to limited research in this direction, it remains unclear if these findings hold true in a broader context.

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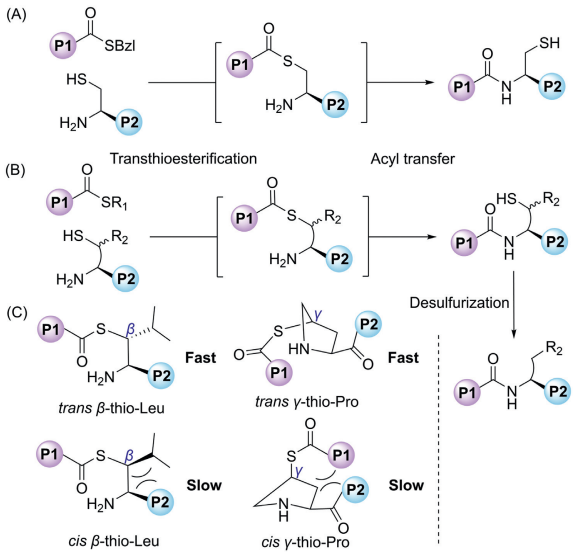
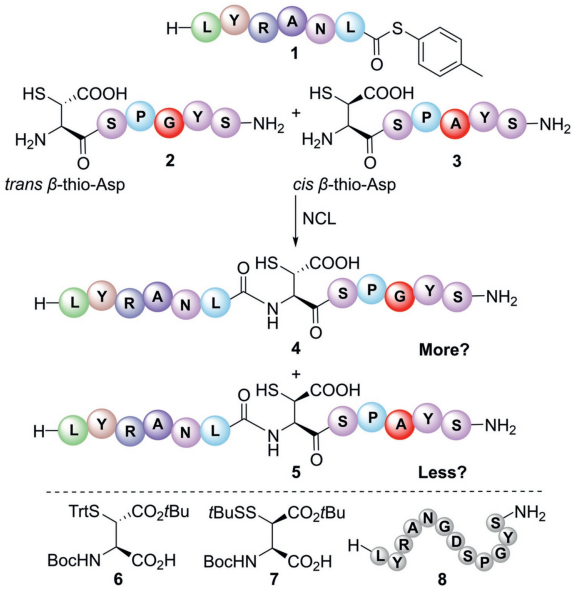


Scheme 1. Influence of stereochemistry on NCL. (A) Original NCL method. (B) Expanded NCL employing thio-substituted amino acids. (C) Effect of stereostructure on NCL reactions. **P1** and **P2** denote the peptide fragments participating in the reactions. R_1 represents aryl groups, while R_2 represents side chains of amino acids.



Scheme 2. Competition reaction for investigating the differences in ligation activity between *trans* and *cis* β-thio-Asp isomers.

To address this existing knowledge gap, we conducted a thorough investigation into the ligation activity differences between the *trans* and *cis* stereoisomers of β-thio-Asp. A previous study by Payne and colleagues did not provide a clear differentiation in their ligation activity, likely due to limited research and estimation without quantitative analysis [24]. In order to overcome this uncertainty, we implemented two improvements in our study. Firstly, we employed ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) in combination with specially designed peptide fragments and an internal standard for precise quantification of the ligation products. Secondly, we utilized preformed active thioesters in the NCL reaction to minimize potential interference caused by the thiol-thioester exchange step (Scheme 2) [25].

To investigate the differences between the *trans* and *cis* isomers, three model peptides, namely **1**, **2**, and **3**, were prepared as illustrated in Scheme 2. Peptide fragment **1**, featuring a C-terminal Leu

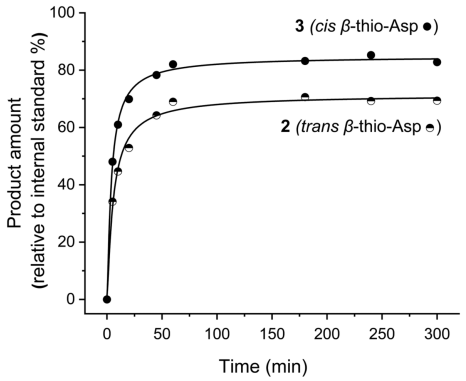


Fig. 1. Ligation activity difference between peptide thioester **1** and peptides **2** and **3**. NCL conditions: 6 mol/L Gdn·HCl, 0.1 mol/L Na_2HPO_4, 50 mmol/L TCEP, pH 7.5, 25 °C, with concentrations of 2.50 mmol/L for **1**, 2.50 mmol/L for **2**, and 2.50 mmol/L for **3**.

