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COMPAS 242. NEW TYPE OF MULTIPLE PEPTIDE SYNTHESIZER UTILIZING COTTON AND TEA BAG TECHNOLOGY

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A new type of multiple solid phase peptide synthesizer is described. Comparative studies utilizing various solid phase materials (cotton, aminomethyl polystyrene, polyoxyethylene-modified polystyrene (TentaGel)) were performed and the versatility of the instrument was demonstrated. COMPAS 242 is the first multiple peptide synthesizer (i) without reaction vessels; (ii) without robotic arm, and (iii) without any solenoid valves.

INTRODUCTION

The rapidly growing demand for synthetic peptides in various fields of biomedical research has led to the development of a variety of multiple peptide synthesis methods. The common feature of all of these methods is the use of compartmentalized solid supports, which are either beaded resins contained in polypropylene mesh bags (1), syringes (2) or columns (3), or segmental carrier materials such as pins (4), paper (5), cotton (6,7) or membranes (8). The multiplicity was accompanied by miniaturization of the synthesis. Whereas single peptides were usually synthesized in a milli-molar scale, multiple peptide synthesis is carried out in a micro- or even nano-molar scale, and still provides sufficient amounts of peptides for most screening assays.

The logical continuation of this development was the construction of automated multiple peptide synthesizers (e.g. 9,10). The principal design of these synthesizers is similar. It was adopted from robotic systems that are used for autosamplers. The robots, together with a workbench carrying a multi-column reactor and the reagent containers, make up a workstation for the simultaneous synthesis of up to 96 different peptides in micro-molar scale. Controlled by a computer, this work station allows for completely automated operation. Zuckerman et al. (11) introduced a system capable of the automated synthesis not only of individual peptides, but also of equimolar peptide mixtures.

COMPAS 242

We have designed and constructed a different type of automated multiple peptide synthesizer without a robotic arm, thus reducing the number of moving parts, and even without reaction vessels. This synthesizer (COMPAS 242, Figure 1) was originally designed for the use of cotton as the solid support. Later on it was found, however, that it works as well with "tea bags" (1), which can be filled with any resin, thus greatly enhancing the versatility of the instrument. The central part of the synthesizer is a centrifugal plate **1**, which either moves in defined steps to position the carrier

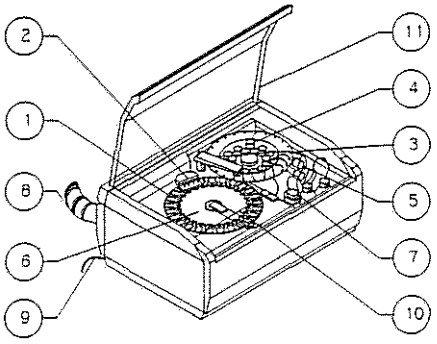

Figure 1: Schematic drawing of COMPAS 242. 1 - Centrifugal plate; 2 - Dosing head (delivery of DMF, DIC/DMF, Piperidine/DMF, Bromophenol blue/DMF by four independent lines); 3 - Pneumatically operated arm; 4 - Carousel with amino acid solutions in vials equipped with spray pumps; 5 - Gear pumps; 6 - Rotor; 7 - Vessels with reagent solutions; 8 - Exhaust; 9 - Liquid waste line; 10 - Safety cover; 11 - Cover.

compartments, or spins fast to remove reagent and wash solutions from the carriers. The first version of this instrument was described earlier (12).

The cotton squares (3x3 cm, approx. 20 μmols per square) or resin filled polypropylene mesh packets (25-75 mg, approx. 20 μmols per bag) are heat-sealed to polypropylene frames and mounted to shallow wells on the perimeter of the centrifugal plate. Fmoc-amino acids, along with HOBt, are dissolved in DMF (0.3 M solutions) and arranged in vials, which are equipped with spray pumps (Figure 2), on a circular rack **4**. The amino acid solutions are dispensed by pushing the spray pumps by a pneumatically operated arm **3**. The dispensed volumes are controlled by the mechanically adjustable volume delivered by one stroke of a spray pump and the number of these strokes.

Wash and deprotection solutions (DMF and 20% piperidine/DMF, respectively), as well as the coupling reagent (2 M DIC/DMF) are dispensed by gear pumps **5** in completely independent lines, avoiding the use of multichannel valves as well as the danger of cross-contamination. They are delivered by the gear pumps. The optical sensors detect the front of the solvent and the delivered amount of the reagents is then controled by the time elapsed.

