Tetrahedron Letters Vol. 21, pp 589 - 592 0040-4039/80/0208-0589$02.00/0

SYNTHESIS OF AN OPEN-CHAIN CYSTINE PEPTIDE CORRESPONDING TO THE ASYMMETRICAL INSULIN INTERMEDIATE A^{1-21}-B^{18-26}

W.Kullmann

Max-Planck-Institut fur biophysikalische Chemie
Am Faßberg, D-3400 Gottingen-Nikolausberg

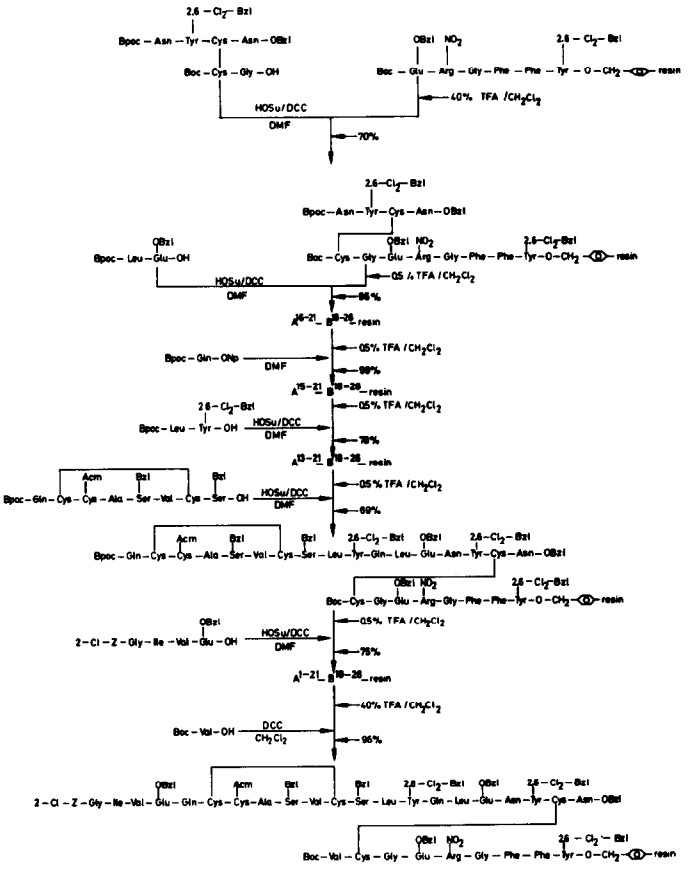
The synthesis via fragment condensation on a solid support of an insulin intermediate containing the A-chain linked by the disulfide bond A20-B19 to the B-chain segment B18-B26 is reported.

To evaluate the accessibility of complex asymmetrical cystine peptides via solid phase fragment condensation, studies toward an unambigious synthesis of insulin analogues were performed. The solid phase fragment approach combines the advantages of both conventional and solid phase methods. Protected fragments of a peptide chain are prepared in solution, thoroughly purified, characterized and finally coupled to the peptide resin. Although the fragment condensations do not proceed with a 100% yield, purification of the product synthetized by this approach seems easier than purification of a peptide assembled stepwise solely on the resin(1) due to the pronounced difference in molecular weight of the by-products and the target-sequence. On the other hand, solubility problems encountered during several conventional syntheses of the insulin chains(2) may be overcome by the solid phase fragment procedure provided the solvation of the carrier-bound protected peptides is not prevented by sterical hindrance (for details see(3)).

To prevent the difficulties inherent in the correct combination of the separately prepared A- and B-chains each disulfide bridge was formed selectively at different stages of the synthesis following the strategy reported by Sieber et al.(4). Since this required the assembly of one chain in the presence of the partial or total sequence of the other chain, protecting groups of different reactivities were needed. To meet this requirement, the synthetic pathway outlined in scheme 1 was chosen. A different approach was recently described by Birr and Pipkorn(5) who reported the synthesis of insulin via stepwise formation of the disulfide bonds between a reduced natural B-chain and a synthetic A-chain the sulfur protecting groups of which were selectively cleavable.

Selective formation of the asymmetrical disulfide A^{18-21}-B^{19-20} was achieved by a procedure described by Brois et al.(6) via a sulfenylthiocarbonate derivative of the A^{18-21} fragment which was subsequently cleaved by the free thiol group of the dipeptide B^{19-20} to give the protected asymmetric tetrapeptide-dipeptide-disulfide(7)(Scheme 2). The second disulfide bond (A^6-A^{11}) was part of the protected intermediate A^{5-12} (Scheme 3). This octapeptide represented a cyclic cystine peptide containing an additional cysteine residue(A^7) which was to remain unaffected by the ring closure between residues A^6 and A^{11}. This strategy would enable the formation of the third disulfide bond(A^7-B^7) in a separate step. Owing to this requirement the thiol moieties of residues A^6 and A^{11} were tritylated whereas the residue A^7 was acetamidomethylated. As described by Sieber et al.(8), the S-trityl groups were oxidized by iodine in trifluoroethanol/water(4:1)(8a) leaving the S-Acm protection largely intact.

