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S- to *N*-Acyl transfer in *S*-acylcysteine isopeptides via 9-, 10-, 12-, and 13-membered cyclic transition states

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***S*-Acyl cysteine peptides containing α-, β- or γ-amino acid residues undergo long-range *S*- to *N*-acyl transfer to give analogs of native tripeptides and tetrapeptides containing additional carbon atoms in the chain. The ease of intramolecular *S* → *N*-acyl transfer relative to intermolecular transacylation is favored increasingly for 9 < 12 < 13 ~ 10-membered cyclic transition states; the observed order is explained on conformational and intermolecular interaction considerations. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.**

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Keywords: *S*- to *N*-acyl transfer; peptides; cysteine; *N*-acylbenzotriazoles; S-acylation

Introduction

Native chemical ligation (NCL) is an important two-step transformation of peptide and protein thioesters, comprising (i) chemoselective transthioesterification of a C-terminal thioester with N-terminal cysteine moiety followed (ii) by *S*- to *N*-acyl rearrangement to form a native amide bond [1–4]. Recent significant achievements of NCL include the total chemical synthesis of both post-translationally modified and unmodified proteins [5], segmental isotopic labeling of proteins for NMR studies [6,7], and the formation of peptide conjugates with macromolecules such as peptide nucleic acid [8,9] and DNA [10]. NCL has been particularly useful for assembling proteins that can probe biomolecular processes *in vitro* [5,11,12] and *in vivo* [13–15].

One major limitation of NCL was its restriction to peptide chains possessing cysteine at the N-terminus. The relative rarity of cysteine (1.3% average content in proteins) has encouraged attempts to overcome this limitation. The requirement of a cysteine residue can be avoided by use of ligation auxiliaries [16,17], but these can be difficult to remove, and synthetic strategies using removable cysteine mimics can sterically hinder the ligation [18,19]. The possibility of long-range acyl migration was demonstrated by Kemp *et al*. utilizing a 4-mercapto-6-oxydibenzofuran template for thiol capture [20–24], Wong group in sugar-assisted glycopeptide ligation [25], and Haase and Seitz utilized internal cysteine residues to accelerate long-range thioester-based peptide ligations but required relatively long reaction times (48–72 h) and did not isolate or characterize ligation products [18].

We previously demonstrated long-range *S* → *N*-acyl shifts of S-acylated cysteine peptides via 11- and 14-membered cyclic transition states [26,27]. Cysteine-containing peptides were selectively S-acylated by *N*-acylbenzotriazoles [28] under mild reaction conditions [26,27]. Deprotection of the resulting *N*-Fmoc-*S*-acyl C-terminal cysteine tripeptides and tetrapeptides afforded *S*-acyl isopeptides possessing free carboxyl groups that underwent microwave-assisted chemical ligations (1 h, 50 °C, at pH 8.2) to form native peptides. Subsequently [29], we found that 8-membered transition states are clearly disfavored, whereas the 11- and 14-membered transition states are relatively favored for ligations in *S*-acyl isopeptides containing non-terminal cysteine residues. The present work studies ligation via other transition state sizes by replacing one or more α-amino acid units by β- or γ-aminoacyl units. We wish to identify sequence and geometry requirements that enable long-range acyl migration by study of 'chemical ligations[1]' of isotripeptides and isotetrapeptides containing α, β, or γ-amino acid units via 9-, 10-, 12- and 13-membered cyclic transition states.

Results and Discussion

The Feasibility of *S*-Acyl Monoisotripeptide[2] 7 to Undergo *S* → *N*-acyl Migration via 9-Membered Cyclic Transition State

We prepared starting monoisotripeptide **7** for study of *S* → *N*-acyl migration via 9-membered cyclic transition state as illustrated

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[1]The term 'chemical ligation' of isopeptide is sometimes used for non-native chemical ligations from *S*-acyl peptides to furnish native peptide analogs in a stepwise approach. A cysteine-containing peptide is converted to an *S*-acyl peptide, deprotected, and then transferred to a native peptide analog by a subsequent *S*- to *N*-acyl migration. In contrast to native chemical ligation, this isopeptide methodology allows the isolation and characterization of the *S*-acyl peptides and migration of the *S*-acyl group in an independent step.

[2]The term 'monoiso' is used to indicate that the S atom has just one amino acid unit attached to it; similarly, 'diiso' would indicate two amino acid units attached to S.

