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Peptide Weinreb amide derivatives as thioester precursors for native chemical ligation†

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Peptide Weinreb amide derivatives with an *N*-substituted mercaptoethyl group are designed as thioester precursors for native chemical ligation. We show that these amides undergo rapid ligation with a cysteinyl peptide under normal NCL conditions to form various Xaa–Cys peptide bonds, including the difficult Val–Cys junction. Facile synthesis of the Weinreb amide linkers allows easy access to this new type of peptide thioester precursor by standard Fmoc solid phase synthesis.

Introduction

Recent years have seen considerable efforts in developing new methods for the solid phase synthesis of peptide thioesters using Fmoc chemistry. These efforts are stimulated mostly by the success of native chemical ligation for protein chemical synthesis.[1] Although the traditional Boc chemistry approach can yield a thioester peptide directly,[2] the repetitive use of TFA and the need for a very strong acid at the final cleavage step make it incompatible with peptides that contain certain acid-sensitive post-translational modifications such as glycosylation. Among the many different Fmoc chemistry-based strategies developed thus far,[3–6] the one using the intramolecular *N*,*S*-acyl transfer principle to convert a C-ter amide to a thioester is particularly appealing as it requires no additional activation steps for thioester generation.[7] Almost all the methods designed on this principle feature an *N*-substituted mercaptoethyl group to enable acyl migration from the N to S through a 5-membered ring intermediate.[4–6] Although both 2° and 3° amide systems have been designed for this purpose, it is more difficult for a 2° amide like the Xaa–Cys peptide bond to undergo *N*,*S*-acyl transfer, presumably because the 2° amide prefers to adopt the more stable but unproductive *trans* form and thus specific mechanisms are needed to drive the process.[4] For the naturally existing protein splicing, this process is catalyzed by the intein.[8] The Cys–Pro ester (CPE) approach by Aimoto's group utilizes diketopiperazine formation to trap the newly exposed amine to drive amide-to-thioester conversion at neutral or slightly basic pH.[4b] Other methods make use of native peptidyl–Cys bonds for *N*,*S*-acyl transfer under acid or mild acid catalysis and heating.[4a,c–e] For a 3° amide, the additional *N*-substitution makes it exist in the *trans* and *cis* forms more equally because the two isomers are more equal in energy. Although *trans*-to-*cis* isomerization may be slow at room temperature, the barrier of activation energy can be easily overcome by heating.[5,6] A symmetrical bis-mercaptoethyl-substituted amide has also been designed for which no such isomerization is needed.[5d,e] Nevertheless, due to the relatively inert nature of most of the tertiary amide systems developed so far, the efficiency of *N*,*S*-acyl transfer is still low and it is usually the rate-limiting step when they are used directly in NCL. Some of the tertiary amides require a separate, acid-catalyzed step for conversion to a new thioester through exchange with an external thiol compound. For those that can be used directly for NCL *via in situ* converted thioesters, their reactivity is typically lower than a normal alkyl thioester. So amide systems enabling more efficient *N*,*S*-acyl transfer are still desired as thioester surrogates for NCL.

In order to increase the efficiency of *N*,*S*-acyl transfer, the carbonyl carbon of the amide should have a more electrophilic character for higher reactivity towards a nucleophile. Weinreb amides **1** have an electron-withdrawing O on its amide N (Fig. 1), which makes them much more electrophilic than

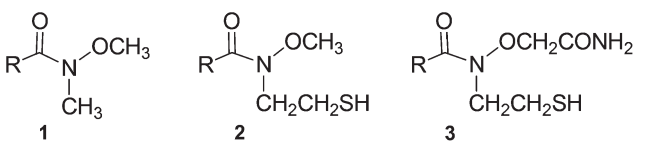

Fig. 1 Weinreb amide **1** and its derivatives, SET-Weinreb amide **2** and CASET-Weinreb amide **3**.

