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Homoserine and Threonine Peptide Assembly

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Abstract Drawing on our recent success with reagent-less peptide bond formation through serine-based assembly reactions in organic solvent, their range has been expanded to threonine and homoserine (an aspartic acid precursor) in the *N*-terminal peptide. Amino acid scope available at the assembly *C*-terminus includes bulky residues not amenable to classical ligation methods, such as cysteine-based NCL in aqueous media. The method was used to assemble a snakebite toxin-neutralizing peptide from opossums.

Key words amides • amino acids • acylation • nucleophilic substitution • trans-esterification

We recently described peptide assembly reactions based on *N*-terminal serine peptides reacting with amino acids mildly activated at their *C*-termini (Scheme 1).¹ Groups effective for such activation include fluorinated alkyl and cyanomethyl esters that are installed by carboxylate *O*-alkylation. The appeal of serine peptide assembly (SPA) includes preparative-scale segment couplings at high concentration in organic solvent. Unlike conventional peptide synthesis with acyl activation reagents, SPA has zero racemization risk at *C*-terminal residues owing to a trans-esterification/acyl transfer reaction pathway analogous to native chemical ligation (NCL). Its *N*-terminal Ser residue is more common in protein sequences² than the rare Cys (6.9% vs. 1.7%) used in NCL, however.

Scheme 1 Past work on serine peptide assembly (SPA)

Recognition that two other hydroxyl amino acids, threonine (Thr) and homoserine (Hse), could follow the same reaction pathway stimulated the work reported here. They also install residues (Thr - 5.8%; Asp - 5.3%) much more prevalent in

protein sequences than Cys. In combination, these three residues expand by 10-fold over NCL the scope of peptide bonds that can be created by preparative segment condensations. Considering threonine for a process analogous to SPA, its secondary alcohol might be less reactive in trans-esterification than the primary alcohol of serine. Yet, in early work on ligation of hydroxyamines with amino acid cyanomethyl esters, 1-amino-2-propanol ligates with valine in high yield.³ Likewise, homoserine has a 1,3-relationship of amine and alcohol that could reduce its reactivity relative to the 1,2-relationship in Ser. We have postulated that an internal H-bond gives special reactivity in trans-esterification to aminoalcohols. Ser has a 5-membered H-bonded ring and Hse has a 6-membered ring, which could make it less effective in trans-esterification. Further, acyl transfer in homoserine assembly must proceed through a 1,5 O→N pathway via a 6-membered ring transition state, which could be less favorable than the 5-membered ring transition state in serine assembly. Yet, our initial work showed that 1-amino-3-propanol ligates effectively.³ After assembly, it was envisioned that the alcohol of Hse could be oxidized to create an unprotected aspartic acid residue (Scheme 2). This idea is preceded in what is formally a methionine ligation,⁴ wherein homocysteine is used in NCL, followed by a selective post-ligation methylation reaction to convert it to methionine.

Scheme 2 Homoserine peptide assembly and oxidation to Asp

Initial screening of Thr assembly with acetyl alanine showed the hexafluoroisopropyl (HIP) ester we earlier found to be preferred undergoes assembly at ambient temperature with threonine methyl ester in high yield (Scheme 3). It was surprising, though, that the cyanomethyl (CM) ester also studied earlier is about as effective, while they have different reactivity in SPA. The

trifluoroethyl (TFE) and pentafluoropropyl (PFP) esters react slower.

Scheme 3 Threonine peptide assembly with Ala esters

Reaction of methyl threoninate, at 1 M concentration, with a 50% excess (recoverable) of Ac-Ala-OHIP active ester, was optimized regarding solvent, catalyst, time, and temperature. A variety of potential catalysts (20 mol %) were examined, including triazabicyclodecene (TBD), hexafluoroisopropyl alcohol, pyridine, and phenol. TBD, a trans-esterification catalyst,⁴ increased the rate substantially but also resulted in a product **4** incorporating two moles of active ester via a second trans-esterification. Their ratio was 5:4. Acetic acid proved most effective as a catalyst, yielding a 16 h reaction time compared to 48 h in its absence.