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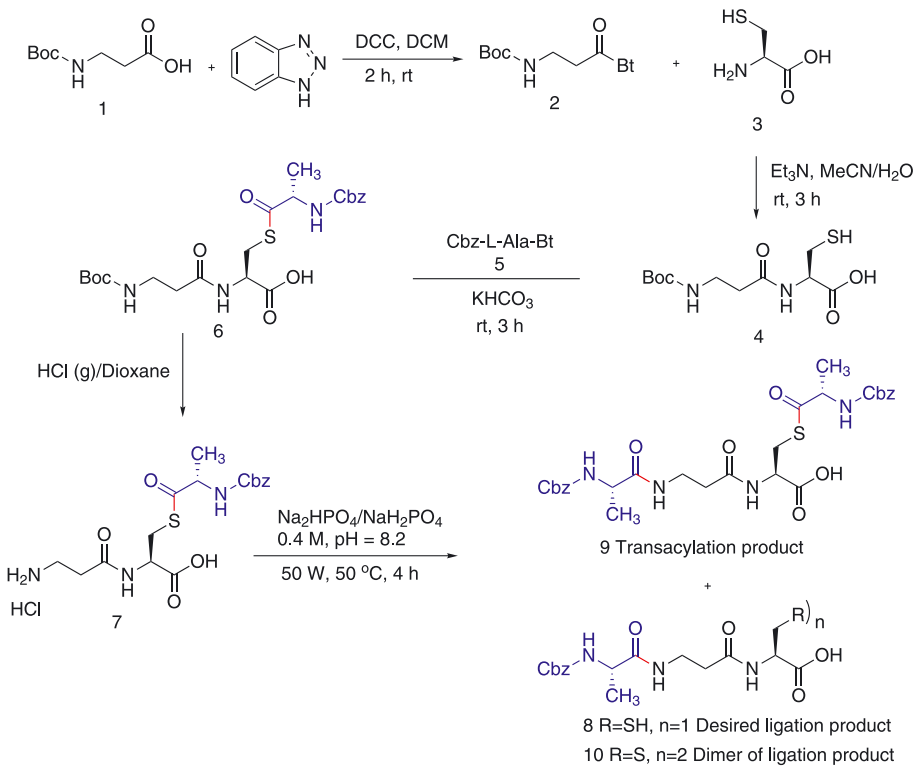


Scheme 1. Chemical ligation of *S*-acyl monoisotripeptide **7**.

in Scheme 1. Boc-protected β-alanine **1** was converted into the corresponding benzotriazolide **2** by the standard method [28]. L-Cysteine was reacted with the Boc-β-alanine benzotriazolide **2** in aqueous acetonitrile (MeCN/H_2O, 7/3) containing 1 equiv of Et_3N for 1 h at 20 °C to give the Boc-β-alanyl-cysteine dipeptide **4** (68%). Subsequent S-acylation of **4** with Z-L-Ala-Bt **5** at room temperature in the presence of $KHCO_3$ furnished the Boc-protected *S*-acyl monoisotripeptide **6** (70%), and Boc-group deprotection of **6** with dioxane saturated with hydrochloric acid gas formed *S*-acyl monoisotripeptide **7** as its hydrochloride salt.

Chemical ligation via the 9-membered cyclic transition state was attempted by microwave irradiation of a solution of **7** at a 2 mM concentration in 0.4 M NaH_2PO_4/Na_2HPO_4 buffer (pH = 8.2) and acetonitrile (24 : 1) for 4 h at 50 °C. However, HPLC-MS (ESI) analysis of the reaction mixture (Scheme 1, Table 1) revealed that the major product **9** arose from intermolecular disproportionation **7** → **9**, thus indicating that intermolecular aminolysis of one molecule thioester **7** by another (**7** + **7** → **9**) is favored over intramolecular attack through a 9-membered ring. Ligated product **8** was also present (Table 1) but was formed in only 4% yield.

