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Orthogonal Coupling of Unprotected Peptide Segments through Histidyl Amino Terminus

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Abstract. We report a new scheme for orthogonal coupling of unprotected peptide segments through the anchimeric assistance of a proximally located histidine at the amino terminus.

Orthogonal coupling is a chemoselective method for amide bond formation.[1] It holds promise for preparing large and complex peptides using their unprotected forms as building blocks. A common strategy is to utilize a strong nucleophile at the N^{α}-amine terminus to capture an activated acyl moiety under aqueous conditions thus allowing a proximity-driven intramolecular acyl shift to form the peptide bond.[1-5] So far, a thiol at the N-terminal cysteine or cysteinyl analogs has been used as nucleophile in the capture reaction by thiazolidine,[3] transthioesterification[1, 4] and acyl disulfide formation.[5] In order to extend the application of the orthogonal coupling scheme to non-cysteinyl N-terminal amino acids, we examine other nucleophiles as potential capture devices. An alternative nucleophile is the imidazole group of the histidine side chain which is known as a weak base at acidic pH and can catalyze acyl transfer in the enzymatic process.[6] In this paper, we describe an orthogonal coupling scheme that uses a histidyl amino terminus for the synthesis of peptides without protecting groups.

In our scheme (Fig. 1), the amine segment contains a histidine at the N-terminus. The acyl segment contains a C-terminal thiocarboxylic acid. Both segments were obtained in their unprotected forms through Boc-benzyl chemistry from solid-phase synthesis. No significant coupling reaction occurred when the segments were mixed together. However, we envisioned that upon addition of a suitable thiophilic promoter, such as an aryl disulfide, the acyl segment would be captured by the imidazole of the N-terminal histidine, subsequently leading to N^{im} to N^{α}-acyl transfer to form a peptide bond.

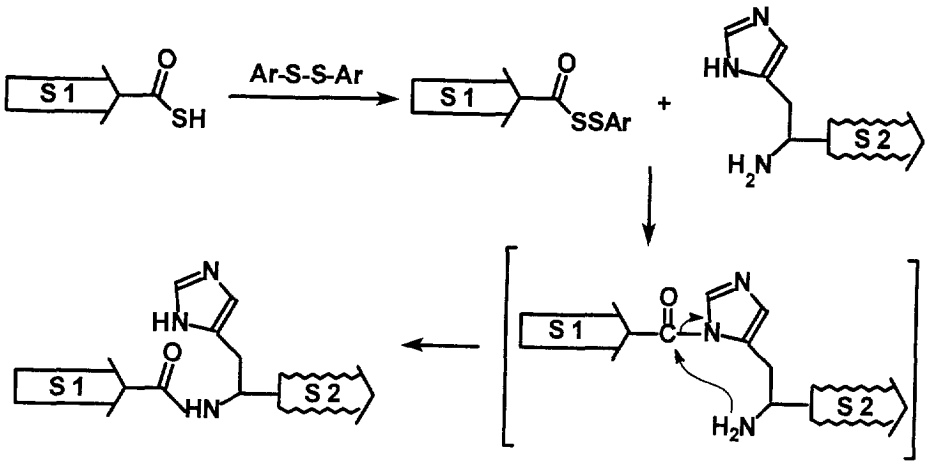

Fig. 1 Proposed scheme for orthogonal coupling of unprotected peptide segments.
S1: a peptide thiocarboxylic acid; S2: an N-terminal histidyl peptide

The selectivity of the orthogonal coupling between N-terminal histidyl peptides and C-terminal thiocarboxylic acid was first studied by a model peptide, HGKA **1**, with simple thiocarboxylic acids: Boc-Gly-SH, Boc-Ala-SH and Boc-Leu-SH, using 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) as a thiolphilic promoter. A control peptide, AGKA **2**, with alanine at the N-terminus, was used for comparison. The desired ligated product and side products resulting from ϵ-monoacylation and α,ϵ-diacylation were monitored by RP-HPLC and compared with authentic samples prepared independently by solid-phase peptide synthesis. At pH 5, 5.7 and 6.5 in aqueous solution, Boc amino thiocarboxylic acids activated by Ellman's reagent preferentially reacted with the N^{α}-amino group rather than with the N^{ϵ}-amino group of lysine in tetrapeptides **1** and **2**. At pH 5.7, a higher selectivity of α-acylation was achieved in the N-terminal histidyl peptide **1** with the assistance of the imidazole side chain (Table 1). Furthermore, acylation of **1** which was completed in 2 hr was much faster than acylation of the alanyl peptide **2**. The activated acyl moiety was readily hydrolyzed, resulting in a low yield in the case of nonhistidyl peptide **2**. At pH 6.5, similar results were obtained. A competitive experiment was used to determine the selectivity of an activated thiocarboxylic acid towards two N^{α}-amines and one N^{ϵ}-amine of lysyl side chain. Equal molar concentrations of **1** and **2** were mixed and allowed to react with Boc-Gly-SH activated by Ellman's reagent. The main product in >90% yield was Boc-GHGKA and less than 10% of Boc-GAGKA was found. This confirmed the selective reactivity of HGKA **1** with activated Boc amino thiocarboxylic acid.