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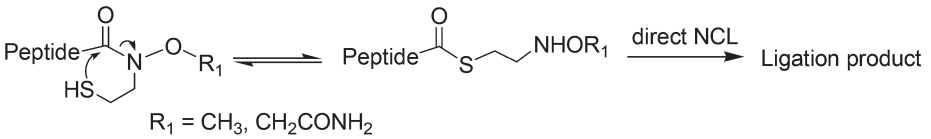


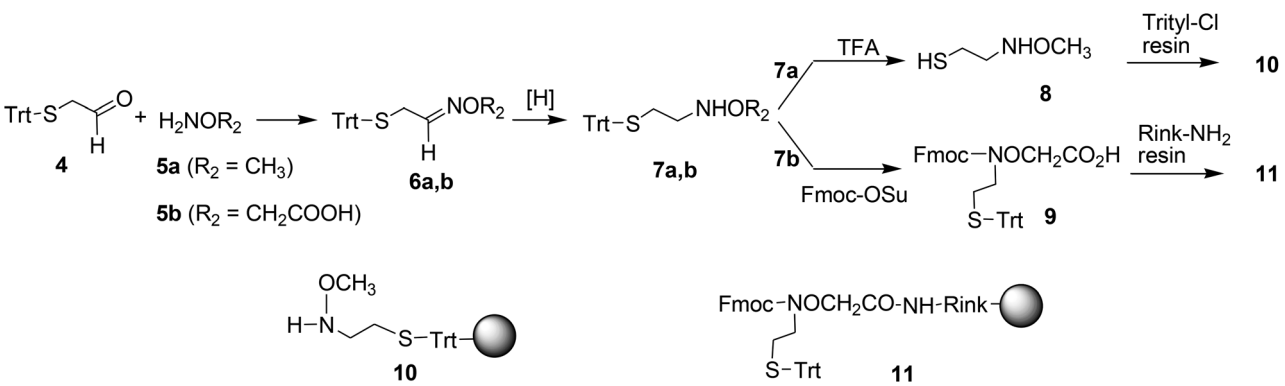
Scheme 1 Weinreb amide systems for NCL. *N,S*-Acyl transfer of peptidyl SET-Weinreb ($R_1 = CH_3$) or CASET-Weinreb ($R_1 = CH_2CONH_2$) amide for thioester formation.

normal tertiary amides.[9] Partly for this reason, Weinreb amides are excellent acylating agents in nucleophilic addition reactions with organometallic or hydride reducing reagents for the preparation of ketones or aldehydes.[9b,c] In fact, solid-phase synthesis of Weinreb amide peptides was realized long ago for the preparation of peptide C-ter aldehydes through on-resin reduction by lithium aluminum hydride.[10] We envision that if the *N*-methyl group of the original Weinreb amide is replaced by the 2-sulfanylethyl group, the new Weinreb amide derivative, SET-Weinreb amide **2** (Fig. 1), would enable intramolecular *N,S*-acyl transfer (Scheme 1). Furthermore, the reactivity would be further increased in the case of CASET-Weinreb amide **3** (Fig. 1) which has another electron-withdrawing carboxamide group on the *O*-methyl group of **2** (Scheme 1). Indeed, we have shown in our present work that both the peptidyl SET-Weinreb amide and CASET-Weinreb amide can be used directly for NCL and that the CASET derivative is significantly more reactive. Interestingly, during the preparation of our manuscript, Dr Yoshiya's group published a report describing a similar design in which a longer *O*-butyramide linker is used to link the peptide to the resin.[11] Nevertheless, in our present study we show a strong effect of the *O*-substitution on the reactivity of the Weinreb amides in NCL and that the ligation reaction of the CASET-Weinreb amides can be completed in 1 h at 42 °C for the average C-ter amino acid residues (Ala, Leu, Phe, Ser) at millimolar concentrations of the two ligation partners. We also show for the first time that an amide-based thioester precursor, the CASET-Weinreb amide, can be directly used for NCL at the Val–Cys junction with satisfactory efficiency.