Table 1. Chemical ligation of *S*-acyl isopeptides **7**, **17**, **23**, and **28**

Cyclic TS size/*b* (N-C)[a]	Total crude yield (%) of products isolated[b]	Product and relative amounts of each product (%)[c,d]				Product characterization by HPLC-MS				
						Structure	$[M+H]^+$ found		TA product	
		R	LM	LD	TA		Ligated peptide monomer (LM)	Ligated peptide dimer (LD)	Structure	$[M+H]^+$ found
9/3.32	85	**7** (5)	**8** (4)	**10** (0)	**9** (91)	**8**	397	792	**9**	602
10/3.19	82	**17** (3)	**18** (12)	**20** (68)	**19** (17)	**18**	411	820	**19**	616
12/3.18	91	**23** (0)	**24** (0)	**26** (46)	**25** (54)	**24**	454	906	**25**	659
13/3.43	87	**28** (1)	**29** (5)	**31** (77)	**30** (17)	**29**	468	934	**30**	673

R, recovered reactant; LM, ligation monomer; LD, ligation dimer; TA, transacylation; TS, Transition state.

[a]The distance between the terminal amino group and the thioester carbon atom.

[b]The combined crude yield was calculated according to the following equation: combined crude yield = [(ligated peptide) + 2 × (transacylation product)]/(starting material).

[c]Determined by HPLC-MS semi-quantitative. The area of ion peak resulting from the sum of the intensities of the $[M+H]^+$ and $[M+Na]^+$ ions of each compound was integrated.

[d]Amounts are corrected for LD = 2 mmol, LM = 1 mmol.

Demonstration of *S* → *N*-Acyl Migration in *S*-Acyl Monoisotripeptide 17 to Give Native Tripeptide Analog 18 via a 10-Membered Cyclic Transition State

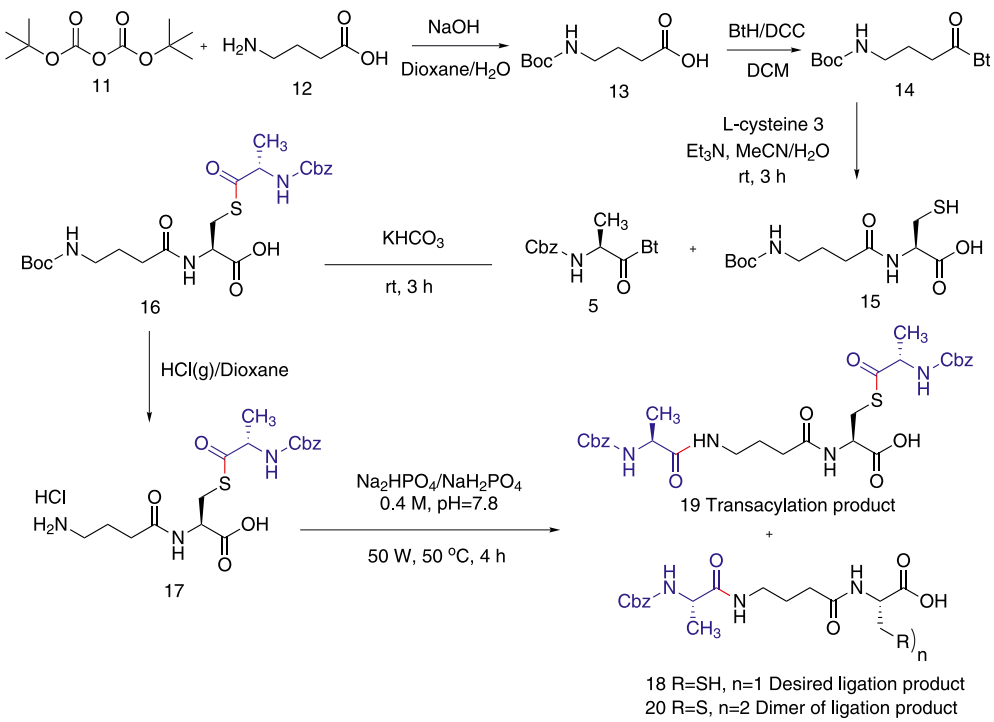
To study chemical ligation via 10-membered cyclic transition state, we coupled Boc-anhydride **11** with γ–aminobutyric acid **12** following a literature procedure [29] (Scheme 2) and converted the resulting Boc-γ-aminobutyric acid **13** (87%) into the corresponding novel Boc-aminoacylbenzotriazolide **14** (80%). Coupling **14** with L-cysteine in CH_3CN–H_2O in the presence of Et_3N for 3 h at 20 °C gave the Boc-protected cysteine dipeptide **15** (64%), which on S-acylation with Z-L-Ala-Bt **5** in aqueous acetonitrile, in the presence of one equivalent of $KHCO_3$ afforded the *S*-acyl monoisotripeptide **16** (71%). Boc-group deprotection of **16** using HCl(g) in dioxane gave amino-S-acylated monoisotripeptide **17** as its HCl salt.

