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Fmoc–Based Synthesis of Glycolate Ester Peptides for the Assembly of *de novo* Designed Multimeric Proteins Using Subtiligase

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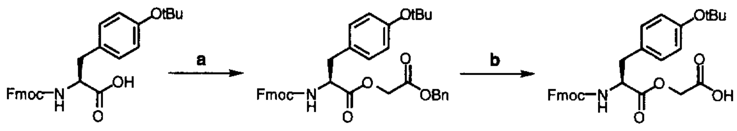
Abstract: An automated method utilizing Fmoc-protected amino acids has been developed for the synthesis of glycolate ester peptides as substrates for subtiligase. As a test of this methodology, peptide esters containing α-helical sequences that specify the association into 3- and 4-helix bundles were synthesized, and used to explore the suitability of subtiligase as a tool for the generation of covalently linked bundles. The feasibility of creating *de novo* designed, single-chain or cyclic structures from simple, readily-synthesized modules has been demonstrated.

Subtiligase, a double mutant of the serine protease subtilisin BPN', is capable of joining peptides via efficient main chain amide bond formation in aqueous solution.[1] Such "peptide ligation" has been developed as a tool for the complete synthesis and semisynthesis of proteins and for the cyclization of peptides.[2-4] Substrates for the enzyme that correspond to the N-terminal peptide are glycolate aminoacylamide esters of the desired sequence. These substrates react with the active-site cysteine of the enzyme to form a thioacyl intermediate. Ligation is achieved upon aminolysis of this intermediate by the α-amino group of the peptide corresponding to the C-terminal sequence. Herein is described an improved, Fmoc-based method for synthesizing glycolate ester peptides, and the synthesis of α-helical peptide esters using this method. The successful covalent linkage of these peptide esters into bundles using subtiligase has demonstrated the feasibility of generating *de novo* designed proteins from preassociated elements of secondary structure.

In previous work, peptide ester substrates were prepared by solid-phase peptide synthesis using α-BOC-protected amino acids, requiring the use of HF or other strong acids for the final side chain deprotection.[2] Clearly it would be desirable to devise a synthetic method that uses the more convenient Fmoc-protected amino acids. A method for the synthesis of peptide ester substrates using Fmoc chemistry in a completely automated, solid-phase format has been developed and used to synthesize peptide esters of up to 36 residues in length. The method relies on the initial solution-phase synthesis of the protected glycolate ester of the C-terminal or P_1 amino acid in the target sequence (Fig. 1). The glycolate ester can then be incorporated into the peptide chain by coupling this key intermediate at the desired location during automated, stepwise solid-phase synthesis of the target sequence. Since the P_1 residue for all peptides in this study was tyrosine, the tyrosyl glycolate intermediate **3** ('Y–glc') was synthesized (Fig. 1). The carboxylate salt of Fmoc-L-Tyr(OtBu)-OH was treated with benzyl 2-bromoacetate[5] to yield ester intermediate **2**. Cleavage of the benzyl ester via catalytic reduction afforded **3** in 75% overall yield for both steps.[6] This procedure could be carried out with any appropriately protected amino acid.

Three peptides, designated CoilSer1, α_1B-G2, and α_1B-G3 (Fig. 2), were synthesized, in which the desired glycolate ester was installed in the peptide chain by the incorporation of **3** using a standard peptide coupling reaction. The syntheses were carried out using automated, stepwise solid-phase methodology on a Milligen 9050 peptide synthesizer with standard Fmoc chemistry using PAL resin (Milligen)[7] on a 0.2 mmol scale. All couplings were performed using the appropriately protected Opfp esters of amino acids and HOBt as an additive, except in the case of Trp, Ser, and **3**, which were coupled as the appropriately protected free acids (Trp as the side chain Boc derivative) using HATU as a coupling agent and DIEA as the base. All residues were single coupled, except in the case of the initial Lys, Ser, and **3**, which were double coupled. Piperidine deprotection times for each step after the coupling of **3** were reduced from the standard 7 minutes to 3 minutes. This was done to minimize the possiblity of hydrolysis or aminolysis of the ester, or cleavage due to diketopiperazine formation after coupling the first amino acid to resin-bound **3** (in this case Pro). The peptides were cleaved from the resin and deprotected using TFA containing 2–3 equivalents of triisopropylsilane per protecting group for 2–4 hours, precipitated with diethyl ether, and purified by reverse-phase HPLC. The identity of the purified products was confirmed by electrospray ionization mass spectrometry (ESI–MS).[8]



1 **2** **3**

FIGURE 1. Synthesis of protected tyrosyl glycolic acid intermediate **3**. Reagents and conditions: (**a**) 1 equiv. of benzyl-2-bromoacetate,[5] 1 equiv. of DIEA, THF, 8 hr, 77%. (**b**) 10% Pd/C (5 mol% Pd), H_2 (atm), THF/H_2O (3:1), 7 hr, 98%.