Results and discussion

The SET-Weinreb amide and CASET-Weinreb amide linkers can be conveniently prepared from the commercially available starting materials using conventional synthetic procedures (Scheme 2). First, 2-tritylthioacetaldehyde, prepared *via* periodate oxidation of 3-(tritylthio)propane-1,2-diol (ESI†), was reacted with methoxyamine **5a** and aminooxyacetic acid **5b**, respectively, to afford the oxime products **6**. Reduction of **6** with $NaBH_3CN$ yielded reductive amination products **7** in excellent yields. The trityl group in **7a** was removed with TFA to give the SET-Weinreb linker **8** which was then reacted with trityl chloride resin to give the SET-Weinreb linker-loaded trityl resin **10**. **7b** was treated with Fmoc-OSu to give the Fmoc protected CASET-Weinreb linker **9**, which was loaded to Rink amide resin using PyBOP to afford **11**. Detailed synthetic procedures of the two linkers and compound characterization data are found in the ESI.† The key to the facile synthesis of our linkers lies with relative ease of oxime formation and reduction, whereas in the synthesis of the Yoshiya linker, the Mitsunobu reaction was used to introduce the 2-sulfanylethyl group on the nitrogen.[11]

The linker-loaded resins, **10** and **11**, could then be used for the standard Fmoc solid phase peptide synthesis. The coupling of the first Fmoc-amino acid to the 2° oxyamine of both linkers was without any difficulty due to the good nucleophilicity of the oxyamine. A series of small model Weinreb amide peptides with the sequence **LSKFXaa** with different C-terminal amino acid residues (**Xaa** = **Ala, Leu, Phe, Ser and Val**) were synthesized using standard Fmoc SPPS procedures. After TFA cleavage, the crude products were purified by reverse-phase



Scheme 2 Outline of synthesis of Weinreb amide linkers for the Fmoc solid phase peptide synthesis. See the ESI† for detailed experimental procedures.

HPLC. A cysteinyl peptide amide **CFAEKSLK**-NH_2 was also synthesized for use in NCL with the above-prepared Weinreb amide peptides.

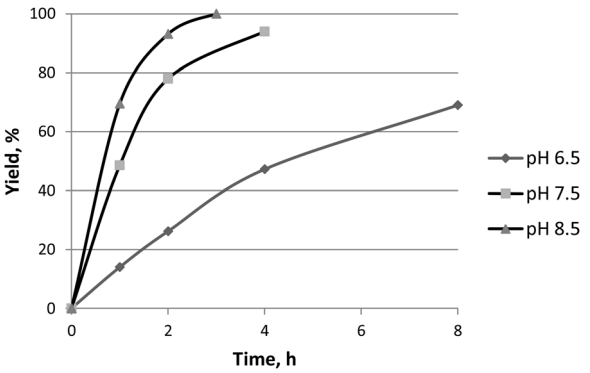


Fig. 2 pH-Dependence of the ligation reaction rate of **LSKFA**-N(OCH_3)CH_2CH_2SH (3 mM) with **CFAEKSLK**-NH_2 (5 mM) at 42 °C in phosphate buffer containing 2% MESNa and 5 mM TCEP.

We first used the C-ter Ala SET-Weinreb amide peptide **LSKFA**-N(OCH_3)CH_2CH_2SH and CASET-Weinreb amide **LSKFA**-N(OCH_2CONH_2)CH_2CH_2SH for NCL with **CFAEKSLK**-NH_2 to evaluate their respective reactivity and establish optimal reaction conditions. The reaction was conducted using 3 mM of the Weinreb amide peptide and 5 mM of the cysteinyl peptide in phosphate buffer containing 2% MESNa (w/v) and 5 mM TCEP. First, we found that the pH had a strong effect on the ligation reaction rate. As seen from Fig. 2, the reaction rate increased dramatically when the pH was increased from 6.5 to 8.5. For instance, for the SET-Weinreb amide of LSKFA, at pH 8.5 and 42 °C, the reaction had a 93% conversion yield (based on the calculations of integrations of UV absorption peaks at 220 nm on analytical HPLC) after 2 h and was completed at 3 h (see also Fig. 3C), whereas at pH 7.5 and 6.5 the reaction had a conversion yield of 78% and 26% respectively in 2 h. At 4 h, the reaction had a yield of 94% at pH 7.5 and 47% at pH 6.5. When the reaction was performed at an even lower pH 5.5, it became very slow and gave a yield of only about 3% after

