COTTON - CARRIER FOR SOLID PHASE PEPTIDE SYNTHESIS

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Cotton is shown to be a suitable carrier for solid phase peptide synthesis. Various ways of attachment of the first amino acid to the carrier are discussed and results obtained with this carrier are shown. Possible uses of cotton as the carrier are shown, including continuous solid phase synthesis.

Recently described methods of multiple peptide synthesis have utilized either various compartmentalization schemes, such as separation of the polymeric carrier in columns (1) or in so called tea bags (2), or alternative carriers such as grafted polyethylene (3) or cellulose paper (4,5) have been used. Cellulose in the bead form was one of the first carriers for solid phase synthesis tested by Merrifield (6) and it was found unsuitable. However, it was shown (4,5), that peptides synthesized on cellulose paper support can be obtained in reasonable yield and purity. The only disadvantage of the paper support is its mechanical instability. Since cotton is the purest form of

cellulose, and since it can be obtained in various forms and shapes we decided to try cotton fabric as the support material for solid phase synthesis.

The first problem of the synthesis is the attachment of C-terminal amino acid to the cotton fabric. We have used a cotton strip of 3 cm width and density 230 g/m^2. At first we attempted direct connection of the protected amino acid to the polysaccharide hydroxy groups via the ester formation. We have shown that the treatment of cotton with protected amino acid, DCC and HOBt in the presence of DMAP can produce 1 to 3 μmol/cm^2 substitution (that is 0.04 to 0.12 mmol/g). (Substitution of 0.1 mmol/g means that one in each 185 available hydroxy groups is modified.) The carrier obtained in this manner can be used as such; in this case the prepared peptide must be cleaved from the carrier by alkaline hydrolysis or ammonolysis. This possibility we tested using some model peptides (Cys(Acm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Ala, Leu-Phe-Pro-Val-Ala, Leu-Phe-Pro-Val-Gly-Ala, Pro-Leu-Gly-Ala, Leu-Pro-Gly-Ala). Some of these peptides were synthesized for the study of the recently described (7) rearrangement of peptides containing glycine in position three. And indeed, after the alkaline treatment (methanolic ammonia) of Pro-Leu-Gly-Ala-cellulose we obtained both Pro-Leu-Gly-Ala-NH_2 and Leu-Pro-Gly-Ala-NH_2 as the major products. Structure of both peptides was determined both by 1H NMR spectroscopy and by the comparison with standards synthesized in a different way. The mechanism of this rearrangement is described elsewhere (7), and this reaction should be considered as the potential danger in all cases where the synthesized peptide contains a suitably located glycine. Alkaline treatment (1M NaOH) also produced peptides bound to the saccharide units at the carboxy terminus as the side product. Therefore, the synthesis of peptides directly bound to cotton by an ester bond cannot be generally recommended for preparative purposes.

Alternatively, we have used the amino acid connected to cotton only as the source of the amino group (2 μmol/cm^2) which was acylated by the handle molecule (8) containing either C-terminal amino acid of the peptide to be synthesized (Fmoc-AA-

O-CH_2-C_6H_4-O-$(CH_2)_3$-COOTcp), or containing only a hydroxymethyl group (HO-CH_2-C_6H_4-O-$(CH_2)_3$-COOTcp). In the first case the synthesis was performed in the usual way (washes EtOH, DCM, deprotection 20% piperidine in DMF, washes DMF, DCM, coupling DCC+HOBt+Fmoc-AA in DMF (all 0.2M), washes DMF, EtOH), in the second case we have to connect the carboxy-terminal amino acid to the hydroxymethyl group. This was performed by means of the treatment of the modified carrier with protected amino acid, DCC and HOBt in DMF (all 0.125 M) in the presence of DMAP (0.04 M). Subsequent treatment of this carrier with TFA has shown that acylation of the cotton hydroxy group reached only (at most) 20% of the protected amino acid coupled to the carrier and that most of the hydroxymethyl groups of the handle have been modified by this procedure (substitution was 1.5 - 1.7 μmol/cm^2). Direct modification of the carrier hydroxy groups is not dangerous from the point of view of product purity (the peptide is not cleaved from the carrier under acidic conditions used for its release from the spacer group), it only decreases its yield. In order to show the applicability of this approach, we have synthesized model heptapeptides I-VIII, containing three-functional amino acids.

