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Peptide Ligations Accelerated by *N*-Terminal Aspartate and Glutamate Residues

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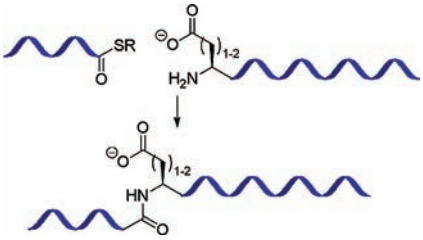
ABSTRACT



A novel application of intramolecular base catalysis confers enhanced reaction rates for aminolysis ligations between peptide thioesters and peptides bearing *N*-terminal aspartate or glutamate residues. The broad scope of this process and its application in the total synthesis of the diabetes drug exenatide is demonstrated.

Ligation chemistry represents an extremely valuable tool for the convergent assembly of peptide fragments to provide polypeptides and proteins for biological study.[1] The most efficient and widely used ligation method, introduced by Kent and co-workers, is native chemical ligation.[1a,2] This method facilitates the chemoselective condensation of a peptide bearing an *N*-terminal cysteine residue and a peptide containing a *C*-terminal thioester moiety to form a native amide bond. The reaction mechanism first involves a transthioesterification step to generate an intermediate thioester which subsequently rearranges via an S→N acyl shift.[1a,2,3] To date, the method has been successfully implemented in the preparation of hundreds of complex protein targets, including those with post-translational modifications.[1a,e] The recent use of thiol-derived proteinogenic amino acids for ligation–desulfurization applications has further expanded the repertoire of this powerful technology.[1k,4]

Thiol-free peptide ligation methods have also been developed[5] and exploited in the synthesis of peptides and proteins.[5c,e,6] These serve as complementary tools to native chemical ligation and have found utility in the synthesis of several large peptide targets. One such method, which utilizes

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the inherent nucleophilicity of *N*-terminal amines for the generation of peptide bonds via reaction with *C*-terminal peptide thioesters as *N*-acyl donors, is direct aminolysis ligation.[5e] A significant benefit of this approach, compared with other thiol-free methods, is that reactions proceed without epimerization of the *C*-terminal amino acid of the peptide thioester.[5a−d] However, as these reactions rely on intermolecular formation of an amide bond, they tend to be significantly slower than native chemical ligation and are not chemoselective in the presence of the ε-amino side chain of lysine.[5e] Nonetheless, such ligation reactions are high yielding with a number of amino acids at the ligation junction, and side chain protection of lysine ensures regioselectivity.[5d,e,6]

Herein, we report that the rates of aminolysis ligations between peptide thioesters and peptides bearing *N*-terminal aspartate (Asp) or glutamate (Glu) residues are enhanced as a result of intramolecular base catalysis facilitated by the side chain carboxylate moiety. The synthetic utility of this reaction is explored, together with its application in the total synthesis of the type II diabetes drug exenatide (Byetta).

Initial studies focused on the reaction of model peptides **1**−**4** with peptide thioester **5** (Scheme 1) under mixed solvent/buffer conditions [4:1 v/v *N*-methylpyrrolidinone (NMP):1.25 M Gn·HCl, 200 mM HEPES, 2 vol % PhSH, pH 7.5].[5e] After 72 h, the reaction mixtures were purified by HPLC to afford ligation products **6**−**9**. Excellent yields of **6** (quant) and **7** (92%) were obtained when peptides **1** and **2**, bearing *N*-terminal Asp and Glu residues, respectively, were reacted with **5**. In contrast, reaction of **5** with **3** and **4** (containing sterically similar *N*-terminal Asp- and Glu-methyl esters, respectively) provided the corresponding ligation products **8** and **9** in poor yields (17−37% isolated yield). In addition, reaction of **5** with peptides containing sterically equivalent *N*-terminal Asn and Gln residues provided poor yields of the desired product, together with a cyclic imide byproduct of the peptide fragment.

