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An Automated Multiple Solid Phase Peptide Synthesizer Utilizing Cotton as a Carrier

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Cotton was found to be a suitable carrier for solid phase synthesis of peptides. Its properties allowed for the design and construction of a multiple peptide synthesizer. The synthesizer is based on the following basic principles: (i) use of cotton as an integrated reaction vessel and a carrier, (ii) liquid removal from the carrier by centrifugation, (iii) coupling monitoring by the noninvasive continuous bromophenol blue method, (iv) gravity and/or nitrogen pressure driven solvent delivery, (v) computer guided stepper motor positioning of the proper synthetic compartment. Model syntheses performed with the machine have shown that (i) sequences which are difficult to assemble on polystyrene can be prepared on cotton, (ii) piperidine can be replaced by an alternative base, (iii) cyclizations can be performed successfully on this carrier, (iv) the new safety-catch handle (SCAL), allowing two step side-chain protecting groups removal and cleavage from the carrier, is compatible with this technology.

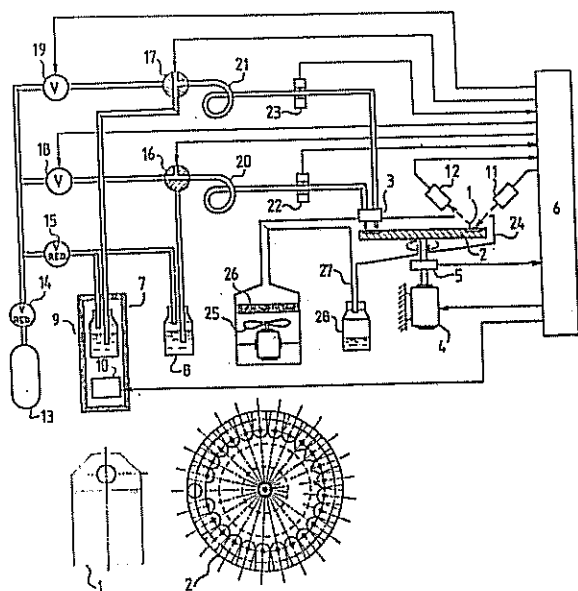
Recently, the interest in multiple peptide synthesis and evaluation has been growing [1-3]. Several multiple peptide synthesizers are now commercially available and are being used in many laboratories all over the world. We have shown [4-7] that cotton has unique features which make this carrier suitable for the design of a multiple peptide synthesizer.

The first and most important feature of this carrier is its fibre structure, permitting easy handling and formation of reaction compartments by simple cutting of the cotton fabric into proper shapes and sizes. Capillary forces hold the substantial amount of solutions inside the textile material, thus making the complete elimination of reaction vessels possible [6,7]. However, cotton pieces placed on the perimeter of the disc-like rotor of the centrifuge release most of the liquid (dimethylformamide) when a centrifugal force of 112 G (1000 rpm on a radius of 10 cm) is applied. Functionalized (amino groups containing) cotton carrier can be stained with bromophenol blue (BB) solution and a colour change from blue to yellow is observed at the final stage of coupling (elimination of free amino groups) [8].

The construction of any automatic peptide synthesizer requires the solution of two basic problems, namely (i) liquid handling and (ii) monitoring of the reaction progress. Most of the synthesizers which are used nowadays do not address the second question at all, and their construction deals only with the solvent handling problem. Textile-like carriers permit a very simple approach to both of these challenges.

^aIt may be stressed here again that there is no colour change during the greatest part of the coupling and the colour change occurs only after there are no more free amino groups available for the interaction with the indicator. Obviously the amount of added indicator determines the sensitivity of the end point detection and at the same time it determines the position of the end point, because the leuco-form of BB is able (due to its high acidity) to decolorize the coloured form. It means that after addition of 1 equivalent of BB (to free amino group), the consumption of 50% of amino groups will be accompanied by decolorization of the carrier. If 0.1 equivalent is added, 95% of the coupling will be observable. We recommend to use 0.01 equivalent of BB (or less) per amino groups present; this amount usually allows a sensitive monitoring (sufficiently coloured carrier) and at the same time the observed end point corresponds to 99.5% (or more) of the coupling.

