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Synthesis of human growth hormone-releasing hormone *via* three-fragment serine/threonine ligation (STL)†

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The synthesis of human growth hormone-releasing hormone (hGH-RH), by the chemoselective serine/threonine ligations (STLs) of three unprotected peptide fragments, is reported. To allow for the multiple-fragment ligation, we chose the Msz (*p*-(methylsulfinyl)benzyloxycarbonyl) group, which is compatible with the preparation of peptide salicylaldehyde esters *via* Fmoc-SPPS and readily removed by reductive acidolysis, to protect the serine and threonine residue at the N-terminus.

Revolutionary work in the development of chemical ligation methods enabling protein chemical synthesis[1] includes native chemical ligation (NCL),[2] which relies on the N-terminal cysteine residue to mediate a chemoselective union reaction of two side-chain unprotected peptide segments with a natural peptidic linkage (Xaa-Cys) at the conjunction site. Since cysteine is one of the lowest abundant amino acids (1.7% occurrence)[1c] in natural polypeptides and proteins, many efforts have been devoted to searching for the chemoselective ligation at other amino acid sites, including a menu of complementary cysteine-free ligation approaches.[3–5] These methods have provided important additions to the toolbox of chemical ligation.

In our own efforts we have recently reported a serine/threonine ligation (STL) enabling peptide/protein convergent synthesis.[6] A side-chain unprotected peptide segment with a C-terminal salicylaldehyde ester chemoselectively reacts with another side-chain unprotected peptide segment containing an N-terminal serine or threonine residue to afford an *N*,*O*-benzylidene acetal linked product. The resulting acetal group, upon treatment with acid, is removed to restore the natural peptide bond (Xaa-Ser/Thr) at the ligation site (Fig. 1). As serine and threonine residues are very abundant in natural polypeptides and proteins (12.7% occurrence in nature),[1c] serine/threonine ligation (STL) may offer new opportunities for convergent protein synthesis.

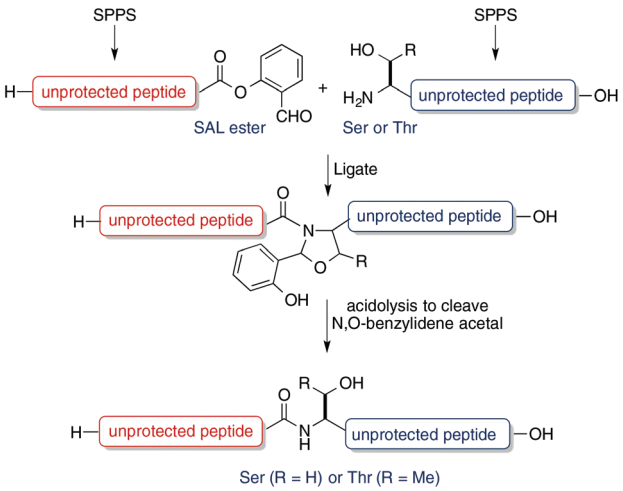

Fig. 1 Serine/threonine ligation (STL).

In this report, we describe the synthesis of human growth hormone-releasing hormone (hGH-RH) *via* serine/threonine ligation. hGH-RH is produced in the arcuate nucleus of the hypothalamus, and it stimulates growth hormone (GH) secretion by binding to the GH-RH receptor.[7] The acylated GH-RH analog tesamorelin has been approved by the US FDA in 2010 for the treatment of lipodystrophy in HIV patients under highly active antiretroviral therapy. Thus, we chose to synthesize acetylated hGH-RH in this study: Ac-YADAIFTN-SYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL-NH_2. Issues associated with the conventional stepwise synthesis of longer peptide therapeutics include the low overall yield and, more importantly, difficulties in obtaining the product in high purity under reproducible conditions, because the deletion sequences and residual protecting groups can cause contamination.[8] Thus, convergent synthesis *via* chemical ligation may offer advantages in this regard.[9] The synthesis of hGH-RH also served as a model to probe the conditions to realize $C \rightarrow N$ multi-fragment serine/threonine ligations.

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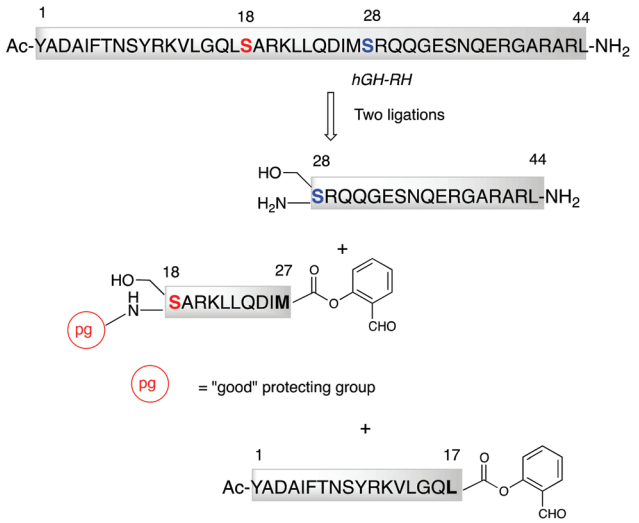

Fig. 2 Retro-synthetic scheme of hGH-RH *via* STL.