Kinetic studies of ligation reactions between **5** and peptides **1**−**4** were performed to rationalize the observed difference in reaction efficiencies (Scheme 1). The data was fitted to a unimolecular rate equation (see the Supporting Information). A greater than 9-fold increase in the reaction rate was observed when **5** was reacted with Asp- or Glu-containing peptides **1** or **2** ($t_{1/2}$: **1** = 13.1 h, **2** = 9.5 h) compared to the analogous *N*-terminal Asp- and Glu-methyl ester-containing peptides **3** and **4** ($t_{1/2}$: **3** = 190 h, **4** = 85 h). The rate enhancement observed for *N*-terminal Asp- and Glu-containing peptides therefore appears to be a direct result of the carboxylate side chain.

Two likely mechanistic pathways are postulated for this rate enhancement, both of which invoke the side chain carboxylate: (1) intramolecular base catalysis (previously proposed for the acceleration of acyl shift-based transformations with peptides bearing *N*-terminal or neighboring Asp, Glu, His, or carboxylate

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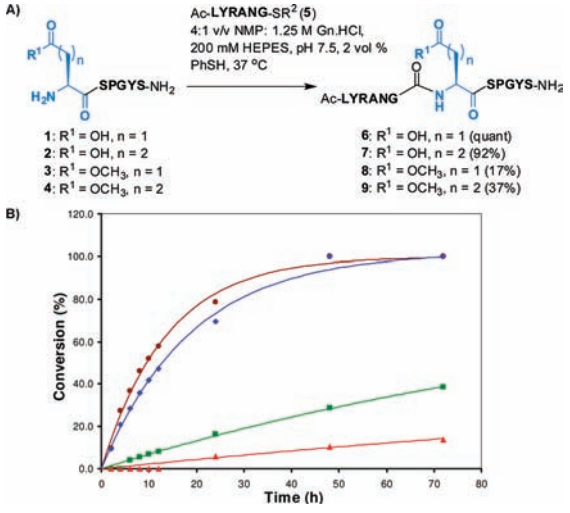
Scheme 1. (A) Aminolysis Ligation Reactions between Peptides **1**−**4** and Peptide Thioester **5**. (B) Kinetics of the Direct Aminolysis Peptide Ligation between Peptide Thioester **5** and Peptides **1**−**4** ($R^2 = (CH_2)_2CO_2Et$)[a]

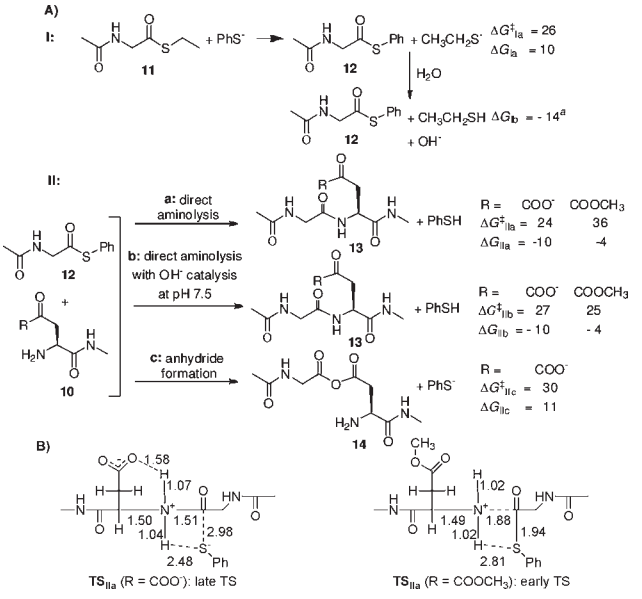


[a] Key: blue diamond = peptide **1**, maroon circle = peptide **2**, red triangle = peptide **3**, green square = peptide **4**.

moieties)[3,7] and (2) generation of an unsymmetrical anhydride followed by an intramolecular O→N acyl shift.[8]

Scheme 2A shows activation free energies $\Delta G^{\ddagger}$ and reaction free energies ΔG calculated for putative pathways for the ligation reaction between model peptide **10** and peptide thioester **11**. Calculations were performed