The centrifugal plate **1** and the circular amino acid rack **4** both can accomodate up to 24 units (carriers and amino acid vials, respectively). They are arranged side by side with an overlap of one unit, so that one carrier is always positioned under one amino acid spray pump outlet at a time.

A typical synthesis cycle begins with the Fmoc-deprotection. The carriers are successively positioned under the outlet of the piperidine line by means of a stepper motor, which is identical to the centrifuge motor, and 0.6 ml of the solution is dispensed to each carrier. Since this volume is

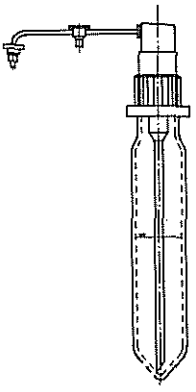

Figure 2: Mechanically actuated spray pump inserted in the amino acid reservoir.

approximately the same as the wetting (cotton) or inclusion (tea bags) volumes, no agitation (shaking, stirring, bubbling etc.) is necessary during operation. The piperidine solution is removed from the carriers by spinning the plate for 60 seconds at 1200 rpm. Then the carriers are repeatedly washed by dispensing 0.6 ml DMF successively on each synthetic compartment, followed by immediate centrifugation. After the last wash, 0.1 ml of bromophenol blue solution (0.05%) in DMF is delivered to all synthetic compartments, and the liquid is removed by centrifugation. Blue coloration of the carrier indicates the presence of free amino groups and its dissappearance signalizes completeness of the coupling (13,14). For the coupling step, the carriers are successively positioned under the overlap with the amino acid rack **4**, which is driven by another stepper motor to place the spray pump with the amino acid to be coupled above the carrier. After the spray pump has been pushed twice (total volume 0.45 ml), the carrier is positioned under the outlet of the DIC line and the DIC solution (0.1 ml) is dispensed. When the coupling is finished, the carriers are centrifuged and repeatedly washed with DMF. The liquid removed by centrifugation is collected in the waste bottle **9**, the vapors are being exhausted to the hood. The centrifugal plate **1** is protected by a safety glass cover which cannot be opened during centrifugation.

The instrument is controlled by a stand-alone IBM computer. The software is self-explanatory and organized in menus and submenus. The program of COMPAS 242 allows one to input the sequences of 24 peptides up to 40 amino acid residues long using the dedicated screen editor. This editor utilizes standard one letter code and all sequences are displayed at the same time. The software allows one to program an individual synthetic protocol; i.e. the operator can choose succession of single steps and their parameters - time delays after reagent addition and centrifugation time. At any moment, the actual status of the synthesis is displayed on the screen. The synthesis can be interrupted anytime, and manual step can be performed or actual status (starting point) can be changed. At the interruption, the active data are stored in a special file and after restarting the synthesis (e.g., the next working day when for any reason the synthesis is not performed continually), the data are retrieved and process continuity is assured. The progress of the synthesis is monitored periodically and stored in the file. Therefore even in the case of unexpected interruption (power failure) the stage of synthesis before the breakdown can be reconstructed. Additional features of the software include information about all volumes of solutions and amounts of amino acid derivatives needed for the synthesis, as well as molecular weights of synthesized peptides. In addition to the twenty natural amino acids, four more amino acids can be used in the remaining positions of the amino acid rack and, after editing their molecular weights, they can also be included in the calculation. To edit a synthesis file, the operator can load one of the two standard files for the cycle step order. The first standard file is for single couplings (deprotection: 20 min, 4 washes; coupling: 90 min, 4 washes), and the second one for double couplings (deprotection: 20 min, 4 washes, coupling: 60 min, 3 washes; recoupling: 60 min, 4 washes). The operator may as well, however, edit an individual step order for each synthesis. After editing the amino acid sequences of the peptides to be synthesized, the computer calculates the amounts of solvents, reagents and amino acids needed, both in terms of volume and weight.

According to the calculated data, the operator fills the reagent bottles and amino acid vials and starts the synthesis, which can run unattended (e.g., overnight). After the synthesis is finished, the carriers are detached from the plastic frames (when using tea bags, the resin is taken out of the bags), and the peptides are cleaved by standard methods.