To investigate chemical ligation, a suspension of **17** in NaH_2PO_4/Na_2HPO_4 buffer at pH = 8.2 was subjected to microwave irradiation at 50 °C for 4 h. HPLC-MS (ESI) analysis revealed the major reaction products as the expected ligation monomer **18**, together with its dimer **20** as evidenced by *m/z* 820 $[M+H]^+$. MS allows unambiguous distinction between the ligation product **18** and starting tripeptide **17** (molecular ion $[M+H]^+$: *m/z* = 411) and dimer **20**. Small peptides containing a cysteine residue easily dimerize to the corresponding disulfide dimer in the absence of reducing agent [30]. The ratio of ligated products (**18** + **20**) : intermolecular transacylation product **19** was 80 : 17 (Table 1). Demonstrating that intramolecular nucleophilic attack by the amino group on the thioester group of **17** through a 10-membered cyclic transition state is preferred over intermolecular acylation between two molecules of **17** to give **19** (Scheme 2).

Demonstration of *S* → *N*-Acyl Migration in *S*-Acyl Monoisotetrapeptide 22 to Give Native Tetrapeptide Analog 23 via a 12-Membered Cyclic Transition State

We investigated *S* → *N*-acyl migration via 12-membered cyclic transition state using *S*-acyl monoisotetrapeptide **22**; coupling N-terminal amino-unprotected *S*-acyl monoisotripeptide **7** with Boc-Gly-Bt **21** afforded **22**. Subsequent deprotection of the Boc group in **22** then gave *S*-acyl monoisotetrapeptide **23** (Scheme 3). The intramolecular *S* → *N*-acyl migration experiment **23** → **24** would proceed through a 12-membered-ring transition state (Figure 1B).

The chemical ligation experiment was carried out at 2 mM concentration of **23** under microwave irradiation at 50 °C for 4 h. Examination of the crude product mixture by HPLC-MS (ESI) revealed two major products in 54 : 46 ratio (Table 1). The most abundant (+) ESI-MS peak *m/z* 929 $[M+Na]^+$ was the Na^+ coordinated oxidized disulfide dimer **26** of the pseudo native tetrapeptide analog **24** (MW 906) ion formed by successful ligation of **23**. The second major product was the intermolecular transacylation product **25** with MS (ESI) 659 (Table 1). The mixture of **26** : **25** in 54 : 46 ratio was purified by semi-preparative HPLC that enabled the isolation and the characterization of the native peptide analog **26**. Ligated product **26** was then further characterized by analytical HPLC and High Resolution Mass Spectrometry (HRMS) analysis (Figure 2). These results indicate that chemical long-range ligation (**23** → **24**) via a 12-membered cyclic transition state is feasible and could be a promising approach for the synthesis of native peptide analogs. It is also in agreement with Kemp's work [23,24] for successful *S* → *N*-acyl shift in thiol capture ligations proceeding through transition states with 12-membered rings (Figure 1A).



Scheme 2. Chemical ligation of *S*-acyl monoisotripeptide **17**.

