CHEMISTRY LETTERS, pp. 881-884, 1993.

Preparation of Partially Protected Peptide Thioesters Containing a Cysteine Residue and Their Segment Condensation

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Methods for the preparation of partially-protected cysteine-containing peptide segments were developed. The peptide thioester, obtained via a solid-phase method of Boc chemistry, was used for segment condensation with an amino component peptide in the presence of silver ions and *N*-hydroxysuccinimide to give a desired product in good yield.

Based on the thiocarboxyl segment condensation strategy by Blake et al.,[1,2] we have developed a method of polypeptide synthesis using partially protected peptide thioesters as building blocks.[3] Highly pure polypeptides could be synthesized by this method.[4] This method retains the best of Blake's method such as selective activation of the carbonyl group in the carboxyl terminal and furthermore overcomes the problems involved in Blake's method such as instability of thiocarboxylic acid and difficulty of the introduction of side-chain amino-protecting groups.

However, a cysteine-containing building block could not be prepared by either method because no route was known to obtain an adequately protected cysteine-containing peptide segment. In this communication, we describe the methods for the preparation of partially protected cysteine-containing peptide segments and also demonstrate the applicability by their segment coupling, referring to the synthesis of hANP(1-28).

hANP(1-28):
Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr.

Preparation of Partially Protected Peptide Segment: We chose the 4-methylbenzyl (MeBzl) group for the protection of the thiol group of cysteine residue, because the MeBzl group is stable toward silver ions under segment coupling conditions. Two methods were examined for the preparation of a peptide segment bearing a MeBzl group on the cysteine residue.

Method A: A protected peptide resin corresponding to hANP(1-9), Boc-Ser(Bzl)-Leu-Arg(Tos)-Arg(Tos)-Ser(Bzl)-Ser(Bzl)-Cys(MeBzl)-Phe-Gly-SC(CH_3)$_2$$CH_2$CO-Ala-O$CH_2$-Merrifield resin,[5] was prepared by a

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solid-phase method. This resin was treated under the low-HF conditions[6)] to give a peptide, Ser-Leu-Arg(Tos)-Arg(Tos)-Ser-Ser-Cys(MeBzl)-Phe-Gly-$SC(CH_3)_2CH_2CO$-Ala.[7)] By this method, *S*-methylbenzylated peptide thioester was obtained and a desired product was easily isolated by reversed-phase HPLC (RPHPLC) with 19% yield based on alanine content in a starting resin. However, low-HF treatment of protected hANP(10-28) on a Merrifield resin, Boc-Gly-Arg(Tos)-Met-Asp(OBzl)-Arg(Tos)-Ile-Gly-Ala-Gln-Ser(Bzl)-Gly-Leu-Gly-Cys(MeBzl)-Asn-Ser(Bzl)-Phe-Arg(Tos)-Tyr(2Br-Z)-OCH_2-resin, gave a partially deprotected peptide mixture and the yield of a desired product, Gly-Arg(Tos)-Met-Asp-Arg(Tos)-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys(MeBzl)-Asn-Ser-Phe-Arg(Tos)-Tyr, was as low as 5%. Therefore this method will be applicable only when the peptide has a few Arg residues.

Method B: A protected peptide thioester prepared on a 4-methylbenzhydrylamine resin (MBHA resin or NH_2-resin) was treated under the high-HF conditions.[6,8)] After purification of a crude peptide by RPHPLC, a MeBzl group was introduced to the thiol group of a cysteine residue using 4-methylbenzyl chloride (MeBzl-Cl).

Ser(Bzl)-Leu-Arg(Tos)-Arg(Tos)-Ser(Bzl)-Ser(Bzl)-Cys(MeBzl)-Phe-Gly-$SC(CH_3)_2CH_2CO$-Nle-NH-resin **(1)**

$\downarrow$ 1) High-HF treatment, 2) RPHPLC

Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-$SC(CH_3)_2CH_2CO$-Nle-NH_2 **(2)**

$\downarrow$ 1) MeBzl-Cl, NMM in aqueous DMF, 2) RPHPLC

Ser-Leu-Arg-Arg-Ser-Ser-Cys(MeBzl)-Phe-Gly-$SC(CH_3)_2CH_2CO$-Nle-NH_2 **(3)**

Scheme 1. Preparation of *S*-methylbenzylated peptide thioester.

Table 1. The yields of peptides bearing a MeBzl group on a cysteine residue

Peptides	Yield / %
hANP(1-9) Ser-Leu-Arg-Arg-Ser-Ser-Cys(MeBzl)-Phe-Gly-$SC(CH_3)_2CH_2CO$-Nle-NH_2	51[a)]
Lysozyme(55-71) Ile-Leu-Gln-Ile-Asn-Ser-Arg-Trp-Trp-Cys(MeBzl)-Asn-Asp-Gly-Arg-Thr-Pro-Gly-$SC(CH_3)_2CH_2CO$-Nle-NH_2	28[a)]
hANP(10-28) Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys(MeBzl)-Asn-Ser-Phe-Arg-Tyr	55[a)]
*c*Myb(60-89) Thr-Asp-Asp-Trp-Lys-Val-Ile-Ala-Asn-Tyr-Leu-Pro-Asn-Arg-Thr-Asp-Val-Gln-Cys(MeBzl)-Gln-His-Arg-Trp-Gln-Lys-Val-Leu-Asn-Pro-Glu-NH_2	6[b)]
gp41(1-12) Ser-Gln-Gln-Leu-Leu-Gly-Leu-Trp-Gly-Cys(MeBzl)-Ser-Gly-$SC(CH_3)_2CH_2CO$-Nle-NH_2	40[a)]

a) Based on the purified peptide before reaction. b) Based on Glu linked to the initial resin.