residue activated with a 4-methylphenyl thioester, was employed in the designed competition reaction with peptides **2** and **3**, each containing a different N-terminal β-thio-Asp. The selection of fragment **1** was based on the favorable reactivity of Leu in ligation reactions and the capability of 4-methylphenyl thioester to circumvent the thiol-thioester exchange reaction step [26,27]. The synthesis of this peptide thioester involved the EDCI-mediated coupling of amino acid thioesters to the fully protected peptide, utilizing the non-epimerizing conditions established by Sakakibara and colleagues (Schemes S2 in Supporting information) [28,29].

The design of peptides **2** and **3** involved the incorporation of two distinct amino acids, Gly and Ala, at the 4th position. This intentional variation in mass allowed for the utilization of mass spectrometry technology to quantify and compare the ligation products, the feasibility of which was established in Fig. S21 (Supporting information). The solid-phase peptide synthesis (SPPS) technique was employed to synthesize these two model peptides using the corresponding building blocks, the *trans* isomer **6** and the *cis* isomer **7** (Figs. S1 and S2 in Supporting information). The synthesis of **6** and **7** followed established methods, with **6** synthesized from HCl·H-Asp(O*t*Bu)-OMe [26], and **7** obtained from Boc-Asp(O*t*Bu)-OAll [24,30]. Notably, the amino acid difference at the 4th position of peptides **2** and **3** was shown to have no impact on their ligation activity (Fig. S22 in Supporting information).

The ligation activity between the *trans* and *cis* β-thio-Asp isomers was compared using a competition reaction between **2** and **3** [21]. The reaction was initiated by mixing peptide thioester **1** with equal amounts of peptides **2** and **3** in a buffer containing 6 mol/L Gdn·HCl, 0.1 mol/L Na_2HPO_4, 50 mmol/L TCEP, and pH 7.5 at 25 °C. The final concentrations of peptides **1**, **2**, and **3** were 2.50, 2.50 and 2.50 mmol/L, respectively. Samples for analysis were collected at different time intervals, and the ligation products were quantified relative to the internal standard **8** using UPLC-MS analysis (Fig. S21). As depicted in Fig. 1, our findings clearly demonstrated the difference in ligation activity between the two β-thio-Asp isomers, with the *cis* isomer exhibiting higher reactivity. The ligation product generated by the *cis* β-thio-Asp was 1.3 times more abundant than that generated by the *trans* β-thio-Asp. After NCL, the desulfurization reaction was successfully accomplished using VA-044 (Fig. S21C).

To investigate whether the observed difference in reactivity between the two β-thio-Asp stereoisomers is influenced by the reaction conditions, a series of competition studies was conducted under varied temperatures, concentrations, and pH values (Table S1 in Supporting information). The results revealed that the ligation activity can be influenced by temperature, with a more pro-

