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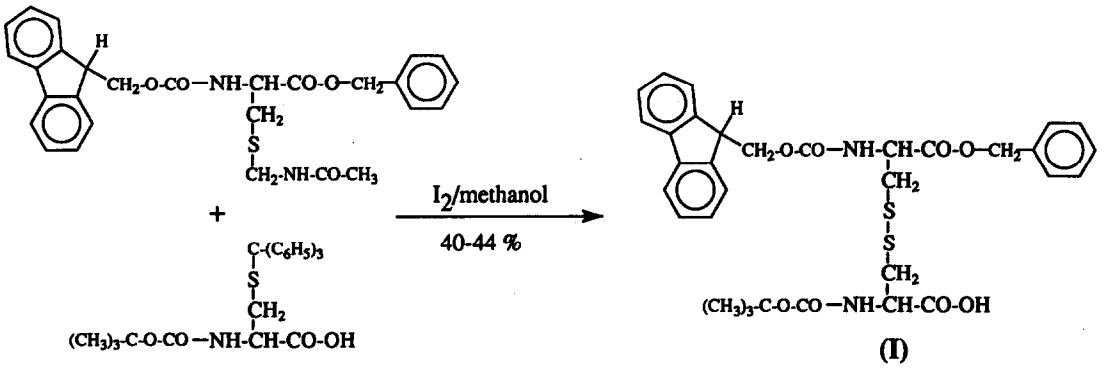
SEQUENTIAL SYNTHESIS OF AN UNSYMMETRICAL TWO-CHAIN DISULFIDE PEPTIDE ON SOLID-PHASE

Erika E. Büllesbach and Christian Schwabe
Medical University of South Carolina
Department of Biochemistry and Molecular Biology
171 Ashley Avenue
Charleston S.C. 29425

The synthesis of an unsymmetrical cystine-derivative (Nα'-9-fluorenyl-methyloxycarbonyl-Nα"-tert.butyloxycarbonyl-cystine-O'-benzyl ester) allowed the sequential synthesis of a disulfide-linked two-chain polypeptide on solid-phase.

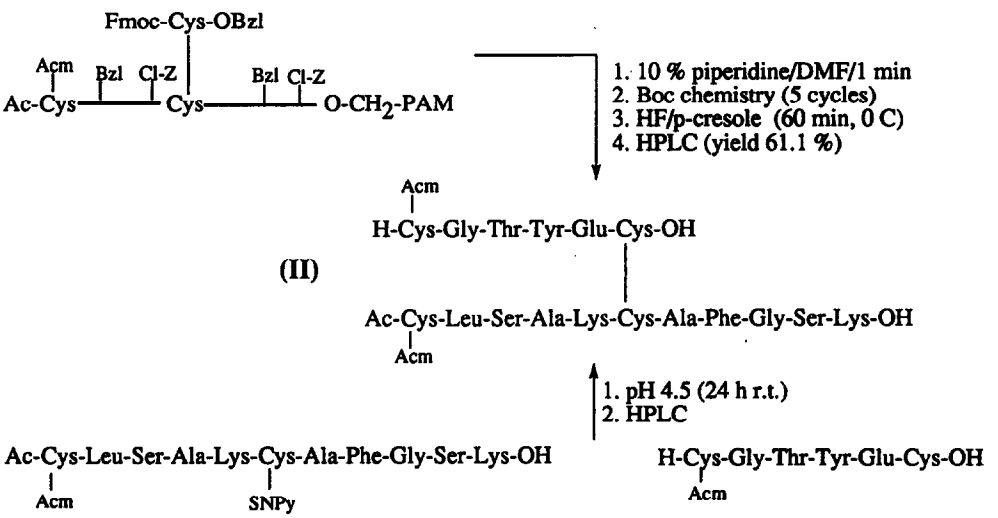
Proteohormones of the insulin family such as relaxin (1), bombyxin (2), and molluscan insulin-related peptide (3), are two-chain polypeptides with at least two interchain and one intrachain disulfide link. Typically, the cysteine at or close to the C terminus of the A chain is crosslinked to a cysteine in the B chain. In order to synthesize peptides of such complex disulfide structure it is adventageous to be able to synthesize each disulfide bond specifically. This approach has been used successfully in the synthesis of fully biologically active insulin (4) and relaxin (5). The distribution of the cysteines in these hormones suggested to us an even more efficient strategy involving an unsymmetrical cystine in which the two amino groups are protected by orthogonally removable protecting groups and wherein one of the two carboxyl groups is semipermanently protected. The only free carboxyl group of the cystine derivative is specifically activated and introduced into a growing peptide chain. Further extension of the peptide is possible by the liberation of one amino group at a time. In the present paper we report on the synthesis of Fmoc-Cys(Boc-Cys-OH)-OBzl (I) and its application in the production of a bis-cystinyl peptide on solid phase.