Figure 1 Side-product from threonine assembly followed by trans-esterification

With AcOH as catalyst, several aprotic and non-polar solvents were examined that our earlier work showed provide the fastest rates, though several were poor solvents for the product and showed incomplete reaction. THF and ethyl acetate are the most effective solvents examined. Foreshadowing later work, DMF required over twice as long for reaction completion. For some peptide reactants and products that are insoluble in THF or ethyl acetate, however, this sacrifice in rate in the name of solubility (at admittedly high concentrations) may be essential.

Table 1 Threonine peptide assembly with HIP esters

Product	Ac-AA-OHIP	Time (h)	Yield (%)
3	Ala	2	92
3	Ala	4	96
5a	Val	5	63
5b	Pro	5	83
5c	Phe	5	90
5d	Ile	5	67
5e	Leu	5	70

We earlier showed that SPA reactions respond well to microwave heating at 70 °C, so threonine assembly under these conditions was examined as shown in Table 1. The prototype assembly with alanine was nearly complete in 2 h, as doubling the reaction time barely increased the yield. Some scope of C-

terminal residues that participate in threonine assembly was examined. For a variety of hindered and unhindered amino acids, these reactions are reliable. The good yield with proline is notable considering a Pro-Thr peptide bond will be assembled in the synthesis of a valuable target (vide infra). Procedures are provided for preparation of **3** using both the ambient and microwave heating protocols.⁵

For the study of homoserine (Hse) assembly, a model reactant was required that was stable under the reaction conditions, given the strong tendency of homoserine derivatives to cyclize to homoserine lactone as well as the strong tendency of dipeptide esters to cyclize to diketopiperazines. Unsuccessful routes thwarted by these side-reactions will not be detailed. The reagent ultimately used was prepared as shown in Scheme 4. A *tert*-butyl amide was chosen to block the C-terminus, as *tert*-butyl groups can be removed from amides with metal catalysis.⁶ The *tert*-butyl amide of Phe was produced in high yield by HATU coupling with Boc-Phe and subsequent Boc removal. A conventional coupling with a commercial Hse derivative **6** gave a dipeptide **7**. The benzyl ether was hydrogenolyzed, and Boc removal was performed at low temperature for a minimal reaction time to suppress lactonization seen otherwise. The free amine was reprotected with a Cbz group that could be removed under completely neutral conditions. The Cbz derivative was carefully purified and then deprotected by hydrogenolysis to give the hydroxyamine (Hse-Phe-NHtBu, **8**) in 52% overall yield from **6**.

Scheme 4 Preparation of a Hse dipeptide model system

Initial investigations of homoserine assembly were performed at room temperature, as summarized in Table 2. As before, the concentration was 1 M with a 50% excess of ester. Due to limited solubility of homoserine peptides in the favored nonpolar organic solvents, DMF was used for these reactions. With the most active ester, Ala-OHIP, the yield of the desired product was modest owing to the formation of a trans-esterification product analogous to **4**. This installs a second alanine as the homoserine ester. Realizing that the unhindered alanine residue and most reactive HIP ester were likely combining to cause this over-reaction, two other esters were examined, CM and TFE. These less reactive esters gave the desired product in excellent yields, though of course the reactions were slower.

Two other amino acid HIP esters were examined under ambient conditions, and they gave moderate yields of tripeptides owing to the formation of side products. A significant steric effect is seen with phenylalanine versus the hindered valine, which requires a far longer reaction time.

Table 2 Homoserine peptide assembly at room temperature

Product	Ac-AA-OR	Time (h)	Yield (%)
9a	Ala-OHIP	10	40
9a	Ala-OTFE	50	84
9a	Ala-OCM	30	89
9b	Phe-OHIP	12	53
9c	Val-OHIP	84	46

Success in homoserine peptide assembly was also achieved using microwave heating at 70 °C, and some scope of the C-terminal residues was established, as summarized in Table 3. Reactions are of course significantly faster, and yields were comparable to those at ambient temperature. Use of the CM ester, which is more easily prepared than the HIP ester, required doubling the reaction time. This ester did usually show a lower yield of the assembly product, possibly due to formation of trans-esterification products. Again, the good performance of proline esters is notable.