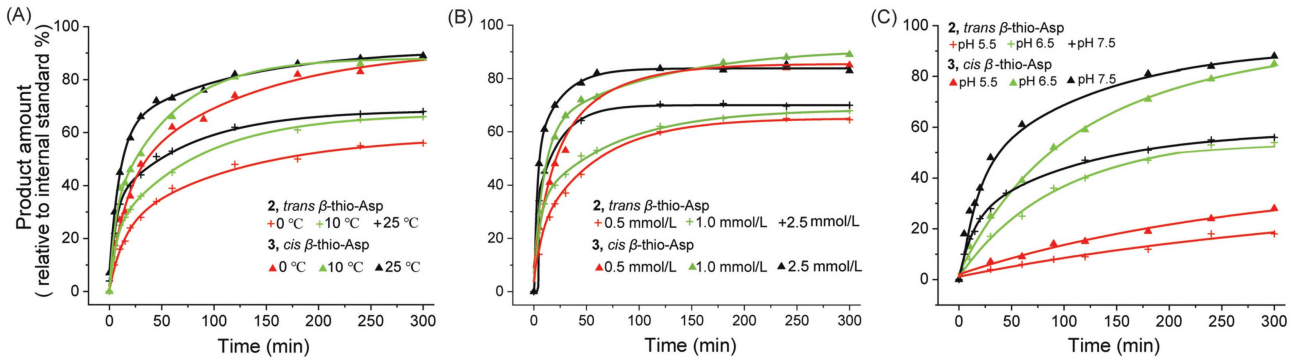


Fig. 2. Impact of reaction conditions on the results of the competition reaction. The competition reaction was conducted at (A) varied temperatures, (B) different concentrations, and (C) distinct pH levels. Unless specified in this figure, the competition reaction adhered to the aforementioned conditions.

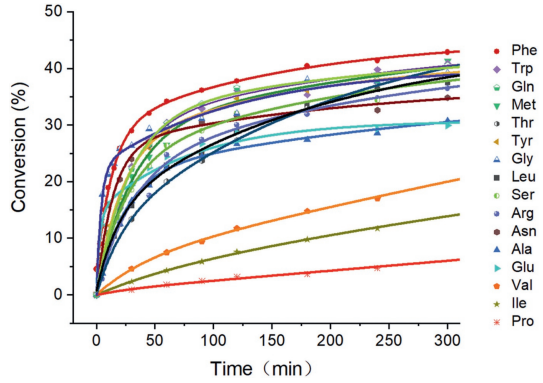


Fig. 3. Comparison of ligation reaction conversions between different thioesters with varied C-terminal amino acids and peptides containing β-thio-Asp **2** and **3** at a 1:1 ratio.

nounced difference between the *cis*- and *trans*-β-thio-Asp isomers observed at lower temperatures (Fig. 2A). Additionally, the concentration of reactants was found to impact the variation in ligation activity, wherein lower concentrations resulted in a greater difference, albeit with a smaller effect compared to temperature (Fig. 2B). Furthermore, pH was found to affect the ligation activity, with higher pH values yielding a more pronounced difference (Fig. 2C). Notably, the difference in ligation activity at a low pH value, such as 5.5, is minimal.

To further assess the factors influencing the difference in ligation activity between the two β-thio-Asp stereoisomers, we conducted a comprehensive study of the competition reaction using a set of 16 peptide thioesters, each incorporating distinct amino acids at the C-terminus (Figs. S5-S20 in Supporting information) [31]. Four naturally occurring amino acids, namely His, Cys, Asp and Lys, were not included in the study due to synthetic challenges or their susceptibility to hydrolysis during the ligation process [32–36]. The results demonstrate that modifying the C-terminal amino acid did not alter the overall trend favoring ligation involving the *cis* β-thio-Asp. However, the magnitude of difference in ligation activity varied with the variation of the C-terminal amino acids. Specifically, Leu, Gln, Thr, and Ala thioesters exhibited relatively larger differences in ligation activity, while Glu, Arg, and Pro thioesters displayed relatively smaller differences (Figs. S29–S43 in Supporting information).

To facilitate the application of β-thio-Asp-based NCL, we also evaluate the suitability of each amino acid for this reaction. This assessment involved comparing the combined conversions of the two stereoisomers in the reaction with different C-terminal amino acid thioesters. The results, presented in Fig. 3, reveal that the amino acids located at the C-terminus of the peptide thioesters can be broadly categorized into two groups. The first group, comprising Val, Ile, and Pro, displayed significantly lower ligation efficiency, potentially attributed to the presence of branched side chains or cyclic structures. The remaining amino acids constituted the second group, demonstrating relatively higher ligation efficiency. This grouping aligns well with a prior investigation conducted by Dawson and colleagues, which involved a similar analysis using a Cys-containing model peptide [31]. It is worth noting that the conversion achieved in our study was relatively low. This can be attributed to the equal amounts of ligation partners employed in the experiments, which likely impacted the overall efficiency of the reaction. Using a larger excess of the thioesters can drive the conversion to completion (Fig. S44 in Supporting information).