I, Arg-Trp-Thr-Lys-Asp-His-Tyr
II, Arg-Trp-Thr-Lys-Asp-His-Ala
III, Arg-Trp-Thr-Lys-Asp-Ala-Tyr
IV, Arg-Trp-Thr-Lys-Ala-His-Tyr
V, Arg-Trp-Thr-Ala-Asp-His-Tyr
VI, Arg-Trp-Ala-Lys-Asp-His-Tyr
VII, Arg-Ala-Thr-Lys-Asp-His-Tyr
VIII, Ala-Trp-Thr-Lys-Asp-His-Tyr

(Protection used: Fmoc-Tyr(Bu^t), Fmoc-His(Trt), Fmoc-Asp(OBu^t), Fmoc-Lys(Boc), Fmoc-Thr(Bu^t), Fmoc-Trp, Boc-Arg; coupling: DCC, HOBt in DMF (all 0.125M)). Peptides containing alanine in the place of all different amino acids were prepared to check the influence of particular amino acid on the purity of the product. The cleavage from the carrier was achieved with the mixture of TFA and ethanedithiol (9:1) containing 2.5% of

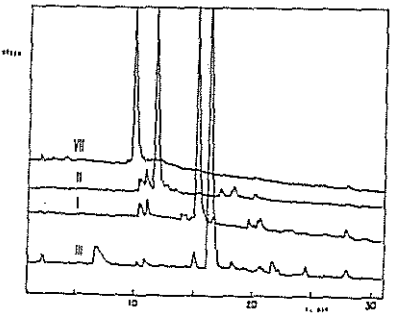

Figure 1 RP-HPLC trace of peptides I-III and VII. Conditions: Vydac C-18, 25 x 0.4 cm, 1.5 ml/min, gradient 0-60% of MeOH in 0.05%TFA in water in 30 min, detection at 220 nm.

indole (2h, r.t.), the obtained solution was evaporated, the residue was dissolved in 1M AcOH, solution was extracted with Et_2O and lyophilized. Peptides were desalted by gel filtration on Sephadex G-10 in 5% AcOH.

As it can be seen from examples given in Fig. 1, quality of all peptides is comparable; significantly better trace can be seen only in the case of peptide not containing tryptophan (VII); this clearly shows that impurities observed in HPLC traces are probably due to the decomposition occurring during acidolytic cleavage and not due to the carrier used for the synthesis. Another set of peptides synthesized employing this approach was derived from the foot-and-mouth disease virus VP1 sequence.

Arg-Gly-Asp-Leu-Gln-Val-Leu-Ala
Arg-Gly-Asp-Leu-Gln-Val-Leu
Arg-Gly-Asp-Leu-Gln-Val
Arg-Gly-Asp-Leu-Gln
Arg-Gly-Asp-Leu
Arg-Gly-Asp

We attempted to use enzymatic reaction for the connection of the first amino acid to the hydroxy group of cotton. Subtilisin catalyzed transesterification of Boc-Gly-OSu in phosphate buffer pH 7.8 (5 days, r.t.) afforded carrier with

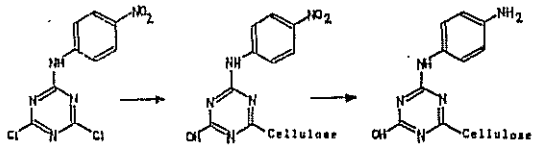

Figure 2 Scheme of the stable modification of cotton.

glycine content 0.09 mmol/g. Identical reaction mixture not containing subtilisin did not result in significant glycine incorporation.