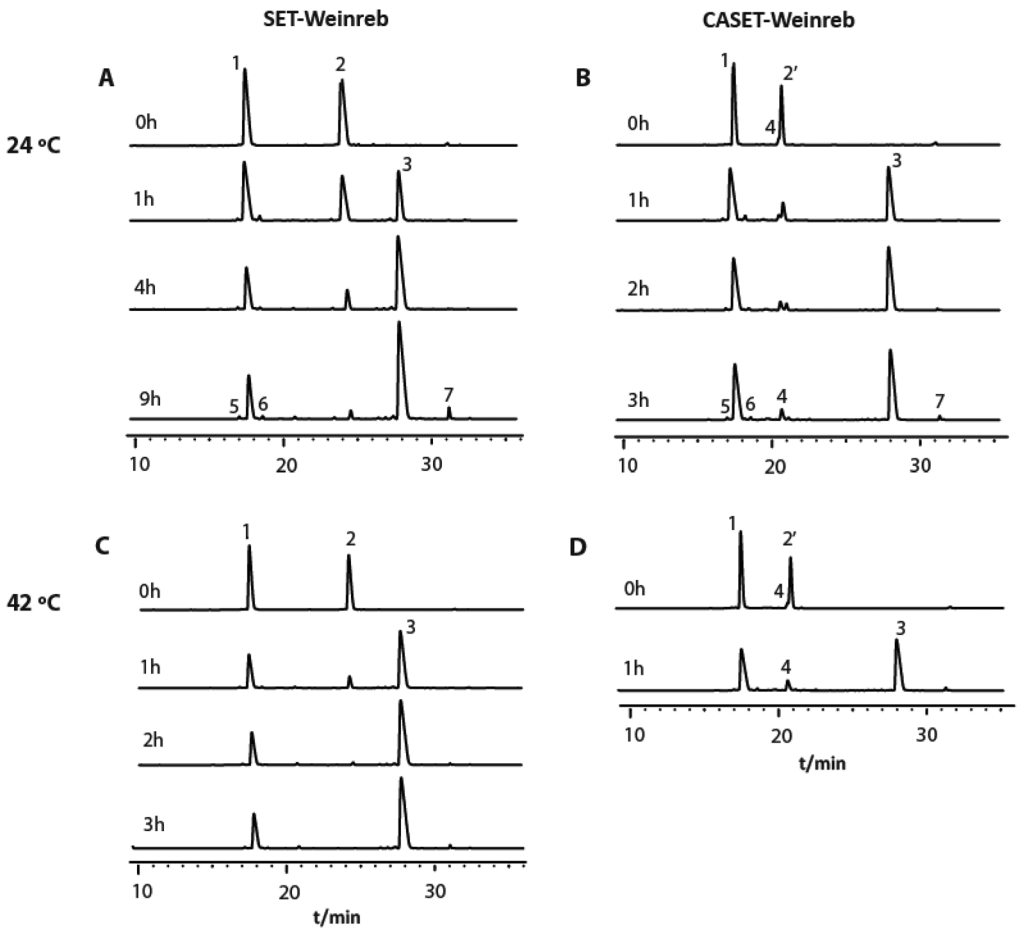


Fig. 3 Comparison of the ligation reactivity between CASET-Weinreb and SET-Weinreb amides. Analytical HPLC monitoring of the ligation reactions (pH 8.5, 2% MESNa, 5 mM TCEP) of the two Weinreb amides of peptide **LSKFA** (3 mM) with **CFAEKSLK**-NH_2 (5 mM) at 24 °C (panels A and B) and 42 °C (panels C and D). Gradient: 0–40% buffer B in buffer A for 40 min. Peak 1: **CFAEKSLK**-NH_2; peak 2: **LSKFA**-N(OCH_3)CH_2CH_2SH; peak 2′: **LSKFA**-N(OCH_2CONH_2)CH_2CH_2SH; peak 3: ligation product; peak 4: impurity **LSKFA**-$NHCH_2CH_2SH$; peak 5: desulfurized product of **CFAEKSLK**-NH_2; peak 6: disulfide product of **CFAEKSLK**-NH_2 with MES; peak 7: disulfide product of the ligation product with MES. See the ESI† for experimental procedures and compound characterization data.