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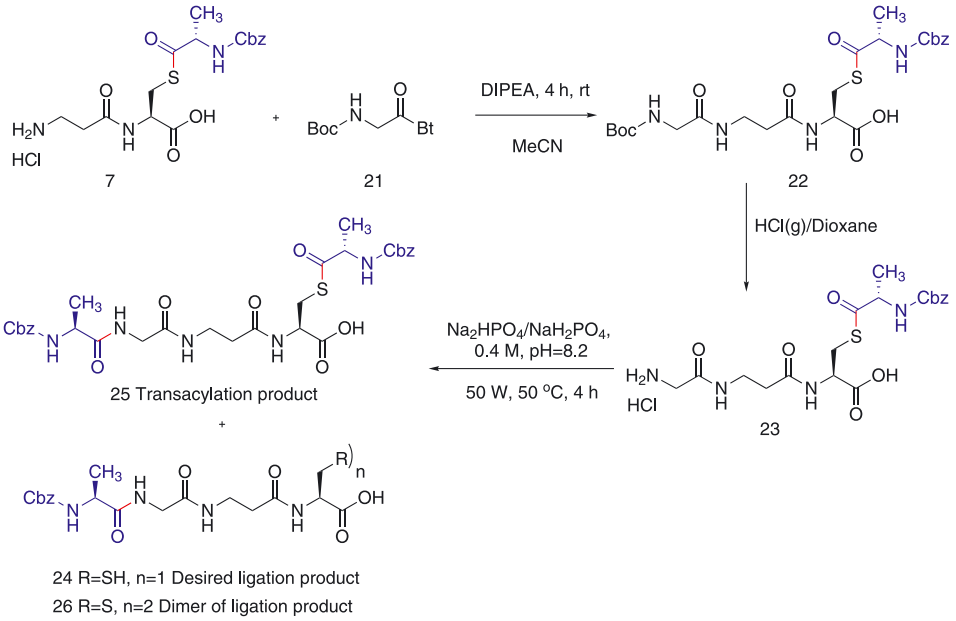


Scheme 3. Chemical ligation of *S*-acyl monoisotetrapeptide **23**.

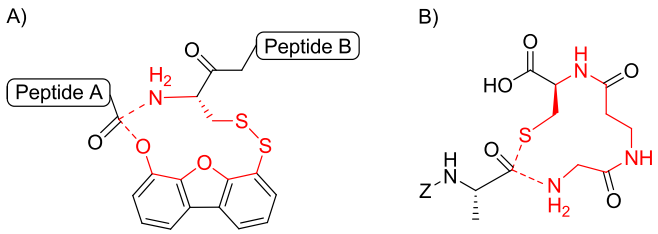


Figure 1. (A) 12-Membered cyclic transition state proposed by Kemp [23,24] for thiol capture ligations. (B) Cyclic transition state needed for chemical ligation of isopeptide **23**.

Demonstration of *S* → *N*-Acyl Migration in *S*-Acyl Monoisotetrapeptide 28 to Yield 29 via a 13-Membered Cyclic Transition State

Coupling of N-terminal amino-unprotected *S*-acyl monoisotripeptide **7** with Boc-β-Ala-Bt **2** gave **27**. Deprotection of the Boc group in **27** produced *S*-acyl monoisotetrapeptide **28** (Scheme 4), which was subjected to microwave irradiation at 50 °C for 4 h in NaH_2PO_4/Na_2HPO_4 buffer at pH 8.2. HPLC-MS (ESI) analysis of the crude ligation mixture revealed the successful ligation of **28** via a 13-membered cyclic transition state. Ligation product **29** was the major component (82%) (molecular ion of the disulfide dimer $[M+H]^+$ *m/z* 934, *versus* 468 for the $[M+H]^+$ molecular ion of the starting *S*-(Z-Ala)tripeptide **28**). The HPLC-MS (ESI) also confirmed that substantial amount of **30** was formed by intermolecular transacylation (Table 1, Scheme 4). Thus, the feasibility of long-range acyl migration via the 13-membered cyclic transition state is confirmed favorable in complete agreement with our previous study [31]. The findings of this investigation hence offer prospects for a convergent assembly of peptides and proteins with β-amino acid architecture.

Computational Analysis

The varying regioselectivity demonstrated earlier in the *S*- to *N*-acyl transfer reactions of isopeptides **7**, **17**, **23**, and **28** is rationalized by analyzing the cyclic transition states required to be formed by the structures to undergo internal *S* → *N*-acyl shift. It follows from obvious steric considerations that larger rings are easier to form