The design of a multiple peptide synthesizer based on cotton as a carrier (cotton multiple peptide synthesizer, COMPAS) is shown in Scheme 1, and its actual appearance can be seen in Figure 1. Cotton squares 1 (3 x 3 cm) are placed on the perimeter of the disc 2 containing 24 shallow wells (2 mm deep). Each cotton square is melted onto the polyethylene handle (2 mm thick) containing a hole through which it is fixed by the small rod inside the well. The disc is fixed on the axis of a stepper motor 4 allowing the precise positioning of any synthetic compartment (well with cotton square) under the delivery head 3. The stepper motor is able to rotate the disc at the speed of up to 2000 rpm, which is more than sufficient for complete removal of any liquid from the carrier. The housing 24 of the rotor has a spiral shape allowing the efficient removal of generated aerosol and its transport to the compartment 26 separating liquid particles from the gas. The rotating disc is the only moving part of the synthesizer and its positioning by the stepper motor can be easily controlled by the computer 6.



Scheme 1. Scheme of the cotton multiple solid phase peptide synthesizer (COMPAS). 1 - Cotton squares; 2 - disc rotor; 3 - reagent delivery head; 4 - stepper motor; 5 - positioning controller; 6 - computer interface; 7 - thermoisolation; 8 - reagent reservoir; 9 - amino acid storage compartment; 10 - cooling device; 11 - light source; 12 - detector of reflected light; 13 - inert gas source; 14, 15 - pressure regulators; 16, 17 - three way valves; 18, 19 - gas valves; 20, 21 - calibrated loops; 22, 23 - sensors; 24 - centrifuge housing; 25 - ventilator; 26 - gas-liquid separation device; 27 - liquid waste outlet; 28 - waste container.

Delivery of the proper liquid is performed by the fixed delivery head 3 placed very close to the surface of the textile carrier on the disc. The volume of the liquid to be delivered is measured in the calibrated tubing. The empty tubing from the three-way valve 17 connected to the appropriate solution is being filled with the solution until the optoelectrical sensor 23 is activated by the front of the liquid. Then the valve 17 is switched and nitrogen flushes the content of the loop 21 (300-400 μ l) to the carrier. There is no danger of cross-contamination since every reagent uses its own liquid path. The solutions are driven either by nitrogen pressure or simply by gravity. Liquids which do not have to be measured precisely (washing solutions) are delivered continuously, without measurement, by slow continuous turning of the disc. This speeds up the washing process significantly. The precise volume measurement is performed in delivering the amino acid and N-hydroxybenzotriazole (HOBt) solution and the solution of the coupling reagent. We have used 0.4 M solution of the Fmoc-amino acid and of HOBt and the same concentration of diisopropylcarbodiimide in dimethylformamide. We were able to demonstrate that the sequential addition of reaction components does not have any influence on the quality of the product in comparison with the process in which the reaction components are pre-mixed and added together. This finding led to a significantly simplified apparatus design. The solutions of

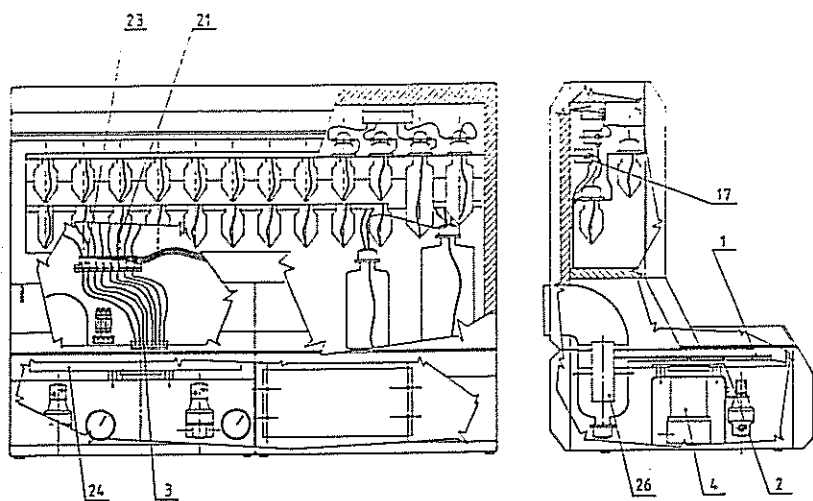
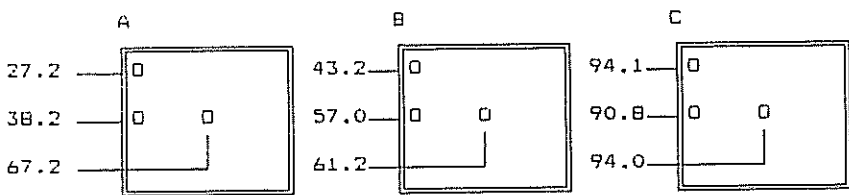