18 h at rt (see the ESI†). The pH-dependent reactivity of the Weinreb amide is likely attributed to a higher amount of the deprotonated thiolate group at higher pH. This behavior of pH-dependence is rather different from that of some other tertiary amide systems which operate optimally at weakly acidic pH.[5d,e,6a,e]

The same pH trend was found for the CASET-Weinreb amide (data not shown); however, the CASET-Weinreb amide was much more reactive than the SET-Weinreb amide (Fig. 3). At pH 8.5 and 42 °C, the reaction of the CASET-Weinreb amide of **LSKFA** was complete in less than 1 h compared to 3 h for the SET-Weinreb amide (Fig. 3C and D). At room temperature (24 °C), the same reaction of the CASET-Weinreb amide was completed at 3 h whereas that of the SET-Weinreb amide took 9 h to be about 95% complete (Fig. 3A and B).

As seen from Fig. 3, there is an unreactive impurity (peak 4, panels B and D) accompanying and eluting immediately before the CASET-Weinreb amide peptide. MS analysis revealed that this impurity was **LSKFA**-$NHCH_2CH_2SH$ due to removal of the OCH_2CONH_2 group from the Weinreb amide nitrogen in **LSKFA**-$N(OCH_2CONH_2)CH_2CH_2SH$. It is unclear how the N–O bond was cleaved. This small amount of impurity was observed in the crude product from the final cleavage step of SPPS and it was difficult to separate it from the desired Weinreb amide peptide by HPLC purification. Although the exact cause of this N–O bond cleavage is not known at this moment, it is plausible that the relatively weak N–O bond (bond energy ≈ 55 kcal mol^{-1}), which is exacerbated by the electron-withdrawing $CONH_2$, might have undergone reductive cleavage in the strong acidic and reducing environment of the final deprotection cocktail. Interestingly, this impurity was not seen with the SET-Weinreb amides. In spite of this, the CASET-Weinreb amide, being much more reactive than the SET-Weinreb amide, is overall still a better choice for NCL.

We also conducted the ligation reaction at stoichiometric amounts of the two ligation partners (both at 5 mM) and in the presence of a higher concentration of TCEP (30 mM). At this reduced nucleophile-to-electrophile ratio, the persistent existence of the thioester exchange intermediate, **LSKFA**-MES, was detected (Fig. S2†). Small amounts of desulfurization side products derived from the cysteinyl peptide and the ligation product were also detected. This side reaction was likely promoted by the presence of a larger amount of TCEP. It is therefore more desirable to conduct the ligation reaction at a low concentration of TCEP to suppress the desulfurization side reaction. Although some side products of disulfide formation may form during prolonged reaction time, a final brief treatment with additional TCEP would reduce all the disulfide-linked side products.

We then continued to demonstrate the native chemical ligation using other CASET-Weinreb amide peptides having different C-ter residues (Leu, Phe, Ser and Val). For Leu, Phe and Ser, the ligation reaction at 42 °C gave >95% yield in 1 h as shown by analytical HPLC (Fig. 4A–C). In all three cases, there was also a small amount of unreacting de-CH_2CONH_2 species, **LSKFXaa**-$NHCH_2CH_2SH$, left-over in the reaction. For Ser, about 5% epimerization was observed. However, the epimerization pre-existed in the CASET-Weinreb amide peptide (Fig. 4C), which most likely occurred during the coupling of Fmoc-Ser(O*t*Bu)-OH, which is well known to be very prone to racemization in the activated form,[12] onto the linker-resin. So the epimer product in the ligation reaction was mostly due to