To test the orthogonal coupling of histidyl peptides, we synthesized SKALHGKLA by coupling two segments between Leu and His. The peptide thiocarboxylic acid, SKAL-SH **3** was prepared by a modified procedure of

Table 1 Acylation Yield and Selectivity of Thiocarboxylic Acid to 1 and 2 Using Ellman's Reagent as Promotor in Aqueous Buffered Solutions Containing 50% DMF at pH 5.7 for 8 hr[7]

Thiocarboxylic acid	Coupling Yield (%)		Acylation Selectivity (α/ε)	
	HGKA 1	AGKA 2	HGKA 1	AGKA 2
Boc-Ala-SH	85	40	16	7
Boc-Gly-SH	82	50	9	5
Boc-Leu-SH	75	20	4	1
SKAL-SH	75	0	50	ND

Yamashiro.[8] The coupling between 3 and HGKA 1 in the presence of Ellman's reagent was performed at pH 5.7. DMF (50% by volume) was used to minimize hydrolysis of the activated acyl moiety. The desired coupling product was obtained in a 75% yield and confirmed by MALDI-MS (Found: 810.5, calcd for $[M+H]^+$: 810.9). The product was also compared with the authentic sample prepared independently. The major byproduct (less than 15%) was the hydrolyzed starting material, SKAL-OH. A 25 residue peptide was obtained by coupling 3 to a 21 residue N-terminal histidyl peptide, HLSSMERVEWLRKKLQDVHNF (HF-21).Coupling was conducted under the same conditions as described above to give an isolated yield of 60 % based on SKAL-SH. The product was characterized by MALDI-MS (Found: 3054.3 ±1, calcd for $[M+H]^+$: 3053.5). The use of a large excess of the histidyl segment greatly improved the yield.

Activation of thiocarboxylic acid by Ellman's reagent yields acyl disulfide 4, thioester 5 and S-symmetric anhydride 6 according to literature (Fig. 2).[8] In our hands, the ratio of 4 to 5 was dependent on reaction conditions and acyl components. For example, in the activation of 3 with Ellman's reagent in 0.1 N acetate buffer at pH 4, three components were obtained in RP-HPLC. Two were identified by MALDI-MS as acyl disulfide, SKAL-SSR' 4 (Found: 631.5, calcd for $[M+H]^+$: 630.6), and the thioester, SKAL-SR' 5 (Found: 599.0, calcd for $[M+H]^+$: 599.6;

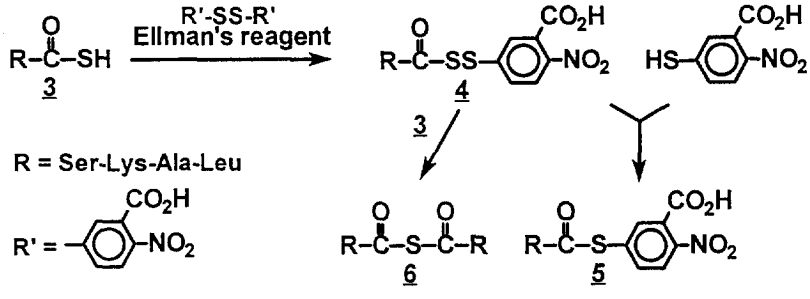

Fig.2 Possible activated species formed by treatment of thiocarboxylic acid with Ellman's reagent

R' = 5'-thio-2'-nitrobenzoic acid). We were unable to determine the third component due to its instability. When the reaction was prolonged for 15 min under the same conditions, the peptide thioester **5** was obtained as a major product, indicating that the acyl thioester was stable. At pH < 2, the main product isolated was acyl disulfide **4** . The formation of **5** was favored at higher pH. When using the peptide thioester **5** as the sole "activated" acyl moiety in orthogonal coupling with **1** in aqueous buffer at pH 6, less than 5% of coupling product was obtained in 18 hr, and 80% of the acyl thioester was hydrolyzed to its carboxylic acid. These results show that acyl disulfide is likely involved in the ligation of histidyl peptides. They also provide a plausible explanation for the selectivity of histidyl peptides over other peptides.

Our results show that N-terminal histidyl peptides can be exploited for orthogonal coupling of two unprotected segments with high regioselectivity. Whether our proposed scheme proceeds through a covalently linked N^{im}-acyl intermediate as shown in Fig. 1 is not clear because we have not been able to isolate this intermediate. However, the rapid hydrolysis of the thiocarboxylic acid implicated its existence due to the instability of the acyl imidazole intermediate. It is possible that regioselectivity is obtained simply because of anchimeric assistance of the proximal imidazole moiety at the ligation site. Such a possibility may open a new avenue for designing new orthogonal coupling schemes of unprotected peptide segments by placing a strong basic nucleophilic moiety at the N-terminus for achieving the required regiospecificity.

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7.Typically, 0.01 mmol model peptide, His-Gly-Lys-Ala and 0.01-0.02 mmol Ellman's reagent were dissolved in 1 ml of 0.2 N phosphate buffer (pH 5.7) and added to 0.01 mmol thiocarboxylic acid. After 8 hr, the reaction was monitored by RP-HPLC, the individual peak identified by MALDI-MS and compared with the authentic standard.
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