The unexpected reversal in ligation reactivity observed between the *cis* and *trans* β-thio-Asp stereoisomers, contrary to previously observed trends in thio-substituted Leu and Pro, implies that the influence of stereostructure on ligation reactivity might extend beyond simple steric hindrance. To investigate the differences between the *cis* and *trans* β-thio-Asp stereoisomers in the NCL reaction, we employed detailed density functional theory (DFT) calculations for the free energy profiles. Our results indicated that the discrepancy in their ligation activity might primarily stem from the difference in activation free energy during the transthioesterification step, which is the rate-limiting step (Fig. 4A), with an activation free energy of 21.16 kcal/mol for the *cis* stereoisomer and 24.20 kcal/mol for the *trans* structure. A comparison of the transition state structures, *cis* TS-I/II and *trans* TS-I/II, revealed an additional intramolecular hydrogen bonding involving the side-chain COO^- group of the *cis* β-thio-Asp with the adjacent amide group. This conformation is likely to lower the energy of the TS-I/II for the *cis* stereoisomer, thereby promoting its ligation activity (Fig. 4B and Fig. S47 in Supporting information). This unique feature of an additional hydrogen bond is also present in the structures of *cis* II, *cis* III, and *cis* TS-III/IV (Fig. S48 in Supporting information), suggesting that it may be unique to the *cis* stereoisomer. This proposed mechanism aligns, to some extent, with the findings from the temperature, concentration, and pH variation study. Lower temperatures and concentrations, along with higher pH, favor the formation of intramolecular hydrogen bonding, thus further accentuating the difference in ligation activity between *cis* and *trans* β-thio-Asp [37–39].

In summary, this study delves into an underexplored aspect of NCL by investigating the impact of stereostructure on the ligation activity of thio-substituted amino acids. The findings from this study reveal a previously unexpected outcome, demonstrating that the *cis* β-thio-Asp exhibits better ligation activity compared to the *trans* isomer. The robustness of this discovery is further validated through variations in reaction conditions and amino

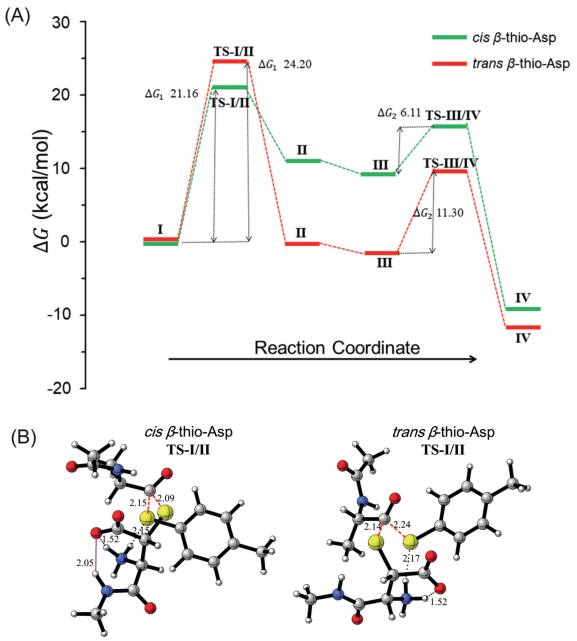


Fig. 4. Proposed mechanism explaining the observed difference in ligation activity between *cis* and *trans* β-thio-Asp. (A) DFT-calculated free energy profiles for the NCL reaction. (B) The optimized transition state structures of the *cis* and *trans* stereoisomers in the rate-limiting transthioesterification steps. The purple line represents the additional intramolecular hydrogen bonding formed between the side-chain COO^- group of the *cis* β-thio-Asp and the adjacent amide group. Bond lengths are in angstroms, and the energies are in kcal/mol.

acid thioesters participating in the ligation reaction. Notably, these results, along with the DFT calculation findings, for the first time indicate that besides steric hindrance, other molecular forces introduced by side chain functional groups, such as hydrogen bonding, may also contribute to the differentiation of ligation capabilities among thio-substituted amino acids in NCL reactions. These findings shed light on the intricacies of non-Cys-based NCL reactions and suggest the need for further investigation in this direction to gain a more comprehensive understanding of this type of reaction. Overall, this research is expected to inspire more in-depth studies of non-Cys-based NCL, potentially leading to the development of new strategies for the more efficient synthesis of proteins and modified proteins using chemical methods [40,41].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2023.109434.

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