Figure 1. Front and side view of COMPAS. (For the numbers see Scheme 1.)

protected amino acids (the machine is dedicated to the Fmoc strategy) stored together with *N*-hydroxy-benzotriazole are stable enough for the syntheses of short peptides. For prolonged storage a decreased temperature is recommended. The only limitation is the solubility - in the case of asparagine and glutamine the side-chain trityl protected derivatives have to be used. The high concentration of the activated species is required due to the fact that the reaction vessel was eliminated and the coupling reaction proceeds in the liquid soaked into the carrier. The excess of carboxy component can be achieved, therefore, only by increased concentration and not by increased amount of the solution.



Scheme 2. Distribution of Fmoc-Gly delivered to the middle of the cotton square. A. 50 μmol Fmoc-Gly delivered in 100 μl DMF; B. 50 μmol Fmoc-Gly delivered in 200 μl DMF; C. 100 μmol Fmoc-Gly delivered in 400 μl DMF. (The samples of the carrier were cut immediately after delivering the solution and the concentration of Fmoc-Gly was measured spectrophotometrically. The values are given in $\mu\text{mol/g}$.)

Due to the fact that the liquid is delivered into one spot and its equal distribution is assured only by the capillary forces of the carrier, it was important to determine the distribution of the active component experimentally. The results are shown in the Scheme 2. It is obvious that an amount of solvent which was barely able to wet the carrier led to an uneven distribution. However, amounts actually used in the synthesis were able to distribute the reaction components in the carrier evenly.

The described arrangement can also be used for the synthesis using classical solid phase

carriers. However, the condition of low substitution has to be fulfilled again. We have placed on the disc of our machine the "tea bags" of Houghten [2], containing aminomethyl resin to which the acid-labile linker [9] was attached (substitution 0.1 mmol/g). Using the same protocol (Table 1) as in the case of the cotton carrier, the test sequence of ACP₆₅₋₇₄ was synthesized and the product was found equivalent (by HPLC) to that synthesized on cotton.

Bromophenol blue monitoring [8] of the coupling reaction is used advantageously in our synthesizer. Bromophenol blue solution in dimethylformamide containing N-hydroxybenzotriazole is delivered (precisely measured) to cotton carrier after the last wash before coupling. The solution is centrifuged away and the carrier is stained blue. (If HOBt is not used, the basicity of dimethylformamide allows the removal of the dye from the carrier by centrifugation. The same observation was reported by Flegel and Stewart [10] in their application of BB monitoring for continuous flow SPPS on polyamide support.) In this way, no additional solvent is added to the reaction, besides the solution of amino acid and coupling reagent. The end of coupling is then easily determined by the decolorization of the carrier. Instead of the subjective assessment of the reaction progress (observation by naked eye), the objective reflectometry method can be employed. In this case, light from the source 11 is reflected to the analyzer 12, the spectral response is evaluated and the result is used as the basis for the computer 6 decision about the next synthetic cycle.