P_1

CoilSer1	H_2N–MKGGEWEALEKKLAALESKLQALEKKLEALEHGGPYglcK–$CONH_2$
α_1B-G2	H_2N–MKGGELEELLKKLKELLKGGPYglcK–$CONH_2$
α_1B-G3	H_2N–MKGGGELEELLKKLKELLKGGGPYglcK–$CONH_2$

FIGURE 2. Sequences of the three glycolate peptide esters synthesized in this study. The underlined sequences indicate the residues recognized by subtiligase. These correspond to residues P_1' to P_4' at the N-terminus, and P_4 to P_1 at the C-terminus. The P_1 tyrosine residue is indicated for CoilSer1. The glycolic acid residue is abbreviated as 'glc'. The ester linkage occurs between the Y and glc residues.

CoilSer1, α_1B-G2, and α_1B-G3 are good substrates for subtiligase, and can be assembled into covalently-linked multimeric structures. For each of the three peptides, the four N-terminal and four C-terminal residues correspond to recognition sequences for subtiligase, and the sequence between these recognition elements corresponds to an α-helix. In the case of CoilSer1, this α-helical segment also causes the individual peptides to associate into an antiparallel 3-helix bundle,[9,10] while in the case of α_1B-G2 and α_1B-G3, the α-helical segment specifies the association into an antiparallel 4-helix bundle.[11] Given this arrangement, individual peptides within the bundles should be capable of ligating to each other up to two times for CoilSer1, or up to four times in the case of α_1B-G2 and α_1B-G3, to form any of a number of potential multimeric (including cyclic) products (Fig. 3). Ligations were carried out at room temperature in 100 mM Tricine pH 8.0 at peptide concentrations at which CoilSer1 exists as a 3-helix bundle and α_1B-G2 and α_1B-G3 exist as 4-helix bundles.[12] Products were purified by reverse-phase HPLC, and characterized by ESI–MS.[13] Table 1 shows that for ligase reactions containing CoilSer1, α_1B-G2, or α_1B-G3 as substrates, 32%, 15%, and 50%, respectively, of the starting material was converted to ligated products (the remainder being converted to hydrolyzed monomer). These yields are good to excellent considering that hydrolysis of the thioacyl enzyme intermediate competes with the aminolysis process that produces ligated products. For comparison, it should be noted that 'tester' peptides AcNH–GGPYglcK–$CONH_2$ and H_2N–MKGG–$CONH_2$ (corresponding to the C and N termini of the helical peptides) were ligated in 55% yield using a 1:1 stoichiometry.[14]

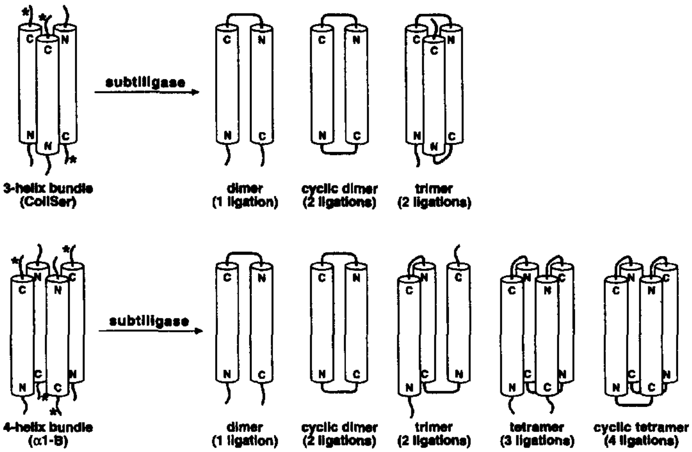


FIGURE 3. Schematic representation of possible products from the ligation of antiparallel 3-helix and 4-helix bundles. Cylinders represent α-helical regions within the peptides, and curvy bold lines indicate recognition sequences at the N and C termini capable of undergoing ligation to form an intervening loop (asterisks indicate the locations of the ester linkages). Adjacent N and C termini from different peptides can potentially be ligated up to two times for the 3-helix bundle and up to four times for the 4-helix bundle to form any of a number of single chain or cyclic products.

The products obtained from the ligation reactions demonstrate the feasibility of covalently connecting elements of secondary structure that self-associate into a defined higher order structure. The concept of using the preassociation of secondary structure elements followed by covalent linkage to generate a specific desired tertiary structure has been applied previously in natural and semisynthetic systems.[15] It is desirable to synthesize *de novo* designed structures by this technique not only because it allows for control of the spatial arrangement of subunits, but also because the construction of complex protein structures, built from readily-synthesized components, is simplified. Further, the covalently linked linear or cyclic products are expected to be more stable than the associated but unlinked subunits.

peptide	noncovalent structure	product distribution (% of total)			% ligated	fraction trimer
		monomer	dimer	trimer		
CoilSer1	trimer	68	26	6.2	32	0.19
α_1B-G2	tetramer	85	15	–	15	–
α_1B-G3	tetramer	50	39[a]	11	50	0.22

TABLE 1. Yields of products obtained from subtiligase reactions. The percentage of (hydrolyzed) monomer, dimer, and trimer generated for each were calculated as the A_{214} peak area from HPLC traces versus the total peak area for the products. The total percentage of products ligated was calculated as the sum of dimer and trimer yields, and the fraction trimer was calculated as the ratio of trimer yield to the sum of dimer and trimer yields. [a]includes 4.7% cyclic dimer.