Scheme 1



The protected fragments A^{13-14} and A^{16-17} were prepared by reacting the respective free amino components with the N-hydroxysuccinimide esters of the carboxyl components. (Yields: 69% and 66%, respectively). Glutamine A^{15} was coupled as Bpoc-Gln-ONp.

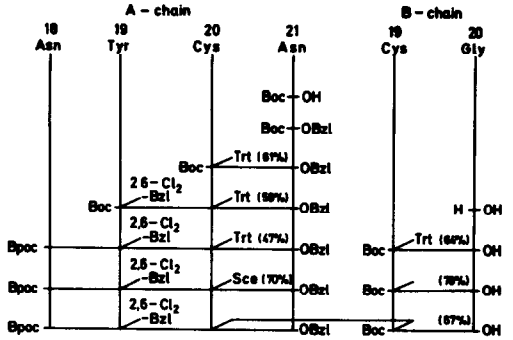
The protected fragment A^{1-4} was prepared stepwise in solution starting from the carboxyl terminus. The couplings were performed using the N-hydroxysuccinimide esters of the carboxyl components and the TFA-salts of the corresponding amino components (Yield: 18.5%).

During the syntheses in solution peptides containing S-trityl groups were deacylated using boron trifluoride etherate(9), otherwise TFA was used to remove Boc and Bpoc protecting groups. Neutralization was achieved with 4-methylmorpholine. Adsorption chromatography on silica gel and gel filtration on Sephadex LH-20 were employed to obtain the protected fragments in homogeneous form.

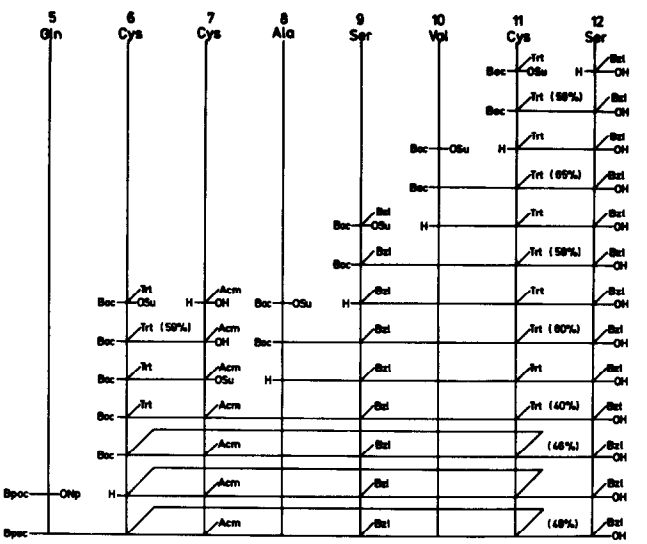
The protected intermediates were added to the B^{21-26}-peptide resin(7) as illustrated in scheme 1. Couplings were performed in dimethylformamide using two equivalents of HOSu and one equivalent of DCC. Bpoc protecting groups were removed with 0.5% TFA in CH_2Cl_2. For neutralization 10% TEA in CH_2Cl_2 was used. To prevent S-acylation of the S-Acm of residue A^7, the deacylation steps following the introduction of fragment A^{5-12} were performed in the presence of 10% anisole. After each coupling the solvated free α-amino groups were treated with acetic anhydride(10). The coupling steps were monitored by amino acid analyses of peptide resin hydrolysates. The coupling yields, calculated with regard to the actually preceding condensation step, were found to be 76% for the solid phase synthesis of B^{21-26}, 70% for fragment A^{18-21}-B^{19-20}(1.4), 95% for fragment A^{16-17}(4.2), 99% for residue A^{15}(2x4.3), 78% for fragment A^{13-14}

Abbreviations:

Bpoc, 2-(4-biphenyl)-2-propyloxycarbonyl; 2-Cl-Z, 2-chloro-benzyloxycarbonyl; Sce, ethoxycarbonylsulfenyl; Acm, acetamidomethyl; Trt, triphenylmethyl; HOSu, N-hydroxysuccinimide; ONp, p-nitrophenylester; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; TEA, triethylamine; DMF, dimethylformamide.



Scheme 2



Scheme 3

(2x4.3), 69% for fragment A^{5-12}(2x2.0), 75% for fragment A^{1-4}(2x3.5), and 96% for Boc-Val-OH residue B^{18}(2x3.0). (The molar excess of each carboxyl component is given in brackets.) The overall coupling yield was thus 19% as compared with 0.19 mmol of Boc-Tyr(2,6-Cl_2-Bzl)-OH esterified to 1 gram of resin.