Scheme 2. (A) Calculated Reaction and Activation Energies (kcal mol^{-1}).[a] (B) Transition-State Structures $\mathbf{TS_{IIa}}$ (Bond Lengths in Å)



[a] CH_3CH_2SH, pK_a = 10.61; PhSH, pK_a = 6.5.

using density functional theory (DFT) by GAUSSIAN-09[9] using the B3LYP[10] density functional, the auc-cc-pVDZ basis set,[11] explicit solvation, and the PCM implicit aqueous solvation model[12] with solution-phase thermodynamic corrections (see the Supporting Information for additional reactions and all optimized structures).[13]

The first reaction stage for aminolysis ligations (reaction **I**, Scheme 2A) is thiolate exchange from alkyl thioester **11** to phenylthio ester **12**.[14] The thiolate exchange is calculated to proceed at pH 7.5 via nucleophilic attack of thiophenolate on the carbonyl over a transition state at $\Delta G^{\ddagger}_{I} = 26$ kcal mol^{-1}, followed by protonation of the displaced alkyl thiolate, in agreement with recent calculations for native chemical ligation.[15] The importance of this step was verified experimentally by conducting ligation reactions of **1** and **2** with **5** in the absence of thiophenol which showed dramatically slower reaction rates (see the Supporting Information).

Three possible mechanisms were considered for the second-stage amide bond formation (reaction **II**, Scheme 2A) between peptide **10** and peptide thioester **12** to afford **13**; namely **IIa**-direct aminolysis, **IIb**-direct aminolysis facilitated by OH^- interacting with the transition state,[16] and **IIc**-reaction via an anhydride intermediate. These were considered (where appropriate) for reaction with both an *N*-terminal aspartate residue ($R = COO^-$) and the corresponding methyl ester ($R = COOCH_3$). Given the high activation barrier ($\Delta G^{\ddagger}_{IIc} = 30$ kcal mol^{-1}) and endothermic nature ($\Delta G_{IIc} = 11$ kcal mol^{-1}) of anhydride **14** formation in **IIc**, this is not considered to be a likely pathway. Furthermore, the operation of such a mechanism would generate a cyclic succinimide (and adducts thereof) in reactions of peptides bearing *N*-terminal Glu residues; however, these byproducts were not observed.

In the case of the direct aminolysis process **IIa**, a significantly faster reaction rate is calculated for $R = COO^-$ compared to $R = COOCH_3$ ($\Delta G^{\ddagger}_{IIa} = 24$ kcal mol^{-1} vs 36 kcal mol^{-1}).[17] A typical transition state ($\mathbf{TS_{IIa}}$ for $R = COO^-$) for reaction pathway **IIa** is shown in Scheme 2B and is late: it is subsequent to the formation of the new CN bond and ionic scission of the CS bond and involves attack by the resulting thiolate on a proton of the produced secondary amide cation. This process is assisted by intramolecular base catalysis by interaction of the side chain carboxylate with the other amide proton. For $R = COOCH_3$ the transition state is early (CN amide bond unformed) and not ionic ($\mathbf{TS_{IIa}}$, see Scheme 2B and the Supporting Information).

Experimentally, the observed rate difference implies a less pronounced difference in transition state energies (ca.1.3 kcal mol^{-1}) than that calculated (12 kcal mol^{-1}) for pathway **IIa**. Alternative calculations, involving simultaneous attack of OH^- from the solvent on the NH_2 hydrogens at the transition state, parametrized for pH 7.5 (**IIb**, Scheme 2A, see the Supporting Information for TS structures) reveal this mechanism to be significantly more favorable ($\Delta G^{\ddagger}_{IIb} = 25$ kcal mol^{-1}) for $R = COOCH_3$, consistent with the observed rate differences. In the case of $R = COO^-$, additional OH^- catalysis provides a less favorable pathway than **IIa** (**IIa**: $\Delta G^{\ddagger}_{IIa} = 24$ kcal mol^{-1} vs **IIb**: $\Delta G^{\ddagger}_{IIb} = 27$ kcal mol^{-1}).