Table 1. Protocol used in the syntheses performed in COMPAS

DMF wash	3x2 min, centrifugation
20% piperidine/DMF	20 min, centrifugation
DMF wash	3x2 min, centrifugation
BB/DMF/HOBt	1 min, centrifugation
Fmoc-AA/HOBt/DMF	no centrifugation
DIC/DMF	until the monitoring spot is decolorized, centrifugation

The software written for this synthesizer permits the repeated coupling in some reaction compartments and therefore flexible "frame shifting" over the sequences. It means that, after n synthetic cycles only, several synthetic compartments contain full peptide sequences of n residues. Some compartments have peptides with $n-1$, $n-2$, ..., $n-m$ amino acid residues due to the fact that some of the couplings were not finished in the preset time and therefore they had to be repeated. With the program written in this way, the operator can decide whether he prefers speed or economy of the synthesis. The standard scenario is as follows: After a given time (30 min) the colour of the synthetic compartments is evaluated. If less than a predetermined percentage (uncoupled threshold limit, e.g. 10%) of compartments have a colour value above the minimal acceptable level, the computer marks these compartments, shifts their frame one amino acid back and performs another synthetic cycle. If more than a predetermined percentage (10%) of couplings are not complete, another colour reading is performed after one additional time unit and the uncoupled threshold limit is increased. If a certain compartment is not completely coupled after two recouplings, either the synthesis is cancelled in this compartment, or acetylation is performed and synthesis continues after shifting the frame appropriately.

To eliminate the unpleasant smell of piperidine used for the cleavage of the Fmoc-protecting group, we have tested alternative deprotection reagents. Surprisingly, the solution of tetraethylammonium fluoride, recommended recently [11], did not work in our case. The following bases were tested: 4-benzylpiperidine, 4-(aminoethyl)piperidine, 4-piperidinopiperidine, 1-(2-aminoethyl)piperazine, tris-(2-aminoethyl)amine. As the only alternative to piperidine (giving satisfactorily high solution concentrations assuring thus fast cleavage of the Fmoc group and producing a well soluble fulvene adduct) we have found 4-benzylpiperidine. Using this base the C-terminal sequence of calcitonin was synthesized and the product was found to be equivalent to that prepared using piperidine. However, routine use of this base is not feasible due to its relatively high price.

To demonstrate the performance of our machine, the syntheses of the peptide sequences given in Table 2 were carried out. The HPLC trace of crude glucagon is given as an example in Figure 2. On cotton as an intramolecular hydrogen bond breaking carrier we were also able to synthesize sequences of polyalanine (up to Ala₂₀) without any remarkable slowing down of the coupling reaction. Cotton also allows the cyclization of the peptide bound to the carrier. We have shown this both in the case of disulfide-forming cyclization and peptide bond forming cyclization. In the first case the Ac_m group was used for the sulfur protection and the cyclization of a model peptide (oxytocin) was performed by thallium tris-trifluoroacetate. The second case is illustrated in Scheme 3 and the HPLC trace of a crude product of cyclization is shown in Figure 3.

Table 2. Examples of peptides synthesized using COMPAS

Enkephalin amides

Tyr-Gly-Gly-Phe-Leu-NH₂

ACP₆₅₋₇₄

Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH₂

Gastrin

Glp-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH₂

[Ser^{4,13}]MCH

Asp-Thr-Met-Arg-Ser-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Ser-Trp-Glu-Val-NH₂