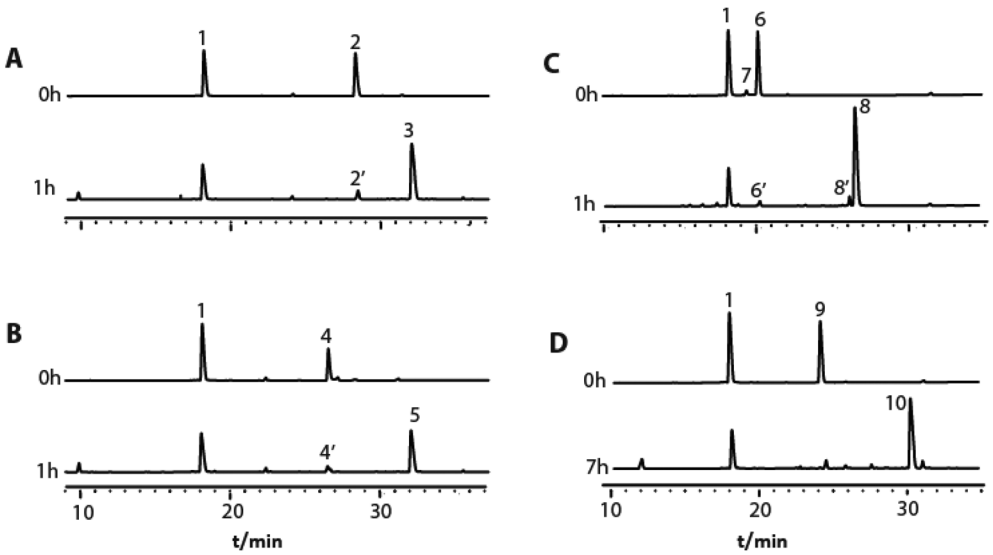


Fig. 4 Analytical HPLC monitoring of the ligation reaction of **LSKFXaa**-CASET Weinreb amide peptides at pH 8.5 and 42 °C (5 mM TCEP, 2% MESNa except for D for which $HSCH_2COOEt$ was used). Panel A: Xaa = Phe; B: Leu; C: Ser; D: Val. Gradient: 0–40% buffer B in buffer A for 40 min. Peak 1: **CFAEKSLK**-NH_2; peaks 2, 4, 6 and 9: **LSKFXaa**-CASET-Weinreb amide with Xaa = F, L, S and V respectively; peaks 3, 5, 8, and 10: corresponding ligation products; peaks 2′, 4′ and 6′: mostly unreactive impurities **LSKFXaa**-$NHCH_2CH_2SH$; peak 7: **LSKFdS**-CASET-Weinreb amide; peak 8′: epimer ligation product **LSKFdS-CFAEKSLK**-NH_2. See the ESI† for experimental procedures and compound characterization data.

the unseparated D-Ser CASET-Weinreb amide peptide ligating with the cysteinyl peptide. The Val CASET-Weinreb amide also gave the ligation product in good yield albeit at a much slower rate. Still, a yield of >85% was obtained after 7 h at 42 °C (Fig. 4D), when using ethyl mercaptoacetate as the thiol additive. Val–Cys is one of the least favored junctions for NCL, and as it is shown in a systematic study by Dawson *et al.* the ligation reaction of a C-ter Val-alkylthioester peptide at 37 °C gave 60% of the product after 48 h.[2b] It is therefore remarkable for an amide-based thioester precursor to have this high level of reactivity in NCL.

Conclusions

The results from this work and from Dr Yoshiya's work corroborate the hypothesis that the Weinreb amide derivatives are excellent electrophiles which can undergo an efficient thiol-initiated intramolecular nucleophilic acyl substitution reaction to form thioesters *in situ* for NCL. In particular, CASET-Weinreb amides are arguably one of the most reactive of all the amide-based thioester precursors that utilize the intramolecular *N*-to-*S* acyl transfer principle. The SET- and CASET-Weinreb amide linkers are easily accessible synthetically and standard Fmoc SPPS protocols are used for peptide synthesis. There is no need for any on-resin or post-SPPS activation procedures to generate thioester peptides since the peptide Weinreb amides are used directly for NCL. A basic pH promotes the ligation reaction of these Weinreb amide peptides, a behavior that is also shared by normal thioester peptides. Altogether, the high reactivity and easy accessibility of these novel thioester surrogates make them highly useful in synthetic protein chemistry. Application of this new technique in the synthesis of histone proteins containing various post-translational modifications is currently pursued in our lab.

Acknowledgements

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