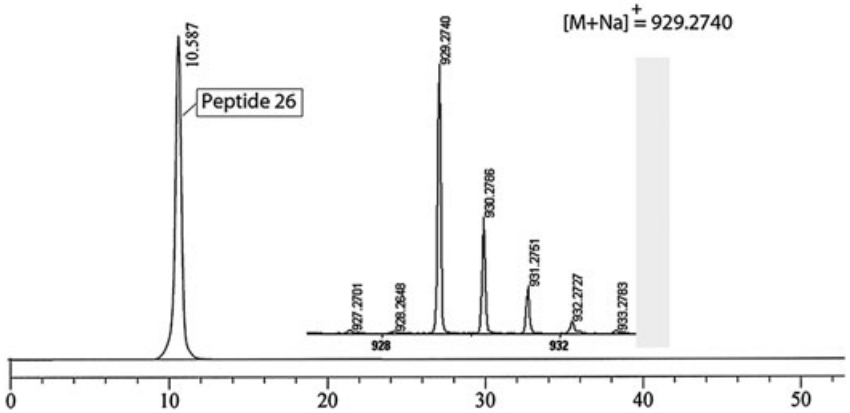
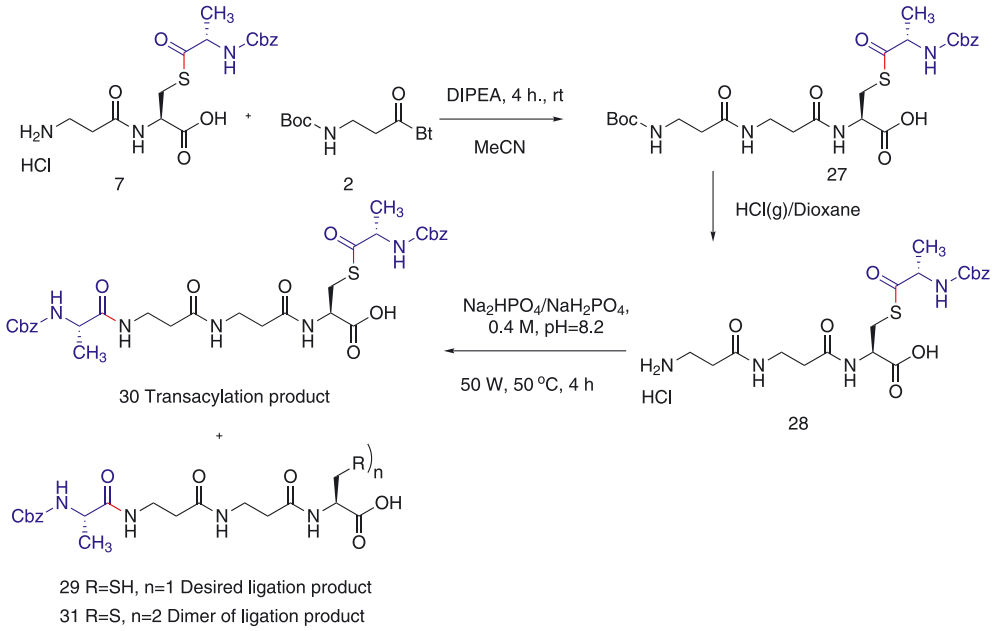


Figure 2. HPLC analysis of the disulfide dimer **26**.



Scheme 4. Chemical ligation of *S*-acyl monoisotetrapeptide **28**.

because the thermodynamic penalty for cyclization gradually decreases as the ring size increases. On the basis of this basic assumption, one would expect isopeptides **7**, **17**, **23**, and **28** (forming 9-, 10-, 12-, and 13-membered transition states, respectively) to be ordered as **7** < **17** < **23** < **28** with regard to their ability to undergo internal *S*- to *N*-acyl transfer relative to intermolecular transacylation. But the experimentally found order is somewhat different: **7** < **23** < **28** ≈ **17**, inferring some other structural features to influence the reactivity. To rationalize the observed reactivity, we applied a computational protocol [32] previously designed by us for the elucidation of similar *S*- to *N*-acyl transfer reactions [26,27]. This protocol includes a full conformational search and subsequently scoring of the generated conformers against a proximity scoring function. The distance between the terminal amino group and the thioester carbon atom denoted as *b*(N-C) is employed here as such a function, because it readily measures the conformational preorganization for the nucleophilic attack.

Full conformational search of isopeptide structures **7**, **17**, **23**, and **28** was performed using the MMX force field, as implemented in PCModel v.9.3 software [33]. As a result, 3–700 conformers were generated for each isopeptide, which were subsequently ranked in the ascending order of the *b*(N-C) proximity function. The best *b*(N-C) scores for structures **7**, **17**, **23**, and **28** are 3.32, 3.19, 3.18, and 3.43 , respectively. It is seen that the geometrical proximity well reflects the better preorganization of the 10-membered ring forming γ-amino acid terminus (**17**) over the 9-membered ring forming β-amino acid terminus (**7**), in accord with the steric arguments. But for structures **23** and **28**, the proximity function taken alone does not fully elucidate the experimental reactivity. However, a closer inspection of the structures of the best preorganized conformers reveals a possible explanation for that: NH–π interactions. The short contacts (2.7–2.8) between the N–H bonds and the plane of the Z π-system displayed in Figure 3 can be identified as a type of intermolecular interaction, which frequently occurs in proteins.