Ester bond to the cellulose carrier is not absolutely stable - we have shown (5) that during the treatment with 20% piperidine in dimethylformamide corresponding to 18 steps of the synthesis using Fmoc protecting groups, up to 6% of peptide chain may be cleaved from the carrier. Treatment with TFA (25% in DCM) corresponding to 12 cycles of synthesis using Boc protecting group resulted in 9% of peptide loss. The stabilization of binding of the amino acid to the carrier was achieved by the utilization of cyanurchloride chemistry, as is shown on Fig.2. Reduction of the nitro group was performed by means of Zn in ethanolic ammonia, and the carrier with the content of 0.05 mmol NH_2/g was obtained. This carrier was used for the evaluation of Nps protecting group compatibility with this hydrophilic carrier. It is possible to acylate up to 80% of the available amino groups using Nps protected amino acid, however cleavage of the Nps group by 0.05M HCl in methanol cannot be followed directly by decolorizatiom of the carrier - hydroxy groups of cotton probably act as an acceptor of generated Nps-Cl. Stronger HCl solution (7.5M) causes very fast decolorization. However, storage of the the aromatic amino group containing cotton led to an inexplicable decrease of amino group content (to 0.003-0.004 mmol/g).

Proper monitoring of the coupling reaction is an important part of successful solid phase synthesis. In all syntheses performed on cotton we have used continuous non-destructive monitoring with bromophenol blue (9). The originally blue color of the carrier (caused by free amino groups) disappears after all amino groups are acylated. Sensitivity of this monitoring

was shown to be higher than sensitivity of the ninhydrin test and it can be used advantageously in cases when destruction of a part of the carrier would eventually lead to its complete consumption. This is the case of multiple synthesis on cotton or paper discs. Bromophenol blue ("BB") method was also found applicable during the synthesis on polyethylene pins (10).

Peptides bound to the solid carrier can be used for example for the RIA or ELISA determination of the given peptide: we tried to apply this approach to detect antibodies against Epstein-Barr determined nuclear antigen 1 (EBNA 1). For this reason peptide Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Gly was synthesized utilizing Fmoc protecting group and direct ester connection to cotton. However, we were not able to block unspecific adsorption of serum components on cotton, and due to high background the test was unsuccessful. Another promising application is the immunization of laboratory animals with cotton modified with the proper ligand. Immunization can be carried out either directly by implantation of the cotton disc (analogously as performed with peptides synthesized on paper (11)) or by injecting animals with disintegrated modified cotton. We have proven the treatment with 1M solution of MSA in TFA in ultrasonic bath, followed by precipitation with water as a suitable way to disintegration. Cotton modified with Met-enkephalin obtained in this way was used for immunization of rabbits. Immunological response was very low, but the same results were obtained with Met-enkephalin coupled to BSA or synthesized on the branched lysine carrier (Lys_4-Lys_2-Lys-Ala (12)).

One of the advantages of the cotton as a carrier (besides simultaneous multiple peptide synthesis) is the possibility of the so called continuous peptide synthesis (13). In this case the carrier in the form of a long (possibly endless) tape is coming through various washes, cleavage (A) and coupling (B) solutions so that all stages of synthesis of the given peptide are performed simultaneously in different part of the carrier. Example of one compartment in which one amino acid residue is coupled to the growing peptide chain is given in Fig. 3. Recently we have described synthesis of several analogs of Met-

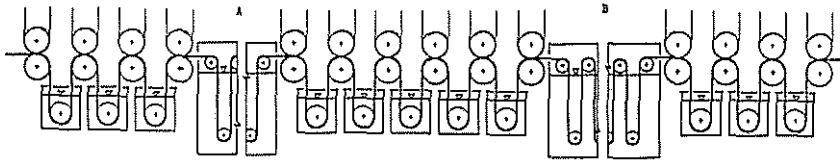

Figure 3 Scheme of the synthetic compartment of continuous solid phase synthesizer in which one amino acid is coupled to the growing peptide chain.

enkephalin under the conditions of simulated continuous solid phase synthesis (14).

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