For the synthesis of Fmoc-Cys(Boc-Cys-OH)-OBzl, Boc-Cys(Trt)OH (1mmol) (Bachem, Torrance, CA) and Fmoc-Cys(Acm)-OBzl (6) (1mmol) in 30 ml of methanol were reacted with 3 mmol of iodine (in 10 ml of methanol) for 30 minutes at room temperature. As previously reported (7) under these conditions a nonstatistical distribution of the unsymmetrical cystines is observed. The reaction was quenched with an excess of aqueous thiosulfate. The cystine derivative was extracted into ethyl acetate (30ml, 3 times), the pooled organic layers washed with water, dried over $MgSO_4$, filtered, and dried *in vacuo*. Symmetrical and unsymmetrical disulfides were



separated on silicagel (2.5 cm x 30 cm; Bio-Sil A, 100-200 mesh, Biorad) in benzene/acetic acid (9:1 v/v). The unsymmetrical disulfide was concentrated *in vacuo* and rechromatographed on Bondapak C_{18}/Porasil B, 37-75 microns (Waters, Milford, MA) (column: 2.0 cm x 30 cm) in acetonitrile/water 8:2 v/v. The yield of the product was 40-44%. The cystine derivative was homogeneous by thin layer chromatography and showed the expected ^{1}H-NMR-spectrum(8).

In order to test the applicability of the Fmoc-Cys(Boc-Cys-OH)-OBzl an unsymmetrical disulfide peptide was synthesized on an automatic peptide synthesizer (ABI-430A, Applied Biosystems, Forster City, CA), using standard Boc-chemistry. The unsymmetrical cystine was introduced on a manual shaker, using a 2.5-fold excess of preactivated 1-hydroxybenzotriazole (HOBT) ester. The condensation was completed after 2 h and the automatic synthesis was continued by Boc-chemistry. The N terminus of the B-chain was acetylated, the Fmoc-group removed by treatment with



10% piperidine in dimethylformamide for 1 min and the A-chain synthesis was continued with Boc-chemistry. After high HF deprotection, using p-cresol as scavenger, the product was isolated by preparative reversed-phase HPLC with an overall yield of 61.1%. The peptide (II) was homogeneous in reversed-phase HPLC in 2 different systems and the amino acid composition (9) was correct. The proper amino acid sequence of the A chain and (after tryptic digest) of the C-terminal region of the B chain was confirmed. The second interchain disulfide bond was formed after treatment with iodine (7) to yield the desired product. The peptide (II) was identical in its properties with a peptide synthesized by an usual chain combination method (overall yield of 27.5%).

Studies on the stability of the disulfide link were performed on the peptide resin either after completion of the B-chain and before liberation of the Fmoc-group or after the synthesis was completed. The remaining A chain was either determined by photometrical methods (10) or by the A to B chain ratio by amino acid composition (Table I). The disulfide link is stable toward nucleophiles like HOBt and tertiary bases like diisopropylethylamine. The low value of A chain in untreated fully synthesized peptide could be caused by incomplete Fmoc deprotection due to the short time of exposure to 10% piperidine in DMF (1 min) prior to condensation. Loss of the A-chain during the piperidine treatment is another possible cause for low values. When the completed resin-bound peptide was exposed to harsher conditions, i.e., 20% piperidine, the A chain loss was about 15% within one minute. This indicates that secondary bases act as nucleophile on the disulfide bond. Other reagents such as 1,8 diazabicyclo[5.4.0]undec-7-ene caused the complete loss of the A chain within 5 min and are therefore not useful. Furthermore, peptides containing a preformed disulfide bond must be deprotected by the high HF procedure in the absence of thiols and thioethers as scavengers (11).

TABLE I: Stability of the disulfide link under conditions of peptide chemistry

treatment	time	% Fmoc	A/B ratio
none	-	100	-
DMF	24 h	104	-
0.2 M HOBt in DMF	48 h	104	-
DIEA/CH_2Cl_2 1:9 (v/v)	24 h	96	-
none	-	-	0.74
piperidine 20% in DMF	1 min	-	0.63
piperidine 20% in DMF	5 min	-	0.32
1,8 diazabicyclo[5.4.0]undec-7-ene 5% in DMF	5 min	-	0.04

The sequential synthesis of disulfide-linked two-chain molecules has significant advantages over chain combination procedures. Chain solubility is often improved by a preformed disulfide link, and the kinetics for subsequent crosslinking formation has been changed to a monomolecular reaction. Our results indicate that unsymmetrical cystine derivatives are a viable approach to this problem.

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8. ^{1}H-NMR (400 MHz, d$_6$-DMSO): 8.05ppm (d, 1H Fmoc-NH-); 7.89ppm (d, 2H Fmoc); 7.70ppm (d, 2H Fmoc); 7.41ppm (t, 2H Fmoc); 7.36ppm (m, 5H phenyl and 2H Fmoc); 7.12ppm (d, 1H Boc-NH); 5.15ppm (dd, 2H, CH_2-phenyl); 4.40ppm (m, 1H, Fmoc-Cys-CH^{α}); 4.31ppm (ds, 2H, Fmoc-CH_2); 4.21ppm (t, 1H, Fmoc-CH); 4.16ppm (m, 1H, Boc-Cys-CH^{α}); 3.17ppm (dd, 1H, Fmoc-Cys-CH_2^{β}); 3.11ppm (dd, 1H, Boc-Cys-CH_2^{β}); 2.98ppm (dd, 1H, Fmoc-Cys-CH_2^{β}); 2.91ppm (dd, 1H, Boc-Cys-CH_2^{β}); 1.35ppm (s, 9H, CH_3-Boc).
9. amino acid composition: Thr 0.90(1), Ser 1.96(2), Glu 0.91 (1), Gly 2.01 (2), Ala 2.08 (2), Leu 1.08 (1), Tyr 0.87 (1), Phe 1.11 (1), Lys 1.97 (2)
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