In some situation, particularly if amide NH bonds are involved, the NH–π interactions can be rather strong, up to 6 kcal/mol [34]. This is the case in the 10-membered ring forming structure **17**,

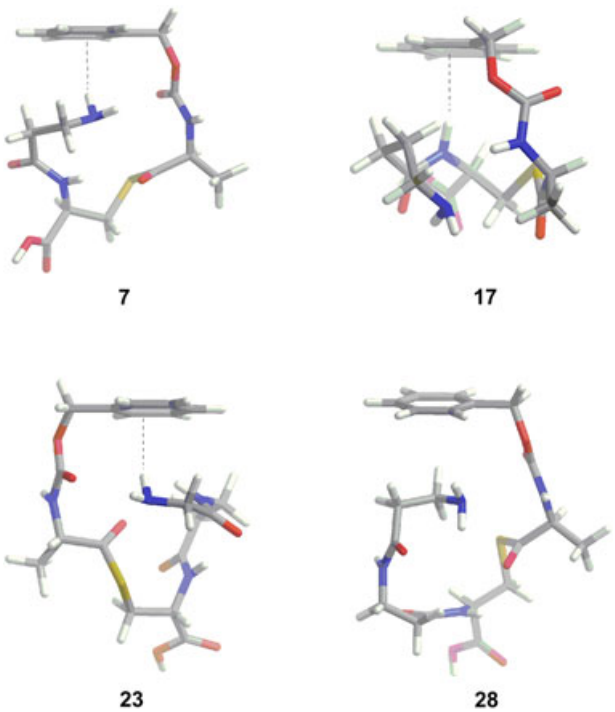


Figure 3. Best preorganized conformers of **7**, **17**, **23**, and **28** (upside down) with NH–π contact visualized.

where one sees an amide NH–π contact capable to provide an additional stabilization to the preorganized conformer. But in the 9-, 12-, and 13-membered ring forming structures **7**, **23**, and **28**, respectively, the NH–π contacts are quite different: they involve not the neighboring amide NH bond as in **17** but those belonging to the terminal amino group. Such engagement somewhat locks the amino group bound to the Z phenyl ring and makes it less available for the nucleophilic attack on the thioester group. Thus, a combination of steric reasons and NH–π interactions explains the observed reactivity of the title reaction.

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Conclusions

Stable, amino-unprotected *S*-acyl-monoisotri- and *S*-acyl-monoisotetra-cysteine-peptides containing α-, β- and/or γ-amino acid residues undergo chemical ligations in which the cysteine *S*-acyl groups migrate to the N-terminal amino acids via 9, 10-, 12-, and 13-membered cyclic transition states to form the corresponding native tripeptide and tetrapeptide analogs. We have already demonstrated [32] by quantum chemical calculations that cross-transition state H-bonding and transition state size can favor or disfavor the intramolecular reaction. Now, we see that not only hydrogen bonding but also NH–π interactions can affect regioselectivity of the $S \rightarrow N$-acyl transfers. The present work describing a range of novel long-range *S*- to *N*-acyl migrations offers evidence that relates to the challenging problem of successfully coupling large peptides and peptide analog fragments. Work is currently in progress on conformational studies of various transition states of size 6–20 to further understand such interactions.

Experimental

General Methods

Details of the syntheses and transformations with related analytical data are reported in the Supplementary Information. Melting points were determined on a capillary point apparatus equipped with a digital thermometer. NMR spectra were recorded in $CDCl_3$, DMSO-d_6 or CD_3OD-d_4 operating at 300 MHz for ^1H and 75 MHz for ^{13}C with TMS as an internal standard. All microwave-assisted reactions were carried out with a single mode cavity CEM Discover microwave synthesizer. The reaction mixtures were transferred into a 10-ml glass pressure microwave tube equipped with a magnetic stirrer bar. The tube was closed with a silicon septum, and the reaction mixture was subjected to microwave irradiation (Discover mode; run time: 60 s; PowerMax-cooling mode).

Acknowledgements

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