Taken together, these calculations suggest that peptides bearing $R = COO^-$ are more likely to react via pathway **IIa**, with the observed rate acceleration facilitated via intramolecular base catalysis by the free side chain carboxylate. In the absence of a carboxylate-containing side chain, reactions are subject to a less effective intermolecular base catalysis (OH^-) process **IIb**.

Given the enhanced reaction rates of aminolysis ligations at *N*-terminal Asp and Glu residues observed above, we next moved to explore the synthetic utility of the transformation for reactions at thioesters bearing a range of *C*-terminal residues. To this end, peptides **1** and **2** were reacted with peptide thioesters (**5** and **15**–**21**) bearing a representative range of *C*-terminal amino acids (Scheme 3). Ligation of **1** and **2** to thioesters with *C*-terminal glycine and alanine residues provided the desired ligation products in excellent yields (86% to quant, entries 1–4, Scheme 3). Ligation reactions to a peptide thioester bearing a *C*-terminal methionine residue also proceeded smoothly to afford the desired products in 70–74% isolated yields (entries 5 and 6). Reaction of these peptides with thioesters possessing *C*-terminal aromatic residues (Tyr and Phe) provided the desired ligation products in good yields (65–84%, entries 7–10). Unfortunately, reaction of **1** and **2** with a peptide thioester containing a *C*-terminal Asn residue led to a diminished yield of the desired product (entries 11 and 12). This was due to the formation of a five-membered cyclic imide byproduct, resulting from facile intramolecular attack of the reactive *C*-terminal thioester by the primary amide side chain of Asn under the ligation conditions. This was not observed when the same peptides were reacted with a peptide thioester bearing a *C*-terminal glutamine residue which provided excellent yields of the desired ligation product (70% to quant, entries 13 and 14). Finally, ligation of **1** and **2** to **21** bearing a sterically encumbered valine residue also provided the desired ligation products in synthetically useful yields (55–58%, entries 15 and 16). It should be noted that no epimerization was detected in the products of any of the reactions studied here.

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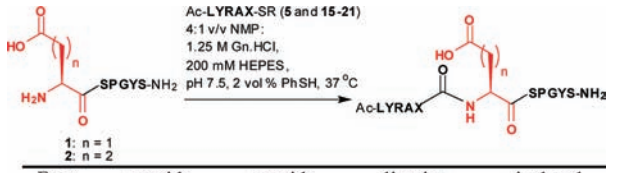
(17) Buildup of the phenylthio ester is observed experimentally, suggesting that $\Delta G^{\ddagger}_{IIa}$ is actually $\geq G^{\ddagger}_{I}$.

Scheme 3. Scope of Direct Aminolysis Ligation Reactions between Peptides Containing *N*-Terminal Asp (**1**) and Glu (**2**) Residues and Peptide Thioesters (**5** and **15**–**21**)[a]