Ala₁₀-Val

Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Val-OH

Calcitonin sequences

Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH₂

Hyaloma influenzae P6 sequence

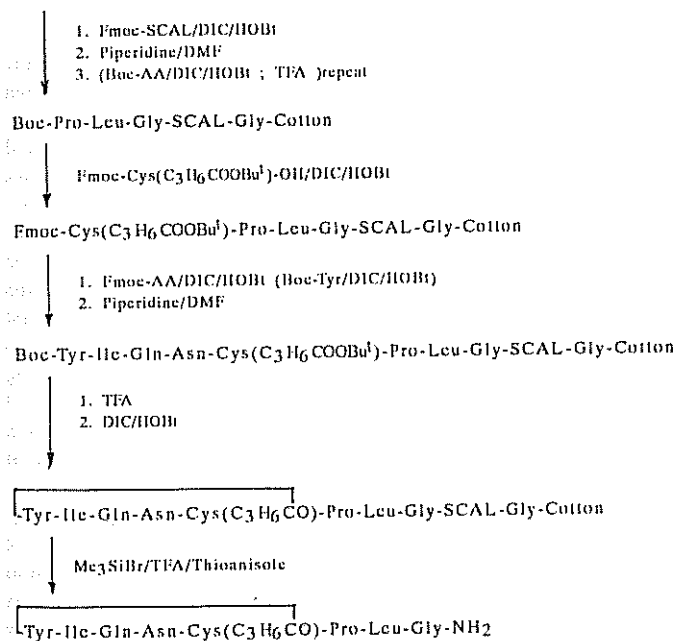
Ala-Val-Leu-Gly-His-Asp-Glu-Ala-Ala-Tyr-Ser-Lys-Asn-Arg-Arg-Ala-Val-NH₂

Magainine II amide

Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile

-Met-Asn-Ser-NH₂

Gly-Cotton



Scheme 3. Synthesis of deamino-1-carba-oxytocin.

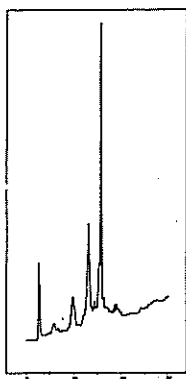


Figure 2. (above, left) HPLC trace of crude gastrin prepared on centrifuge based synthesizer. Column Vydac C18 (25x0.4 cm), 50 to 100% MeOH in 0.05% TFA in 30 min, 280 nm.

Figure 3. (above, right) HPLC trace of crude deamino-1-carba-oxytocin prepared according to Scheme 3. Column Vydac C18 (25x0.4 cm), 30 to 50% MeOH in 0.05% TFA in 30 min, 222 nm.

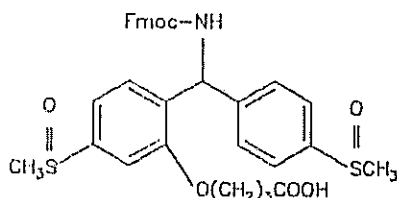


Figure 4. Structure of safety catch amide forming acid labile (SCAL) handle.

Scheme 3 also illustrates the use of a new handle developed in our laboratory. This safety-catch amide forming acid labile (SCAL) handle [12], the structure of which is shown in Figure 4, is completely stable both under the conditions of Boc and Fmoc synthetic strategies in its sulfoxide form. At the same time it can be reduced to the sulfide form and then easily cleaved by mild acidolysis affording the peptide amide. Due to these properties the solid phase synthesis may start with Boc strategy and it can continue by Fmoc strategy. At the end of the synthesis all *tert*-butyl based protecting groups can be cleaved by trifluoroacetic acid and after the reduction of sulfoxide groups, the same reagent can be used for the cleavage of the peptide from the carrier in the form of an amide. We have used this approach in the model synthesis of deamino-carba-1-oxytocin shown in Scheme 3. The first three steps of the synthesis were performed manually using Boc strategy and the rest was performed using the centrifuge and Fmoc strategy. *Tert*-butyl based side-chain protecting groups were cleaved by TFA, and the cyclization was performed with the peptide still attached to the carrier. The reduction of the sulfoxide and acidolytic cleavage forming carboxy terminal amide was performed in one step using 1M trimethylsilylbromide in TFA/thioanisole.

In general, the two step deprotection and cleavage from the solid support is advantageous since it is not necessary to expose the peptide in solution to the large number of cationic species generated during the cleavage of protecting groups. The only cation generated during the splitting of the peptide from the carrier is bound to the solid support and may be effectively trapped with thioanisole [13]. Therefore we believe that the new SCAL handle will find wide application in peptide synthesis methodology.

Conclusion

Textile-like carriers are viable alternatives to the classical pellicular ones. The design of an automated machine is substantially simpler and the feedback monitoring of the reaction steps is relatively straightforward. The developed machine should be commercially available soon.

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