Table 3 Homoserine peptide assembly of HIP and CM esters with microwave heating

Product	Ac-AA-OR	Time (h)	Yield (%)
9a	Ala-OCM	2	62
9b	Phe-OHIP	2	52
9b	Phe-OCM	2	38
9c	Val-OHIP	2	46
9c	Val-OCM	4	41
9d	Pro-OHIP	2	76
9d	Pro-OCM	4	52
9e	Leu-OHIP	2	34
9e	Leu-OCM	4	37
9f	Ile-OHIP	2	56
9f	Ile-OCM	4	34

With this process in hand, we examined the utility of homoserine as a surrogate for aspartic acid in peptide assembly, since the oxidation of homoserine creates an unprotected aspartate residue. Two assembly products, **9b** and **9c**, were treated under oxammonium salt oxidizing conditions that we

have earlier used for peptide alcohols (Scheme 5). After a 1 h reaction time, the aspartic tripeptide products were obtained in good yield. While the tolerance of all amino acids to these oxammonium salt oxidation conditions has not been established, because most reactive functional groups will be protected during this stage, the applicability of this reaction to many peptide sequences should be broad.

Scheme 5 Oxidation of homoserine peptides to achieve formal aspartic acid peptide assembly

Humans intrinsically fear snakebite, but some animals show natural resistance to snake venoms, both neurotoxic and hemorrhagic. The rivalry between snake and mongoose is the stuff of literature; the latter has an acetylcholine receptor that is not much affected by neurotoxic venoms and a serum factor that deactivates hemotoxins. Marsupials such as opossums have innate resistance to animal venoms and toxins that has been traced to a serum component called lethal toxin neutralizing factor (LTNF), a 68 kDa protein.⁷ Further, peptides derived from the N-terminal 10-15 residues of LTNF retain much of its activity; LTNF-10 has the sequence LKAMDPTPPL.⁸ Biotechnology has been used to produce LTNF-derived peptides,⁹ but we viewed LTNF-10 as an excellent proving ground for chemical synthesis using threonine peptide assembly. It involves a challenging junction with a C-terminal proline that is hindered and poorly reactive in native chemical ligation but has good performance in assembly.

LTNF-10 was prepared from two sub-peptides, LKAMDP and TPPL. Using Fmoc SPPS on 2-chlorotrityl resin, these two fragments were synthesized and freed from the support, the former in side-chain and N-terminus protected form (Scheme 6). The N-terminal fragment was converted to HIP ester **11** using a carbodiimide coupling. While we typically prefer to form such fluorinated esters using carboxylate O-alkylation with a fluorinated triflate owing to the inability of that step to trigger racemization of the C-terminus, racemization is not an issue here - proline is widely recognized as a racemization-resistant residue. The C-terminal fragment was converted to ester **12** by O-alkylation with benzyl bromide and Fmoc removal.

Scheme 6 Synthesis of LTNF-10 by threonine peptide assembly

The assembly of **11** and **12** was initially studied in THF. Reactant solubility was poor at a 0.25 M concentration, but reaction still proceeded, albeit giving only a 25% yield of **13** over 5 d at ambient temperature. This assembly did not respond to microwave heating as others have, giving **13** in only 10% yield after 5 h. The solvent was changed to DMF, which we have

earlier used when reactant solubility is an issue. Here, full dissolution could be achieved at a 0.33 M concentration, and reaction proceeded in a 30% yield over 5 d at ambient temperature. As in our past experience with assemblies, the unreacted starting materials are readily recovered. A straightforward three-step process deprotects the assembly product **13**, giving LTNF-10 in 54% overall yield, whose structure was verified by mass spectrometry and purity was verified by HPLC.