Scheme 1 shows a typical example. A protected peptide on an MBHA resin **1** was treated with HF containing 10% anisole at 0°C for 90 min. A crude peptide thioester was purified by RPHPLC and freeze-dried to give a powder **2** in a yield of 49% based on Gly in the starting resin. The purified peptide was dissolved in 90% aqueous *N,N*-dimethylformamide (DMF) containing 6% 4-methylmorpholine (NMM). MeBzl-Cl was added to a concentration of 0.25 mol dm^{-3}. After completion of the reaction, a desired product **3** was isolated by RPHPLC in a yield of 51% based on peptide **2**. Under similar conditions, the MeBzl group was introduced to several peptides, which contain tryptophan, histidine or methionine. Thus, method B is more widely applicable to the preparation of *S*-methylbenzylated peptide than method A, if a peptide containing a free mercapto group is well soluble in DMF or aqueous DMF (Table 1).

Segment Condensation: Cysteine-containing peptide segments were condensed according to scheme 2. Peptide **4** (13.5 mg, 5.0 µmol), obtained by the introduction of a Boc group to peptide **3**, and peptide **5** (18.5 mg, 4.3 µmol) were dissolved in dimethyl sulfoxide (DMSO)(0.5 ml) followed by the addition of silver nitrate (3.5 mg, 20 µmol), *N*-hydroxysuccinimide (HONSu) (11 mg, 96 µmol) and NMM (3 µl, 27 µmol). The condensation reaction proceeded almost quantitatively without any serious side reaction (Fig. 1). To avoid the possible *t*-butylation of the sulfur atom of a methionine residue under the HF treatment conditions of a peptide containing a Boc group, product **6** (38 mg) was first treated with TFA containing 10% 1,4-butanedithiol. After freeze-drying, peptide **7** was further treated with HF containing 1,4-butanedithiol (7.5%) and anisole (7.5%). Product **8**, the reduced form of hANP(1-28), was oxidized according to the method described by Chino et al.[9] to give hANP(1-28) (**9**) in 50% yield based on peptide **5**, which was confirmed by coelution with an authentic sample (Peptide Institute Inc., Osaka), by mass measurement and by amino acid analysis.[10]

Boc-Ser-Leu-Arg-Arg-Ser-Ser-Cys(MeBzl)-Phe-Gly-SC(CH$_3$)$_2$CH$_2$CO-Nle-NH$_2$ (**4**)

↓ Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys(MeBzl)-Asn-Ser-Phe-Arg-Tyr (**5**)

↓ AgNO$_3$ + HONSu + NMM in DMSO

Boc-Ser-Leu-Arg-Arg-Ser-Ser-Cys(MeBzl)-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys(MeBzl)-Asn-Ser-Phe-Arg-Tyr (**6**)

↓ TFA containing 1,4-butanedithiol (10%)

Ser-Leu-Arg-Arg-Ser-Ser-Cys(MeBzl)-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys(MeBzl)-Asn-Ser-Phe-Arg-Tyr (**7**)

↓ HF containing 1,4-butanedithiol (7.5%) and anisole (7.5%)

Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr (**8**)

↓ Oxidation by K$_3$[Fe(CN)$_6$]

Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr (**9**)

Scheme 2. Preparation of hANP(1-28) by thioester method using cysteine-containing peptide segments.

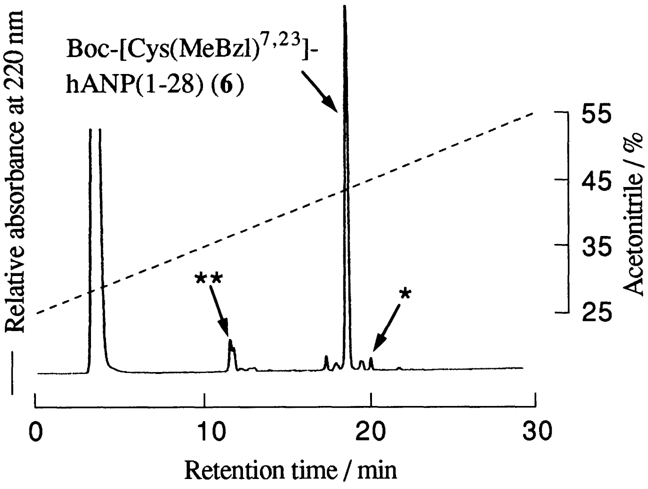

Fig. 1. HPLC profile of crude Boc-[Cys(MeBzl)^{7,23}]-hANP(1-28) (**6**); * shows the peak of the active ester, ** shows the peak of the amino component, peptide **5**.

This research was supported in part by a Grant-in-Aid for Scientific Research No. 04640527 from the Ministry of Education, Science and Culture.

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7) Found: m/z 1611.7 $(M+H)^+$. Calcd: m/z 1611.6 $(M+H)^+$. Amino acid composition: $Ser_{2.63}Gly_{1}Ala_{1.07}Leu_{0.93}Phe_{1.00}(Arg+Cys(MeBzl))_{2.77}$.
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10) Found: m/z 3079.7 $(M+H)^+$. Calcd: m/z 3079.5 $(M+H)^+$. Amino acid composition: $Asp_{2.07}Ser_{4.19}Glu_{1.05}Gly_{4.93}Ala_{1}Cys_{0.71}Met_{0.80}Ile_{0.96}Leu_{1.97}Tyr_{0.85}Phe_{1.99}Arg_{4.70}$.

(Received February 17, 1993)