Table 1 shows the relative amounts of the products obtained from ligations of CoilSer1, α_1B-G2, and α_1B-G3. In the case of CoilSer1, the target trimeric structure was obtained, accounting for nearly 20% of the ligated products, the remainder being dimer.[16] For the α_1B-G2 ligation reaction, dimer was obtained in modest yields. The peptide α_1B-G3 contains an additional Gly residue in each loop sequence compared with α_1B-G2. The results of the α_1B-G3 ligation reaction show that the inclusion of these additional Gly residues improved the yield of dimer significantly, and increased the total amount of ligated products from 15% to 50%. Furthermore, the change enabled the production of trimer and cyclic dimer, products not obtained from the α_1B-G2 ligation reaction. This result suggests that additional Gly residues between the 4-residue recognition sequence and the helical region of the peptide act as spacers that either *i.*) alleviate unfavorable steric interactions between the enzyme and the structured helical bundle, or *ii.*) provide additional flexibility that may be required to bring the recognition sequences of two adjoining helices into the enzyme active site simultaneously. In this way, the presence of the Gly spacers could increase the rate of ligation relative to hydrolysis of the thioacyl enzyme intermediate, thereby increasing the total number of ligations that occur during the course of a reaction. The result would be an increase not only in the yields of products, but also in the number of ligations per bundle. Inclusion of additional spacer elements in these sequences should make possible the synthesis of the single chain and cyclic 4-helix bundles.

In summary, a method has been developed that allows the synthesis of glycolate peptide esters as substrates for subtiligase using standard Fmoc chemistry in a completely automated format. The desired peptide esters can be synthesized rapidly and cleanly, and have been shown to act as substrates for subtiligase, despite the existence of considerable secondary structure. The use of the Fmoc-based synthesis method promises to extend the utility of subtiligase as a tool in the complete and semisynthesis of proteins and the cyclization of peptides. In addition, a means has been established to assemble secondary structural elements into desired higher order structures in a controlled fashion from smaller, readily synthesized units. This approach has the potential to greatly facilitate the assembly of *de novo* designed protein structures.

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5. The benzyl 2-bromoacetate used in this study was prepared in 57% yield by treatment of bromoacetic acid with DCC (1.2 equiv.), DMAP (0.3 equiv.), and benzyl alcohol (2 equiv.) in THF for 16 hr. Benzyl 2-bromoacetate is also available commercially from Aldrich.
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8. CoilSer1– expected: 4181.3, found: 4181.4. α_1B-G2– expected: 2658.6, found: 2658.3. α_1B-G3– expected: 2772.6, found: 2772.5.
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12. The CoilSer1 reaction was run at a peptide concentration of 2.5 mM and a subtiligase concentration of 3.0 μM for 2.5 hr. The α_1B-G2 and α_1B-G3 reactions were run at a peptide concentration of 2.0 mM and a subtiligase concentration of 5.8 μM for 3.0 hr. Reactions were terminated by the addition of neat TFA to a final concentration of 2%(v/v). In each case, all or nearly all of the unligated peptide ester had hydrolyzed after the specified reaction time. Control reactions in which subtiligase was omitted yielded only hydrolyzed and unhydrolyzed monomer.
13. CoilSer1– hydrolyzed dimer expected: 7974.6, found: 7974.4; hydrolyzed trimer expected: 11952.6, found: 11954.3. α_1B-G2– hydrolyzed dimer expected: 4929.2, found: 4927.7. α_1B-G3– hydrolyzed dimer expected: 5157.2, found: 5158.3; hydrolyzed trimer expected: 7726.8, found: 7728.6; cyclic dimer expected: 5139.2, found: 5140.8.
14. The tester peptides were synthesized by the same methods described for the full length peptides. Acetylation was carried out using 0.5 M acetic anhydride and 0.5 M pyridine in DMF.
15. See for example: (a) Wuttke, D. S.; Gray, H.; Fisher, S. L.; Imperiali, B. *J. Am. Chem. Soc.* **1993**, *115*, 8455-8456. (b) Hoogerhaut, P.; Kerling, K. E. T. *J. R. Neth. Chem. Soc.* **1981**, *100*, 215-216. (c) Hoogerhaut, P.; Kerling, K. E. T. *J. R. Neth. Chem. Soc.* **1982**, *101*, 246-253. (d) Vogel, K.; Chmielewski, J. *J. Am. Chem. Soc.* **1994**, *116*, 11163-11164. (e) Graf, L.; Li, C. H. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 6135-6138.
16. This result is obtained for ligation reactions performed at CoilSer1 concentrations of 2.5-10 mM. As the peptide concentration is lowered to 1 mM and below, yields of dimer and trimer decrease; at a peptide concentration of 10 μM, no ligated products can be detected by HPLC.

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