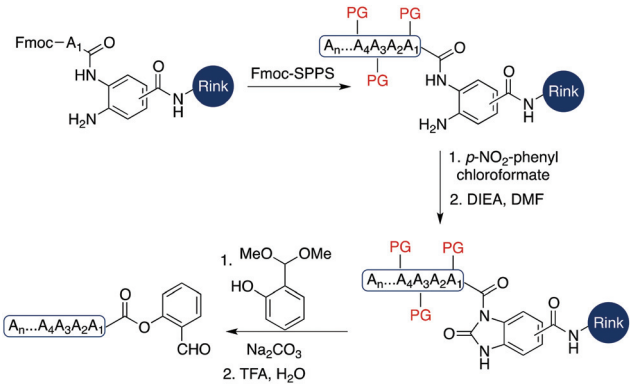

Fig. 3 Synthesis of peptide SAL-esters *via* Fmoc-SPPS.

A three-fragment ligation was devised to synthesize hGH-RH by ligating three segments at Leu[17]–Ser[18] and Met[27]–Ser[28], respectively (Fig. 2). We encountered a critical issue and challenge: a $C \rightarrow N$ multi-fragment ligation strategy required the N-terminal Ser/Thr of the middle fragment to be temporarily protected. Such a temporary "good" protecting group should be orthogonal to the solid phase peptide synthesis (SPPS) of the peptide salicylaldehyde esters, and readily removable for the subsequent ligation. Furthermore, a hydrophilic protecting group would be advantageous to help solubilize the peptide.

According to the retrosynthetic plan (Fig. 2), we first chose to use the Fmoc group as the N-terminal serine residue protecting group, although its hydrophobicity made it not ideal. To prepare the necessary peptide salicylaldehyde (SAL)-ester, we adopted an on-resin phenolysis of the Fmoc-SPPS generated peptide *N*-acyl-benzimidazolinone (Nbz).[10] Such a strategy could provide peptide SAL-esters efficiently and cleanly, without observing epimerization (Fig. 3).[6b] However, when we synthesized peptide *N*-acyl-benzimidazolinone (Nbz) with N-terminal serine protected with an Fmoc group prior to the on-resin phenolysis, we observed a significant amount (~25%) of the peptide product without the N-terminal Fmoc group. It was likely that the Fmoc group was removed by the excess of DIEA used during the cyclization step.[11,12] Thus, the Fmoc group was not a proper terminating group to serve our purpose.

We next started with using an azide group as the temporary protecting group (N_3-SARKLLQDIM-SAL ester). The azide group has been previously used in peptide synthesis.[13] It is rather stable, while readily removable under reducing conditions. However, during the synthesis of hGH-RH (18–27) with N_3-Ser[18](*t*Bu)-OH, two peaks with the desired molecular weight were observed, likely due to the epimerization.

As both Fmoc and azide were not "good" temporary protecting groups, we continued to explore the utility of Msz (*p*-(methylsulfinyl)benzyloxycarbonyl)[14] in the serine/threonine chemical peptide ligation. Msz for amino group protection is stable under both basic and acidic conditions, but readily cleavable under reductive acidolysis conditions *via* the Mtz group (*p*-(methylthio)benzyloxycarbonyl). According to the general procedure, we prepared the peptide SAL ester (Fig. 3). Surprisingly, when we checked the quality of the peptide (Msz-SARKLLQDIM)-Nbz prior to the on-resin phenolysis, a significant amount of the peptide product without Msz but with a molecular weight 16 Da higher was observed, which was presumed to be compound **4**. It is likely that the Msz group underwent oxygen exchange with the methionine residue (**2** ⇌ **3**),[15] thereby leading to the removal under acidolysis (Fig. 4).

To avoid the abovementioned problem, we decided to use a methionine sulfoxide residue for the synthesis of the peptide (18–27)-SAL ester. Thus, peptide Msz-SARKLLQDIM(O) SAL-ester **5** was synthesized with ease *via* on-resin phenolysis of peptide Nbz. Next, peptide SAL ester **5** and peptide **6** SRQQGESNQERGARARL-NH_2 were dissolved in pyridine acetate buffer (1 : 1, mole : mole). The progress of the reaction was monitored by LC-MS. After 13 h at room temperature, the pyridine acetate was removed and the reaction mixture was treated with TFA–H_2O to afford hGH-RH (18–44) (Fig. 5).