To monitor the progress of the synthesis and to investigate the possibilities of purification, aliquots (ca. 100mg) of the A^{18-21}-B^{19-26}-(7), A^{13-21}-B^{19-26}-, and A^{1-21}-B^{18-26}-peptide resins were removed and the peptides were cleaved from the solid support and deprotected by liquid HF in the presence of anisole (yield 60%). The fragments A^{13-21}-B^{19-26} and A^{1-21}-B^{18-26} were purified by fractionation on a Biogel P-4 column, by partition chromatography on silica gel and by ion exchange chromatography on DEAE-Sephadex A-25. The yields (by weight) based on the total amount of resin bound peptides were 10.5% for A^{13-21}-B^{19-26} and 8.4% for A^{1-21}-B^{18-26} The results of amino acid analyses of acid hydrolysates are given in the following table:

	A^{5-12} +	A^{18-21}-B^{19-26} +	A^{13-21}-B^{19-26} +	A^{1-21}-B^{18-26} *	A^{1-21} +
Cys(O_3H)	2.83(3)	2.03(2)	1.86(2)	4.71(5)	4.07(4)
Asp		2.07(2)	1.95(2)	1.95(2)	2.03(2)
Ser	1.60(2)			1.71(2)	1.84(2)
Glu	0.94(1)	1.04(1)	3.15(3)	4.77(5)	3.95(4)
Gly		2.00(2)	2.00(2)	3.00(3)	1.00(1)
Ala	1.00(1)			1.03(1)	1.03(1)
Val	1.04(1)			3.06(3)	1.67(2)
Ile				0.95(1)	0.60(1)
Leu			1.85(2)	1.89(2)	1.96(2)
Tyr		1.87(2)	2.65(3)	2.64(3)	1.85(2)
Phe		2.08(2)	1.90(2)	1.96(2)	
Arg		1.06(1)	0.94(1)	1.04(1)	

\+ 24-h hydrolysate, * 72-h hydrolysate

A sample of the A^{1-21}-B^{18-26}-insulin fragment was treated with iodine followed by performic acid to remove the S-Acm group and to oxidize the disulfide bonds,respectively. After removal of the cysteic acid derivative of the B^{18-26}-fragment, the chromatographic and electrophoretic

data of the tetra-cysteic acid derivative of the A-chain as well as a peptide map of its peptic hydrolysate(11) were found to be in close agreement with those of the oxidized natural A-chain.

It was the first time that two distinct peptides connected by a disulfide bond were synthetized side by side on a solid support, and the success of this approach depended largely on the stability of the disulfide bridges during the various steps of the solid phase procedure. During this synthesis the disulfide bonds were neither affected seriously by TFA and TEA in methylene chloride nor were they found to be unstable in the presence of liquid HF. These findings were in agreement with those of Lunkenheimer and Zahn(12), Kullmann and Gutte(7) and Katz(13) for resin-bound symmetric cystine peptides, resin-bound asymmetric cystine peptides and natural insulin, respectively. The amount of resin-bound peptide after HF-treatment is an obvious drawback,which may be overcome by HBr/TFA cleavage of the benzylester link to the solid support and subsequent removal of the remaining protecting groups by HF/pyridine, the usefulness of which was recently described(5).

In summary, fragment condensation on a solid support proved to be a feasible approach for the synthesis of complex cystine peptides. Due to the differences in molecular size and electrophoretic and solubility properties the desired free peptides could be easily separated from by-products that originated from incomplete coupling of the protected fragments.

References:

1.Weber,K. & André,M.(1975) Hoppe-Seyler's Z.Physiol.Chem. 365,701-714
2.Lubke,K. & Klostermeyer,H.(1970) Advan.Enzymol.Areas Mol.Biol. 33,445-525
3.Birr,C. Aspects of the Merrifield Peptide Synthesis. Reactivity and Structure Concepts in Organic Chemistry Vol.8. Hafner,K.,Rees,C.W., Trost,B.M., Lehn,J-M., v.Ragué Schleyer,P. & Zahradnik,R. eds.,Springer-Verlag,Heidelberg(1978)
4.Sieber,P., Kamber,R., Hartmann,A., Johl,A., Riniker,B. & Rittel,W.(1974) Helv.Chim.Acta 57,2617-2621
5.Birr,C. & Pipkorn,R.(1979) Angew.Chem.Int.Ed.Engl. 18,536-538
6.Brois,S.J., Pilot,J.F. & Barnum,H.W.(1970) J.Amer.Chem.Soc. 92,7629-7631
7.Kullmann,W. & Gutte,B.(1978) Int.J.Pept.Prot.Res. 12,17-26
8.Sieber,P., Kamber,B., Eisler,K., Hartmann,A., Riniker,B. & Rittel,W.(1976) Helv.Chim.Acta 59,1489-1497; 8a. Rittel,W. personal communication(1976)
9.Hiskey,R.G., Beacham,III,L.W. & Matl,V.G.(1972) J.Org.Chem. 37,2472-2478
10.Bayer,E., Eckstein,H., Hagele,K.,Konig,W.A., Bruning,H. & Parr,W.(1970) J.Amer.Chem.Soc. 92,1735-1738
11.Sanger,F. & Thompson,E.O.P. (1953) Biochem.J. 53,366-374
12.Lunkenheimer,W. & Zahn,H.(1970) Justus Liebig's Ann Chem. 740,1-17
13.Katz,J.J. (1954) Arch.Biochem.Biophys. 51,293-305

(Received in Germany ?? October 1979)