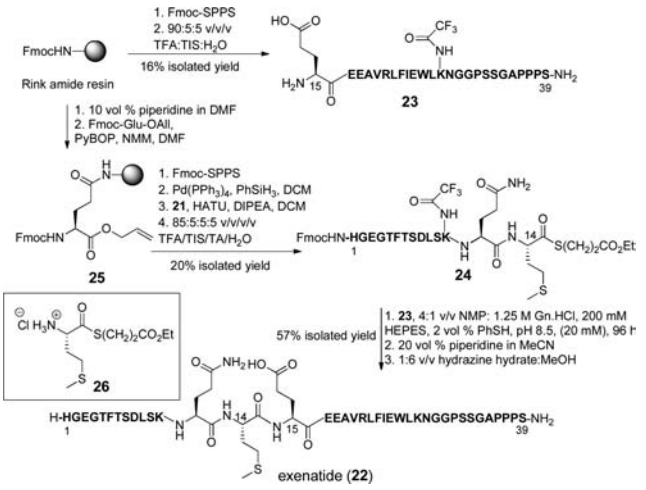


Entry	peptide thioester X =	peptide	ligation Junction AA-Asp/Glu	isolated ligation yield
1	**5**:AsnGly	**1**	Gly-Asp	quant[a]
2	**5**:AsnGly	**2**	Gly-Glu	92%[a]
3	**15**:AsnAla	**1**	Ala-Asp	86%[a]
4	**15**:AsnAla	**2**	Ala-Glu	quant[a]
5	**16**:AsnMet	**1**	Met-Asp	70%[b]
6	**16**:AsnMet	**2**	Met-Glu	74%[b]
7	**17**:Tyr	**1**	Tyr-Asp	68%[b]
8	**17**:Tyr	**2**	Tyr-Glu	78%[b]
9	**18**:AsnPhe	**1**	Phe-Asp	84%[b]
10	**18**:AsnPhe	**2**	Phe-Glu	65%[b]
11	**19**:Asn	**1**	Asn-Asp	11%[c]
12	**19**:Asn	**2**	Asn-Glu	12%[c]
13	**20**:Gln	**1**	Gln-Asp	70%[b]
14	**20**:Gln	**2**	Gln-Glu	quant[b]
15	**21**:AsnVal	**1**	Val-Asp	55%[c]
16	**21**:AsnVal	**2**	Val-Glu	58%[c]

[a] R = $-(CH_2)_2CO_2Et$; (a) = 72 h, (b) = 96 h, (c) = 120 h reaction time.

Having demonstrated the scope of the aminolysis ligation reactions, we next sought to exploit the technology in a ligation-based synthesis of the peptide therapeutic exenatide **22** (marketed as Byetta), a type II diabetes drug (Scheme 4). Exenatide is a synthetic version of the 39 amino acid peptide exendin-4, a structural analogue of the incretin hormone glucagon-like peptide-1, found in the venom of the Gila Monster desert lizard (*Heloderma suspectum*).[18] We envisaged the synthesis of exenatide via disconnection between Met14 and Glu15, thus enabling aminolysis between peptide fragment **23** and peptide thioester **24**. Fragment **23** was synthesized by Fmoc-strategy SPPS from Rink amide resin (Scheme 4, see the Supporting Information). Incorporation of a trifluoroacetamide-protected Lys27 residue was necessary to prevent unwanted aminolysis on the ε-amino group during the ligation reaction. Peptide thioester **24**, bearing *N*-terminal Fmoc-protection and trifluoroacetamide side-chain protection of Lys12, was synthesized via the use of a side chain anchoring strategy (Scheme 4).[19] Briefly, Fmoc-Glu-OAll was immobilized onto Rink amide resin to provide **25** and the peptide elongated via standard Fmoc-strategy SPPS (see the Supporting Information). At this point, removal of the *C*-terminal allyl ester, thioesterification

Scheme 4. Total Synthesis of Exenatide (Byetta) via Glu-Assisted Aminolysis Ligation Reaction



with methionine thioester **26**,[19b] acidolytic cleavage from the resin, and purification by HPLC afforded **24** in 20% overall yield over 23 linear steps. With the requisite fragments **23** and **24** in hand, ligation was performed under our intramolecular base-catalyzed conditions. The reaction progress was monitored by HPLC–MS and, after 96 h, was treated with piperidine followed by methanolic hydrazine to remove the Fmoc carbamate and two side-chain trifluoroacetamide moieties. Gratifyingly, purification by HPLC afforded exenatide **22** in a succinct three steps with an impressive 57% overall yield.

In summary, we have demonstrated a significant rate enhancement when peptide thioesters and peptides bearing *N*-terminal Asp or Glu residues are reacted under aminolysis ligation conditions. This rate enhancement is likely facilitated by intramolecular base catalysis by the side chain carboxylate of Asp and Glu stabilizing the transition state. The transformation was shown to be synthetically useful with a range of peptide thioester coupling partners and the utility of the methodology was further exemplified in the efficient total synthesis of the 39 amino acid type II diabetes drug exenatide. It is anticipated that these aminolysis ligation reactions will find future application in the assembly of a range of peptide and proteins, including other peptide drugs.

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Note Added after ASAP Publication. The corrected version of this Letter was reposted August 12, 2011.

Supporting Information Available. Experimental procedures and characterization of peptide products, computational procedures, and optimized structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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