Discussion

This work reports peptide assembly with two additional hydroxyl amino acids, threonine and homoserine, the latter enabling segment condensations to introduce aspartic acid at the C-terminal side of assembly junctions. The generation of homoserine peptides in KAHA native chemical ligation has been reported by Bode.¹⁰ However, because that process yields unprotected peptides, it offers limited potential for oxidation of the homoserine at the ligation junction to a native aspartic acid residue. Threonine (and serine) peptide ligation technologies are also known, but typically for unprotected peptides in dilute denaturing aqueous media.¹¹ Combined with past work on serine peptide assembly (SPA),¹ hydroxylated amino acid assembly reactions are now applicable to ca. 18% of the residue positions among known protein sequences. This feature should facilitate their use in assembly of a broad range of targets.

Our long-term goal for hydroxylated amino acid assembly is the preparative production of peptide therapeutics via segment condensations. Segments can be prepared by SPPS, which is more reliable with relatively short sub-sequences, or other technologies under development.³ The manufacture of the 36 amino acid HIV drug enfuvirtide (Fuzeon®) by Trimeris/Roche is the pinnacle achievement of synthetic production of therapeutic peptides, and uses SPPS methods with following segment condensations.¹² Establishing methods for threonine assembly makes its application to LTNF-10 pertinent even though it could also be made efficiently by conventional SPPS.

The utility of threonine peptide assembly was demonstrated in the preparation of a useful target with a particularly challenging Pro-Thr junction. As peptide assembly technology matures, such demanding junctions should become more synthetically tractable. As in our past work on serine assembly, the sustainability of threonine and homoserine assembly is high owing to reagent-less coupling reactions at high segment concentrations.

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Supporting Information

Supporting information for this article (experimental procedures and NMR spectra, 117 pages) is available online at XXXXX

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- (5) **Ac-Ala-Thr-OMe (3). A:** Threonine methyl ester (1 equiv) was dissolved in THF (1 M) and set to stir at room temperature. Acetyl alanine HIP ester (1.5 equiv) was added, followed by AcOH (0.2 equiv). The reaction was monitored by TLC. After completion, the mixture was concentrated in vacuo to afford a crude product that was purified by column chromatography. **B:** Threonine methyl ester (1 equiv) was added to a microwave reaction vessel and dissolved in THF (1 M). Acetyl alanine HIP ester (1.5 equiv) was added, followed by AcOH (0.2 equiv) and sealed under air. The reaction was irradiated at 70 °C (300W, 5 min ramp time, 250 psi max, with stirring on). The mixture was concentrated in vacuo to afford a crude product that was purified by column chromatography. Protocol A: 16 h (0.1508 g, 96%). Protocol B: 4 h (0.1606 g, 96%). Mp: 146-149 °C. $[\alpha]_D^{25}$ -56.0 (c 0.519, MeOH). IR (neat): 3302, 3158, 3086, 2996, 2950, 2916, 2868, 2758, 1753, 1641, 1556, 1528, 1440, 1379, 1338, 1313, 1267, 1209, 1177, 1151, 1121, 1082, 1065, 1040, 1007, 992, 953, 937, 917, 896, 844, 778, 750, 699, 649, 602, 529, 460, 449 cm⁻¹. ¹H NMR (400 MHz, CDCl₃) δ 6.62 (d, *J* = 7.3 Hz, NH), 4.68 - 4.52 (m, 2H), 4.41 - 4.31 (m, 1H), 3.78 (s, *J* = 6.2 Hz, 3H), 2.01 (s, *J* = 8.4 Hz, 3H), 1.92 (br s, OH), 1.45 (d, *J* = 7.0 Hz, 3H), 1.22 (d, *J* = 6.5 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 173.0, 171.4, 170.8, 68.3, 58.0, 52.8, 49.3, 23.3, 20.2, 19.0. HRMS calcd. for C₁₀H₁₉N₂O₅ [M+H]⁺ 247.1288, found 247.1286.
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