Next, we explored the condition to remove the Msz group. Some commonly used conditions (*i.e.* NH_4I–TFA–DMS,[16] TMSCl–DMS,[17] and TMSBr–thioanisole[18]) gave either a messy or no reaction. Gratifyingly, a cocktail of TMSOTf (1.0 M)–thioanisole (1.0 M)–TFA[19] cleanly removed the Msz group; at the same time, methionine sulfoxide was also reduced. When TMSOTf was reduced to 0.2 M, the cocktail was still effective. As a control, 0.2 M TMSOTf in TFA did not work. Thus peptide **7** was obtained in 33% yield over two steps (ligation and reductive acidolysis) after one reverse-phase HPLC purification.

Having hGH-RH (18–44) (SARKLLQDIMSRQQGESNQERGARARL-NH_2) in hand, we continued to ligate it with hGH-RH (1–17) (Ac-YADAIFTNSYRKVLGQL SAL-ester) between Leu and Ser to afford the full-length hGH-RH (Fig. 5) in 25% after reverse-phase HPLC purification.

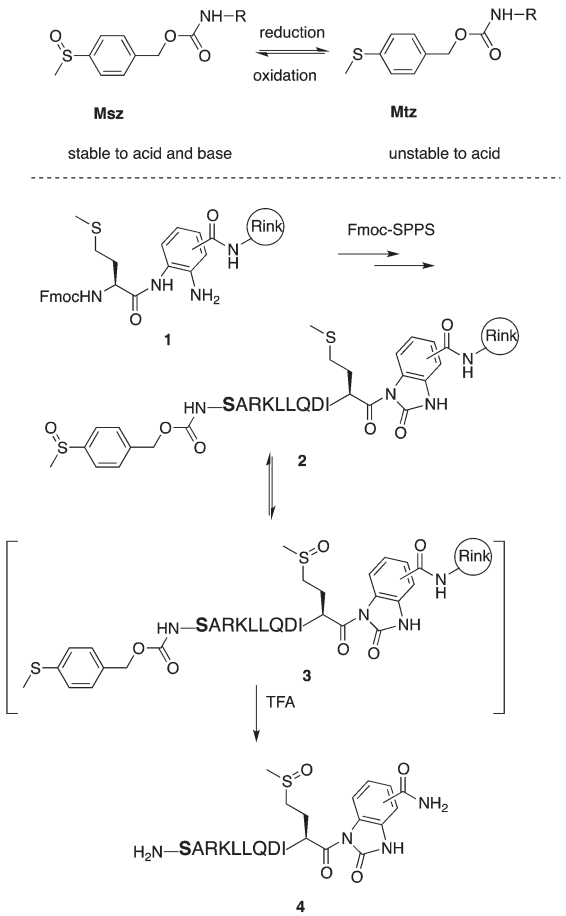

Fig. 4 Methionine reducing Msz cleavage.

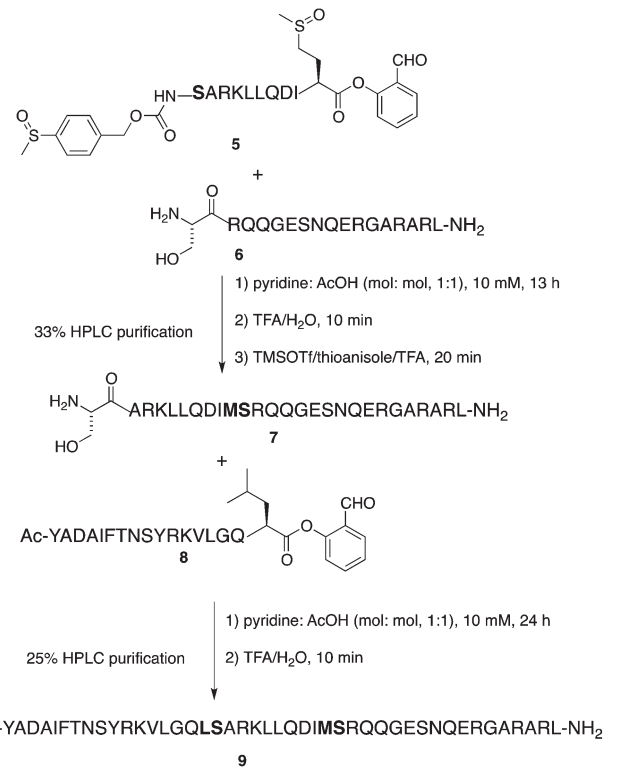

Fig. 5 Synthesis of hGH-RH.

Conclusions

In summary, we have synthesized human growth hormone-releasing hormone *via* $C \rightarrow N$ three-fragment serine ligation. Msz (*p*-(methylsulfinyl)benzyloxycarbonyl), which is compatible with the preparation of peptide SAL esters, is found to be a proper temporary protecting group for the N-terminal serine and threonine residues of the middle peptide fragment. Furthermore, its hydrophilic properties provide advantage in the purification of the peptide. The current studies pave the way for the application of serine/threonine ligation in the synthesis of peptides/proteins with increased complexity and length.

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