Over a one year period, three prototypes of COMPAS 242 were evaluated in four independent laboratories (Prague, San Diego and Tucson). We have synthesized and analyzed more than one thousand peptides ranging in length from 6 to 20 amino acids. As the solid supports we used cotton squares (9 cm^2, 0.12 mmol/g, 20 μmols per square), as well as "tea bags" filled with either 25 mg of aminomethyl polystyrene/1% divinylbenzene resin (0.6 mmol/g, 15 μmols per bag) or 75 mg of TentaGel resin (15) (0.24 mol/g, 18 μmols per bag). To provide amino groups, the cotton was acylated with glycine prior to the synthesis (6). Before starting the amino acid assembly, a TFA

cleavable linker (Fmoc-2,4-dimethoxy-4'-(carboxymethyloxy)-benzhydrylamine) was coupled to the amino groups of the carriers. Alternatively we have synthesized the peptides on glycine-cotton and applied ammonolysis or hydrolysis for their detachment after side chain deprotection. In general, the single coupling protocol is used for up to octa-peptides, and the double coupling protocol for longer peptides.

The peptides listed in Table 1 were synthesized for comparison on all three carriers mentioned above. Whereas the standard double coupling protocol was used for cotton and TentaGel, another cycle step order that takes into consideration the swelling properties of polystyrene resin, was used for the aminomethyl resin (deprotection: 2+20 min, 3 DMF washes, 1 methanol wash, coupling: 60 min, 1 DMF washes, 1 methanol wash, re-coupling: 60 min, 3 DMF washes). Since the DMF removal by centrifugation is much less efficient for polystyrene/divinylbenzene resin than it is for cotton and TentaGel resin, the centrifugation time was doubled (60 vs. 30 sec, 120 vs. 60 sec).

The peptides were cleaved as their C-terminal amides with TFA/DCM/triisobutylsilane/water 70:20:5:5 (v/v/v/v) for 3 hours, precipitated in cold tert.butyl methyl ether, centrifuged, re-suspended in tert.butyl methyl ether, re-centrifuged, and lyophilized from water, generally yielding 10-20 mg of crude peptide (for 6 peptides, however, the yield was less than 5 mg). When cotton was used as carrier, the solution was filtered before lyophilization in order to remove cotton fibres.

The crude peptides were analyzed by analytical HPLC and MS. As determined by HPLC detection at 215 nm), 4 out of the 27 peptides (alytesin and neurokinine A from aminomethyl resin, as well as allatotropin from aminomethyl resin and cotton) had a purity less than 50% and were contaminated with side products of up to 32%. According to the mass spectra, some of these were caused by incomplete deprotection of Arg(Pmc), or, in case of alytesin, Pmc-acylation of the tryptophan indole ring. Six peptides (adipokinetic hormone from both TentaGel and cotton, allostatin from both TentaGel and aminomethyl resin, as well as substance P and the ACP fragment from TentaGel) had a purity of more than 80%. The purity of the remaining 16 peptides was between 50 and 80%. Figure 3 shows HPLC chromatograms of some of the peptides and compares the three different carriers. In general, peptides synthesized on TentaGel, the most expensive of the three carriers, tend to be of higher purity than peptides synthesized on the other two carriers. In particular, this is true for longer peptides (bombesin). For shorter peptides, however, these differences are negligible. Two peptides (adipokinetic hormone and LH-RH) were of highest purity when synthesized on cotton.

It was also shown that polystyrene/divinylbenzene resin can be used with COMPAS 242, yielding peptides with good purity. Due to the special protocol, however, the synthesis is prolonged, and we therefore do not favor this type of resin for use with this instrument.

Figure 4 illustrates typical results from one synthetic run. In the same synthesis both short and long

Table 1: Peptides synthesized for comparison on three different carriers.

I : Adipokinetic hormone	(pGlu)LNFTPNWGT-NH_2
II : Allostatin 1	APSGAQRLYGFGL-NH_2
III : Allatotropin	GFKNVEMMTARGF-NH_2
IV : Alytesin	(pGlu)GRLGTQWAVGHLM-NH_2
V : Bombesin	(pGlu)QRLGNQWAVGHLM-NH_2
VI : LHRH (salmon)	(pGlu)HWSYGWLPG-NH_2
VII : Neurokinin A	HKTDSFVGLM-NH_2
VIII: Substance P	RPKPQQFFGLM-NH_2
IX : ACP (65-74)	VQAAIDYING-NH_2

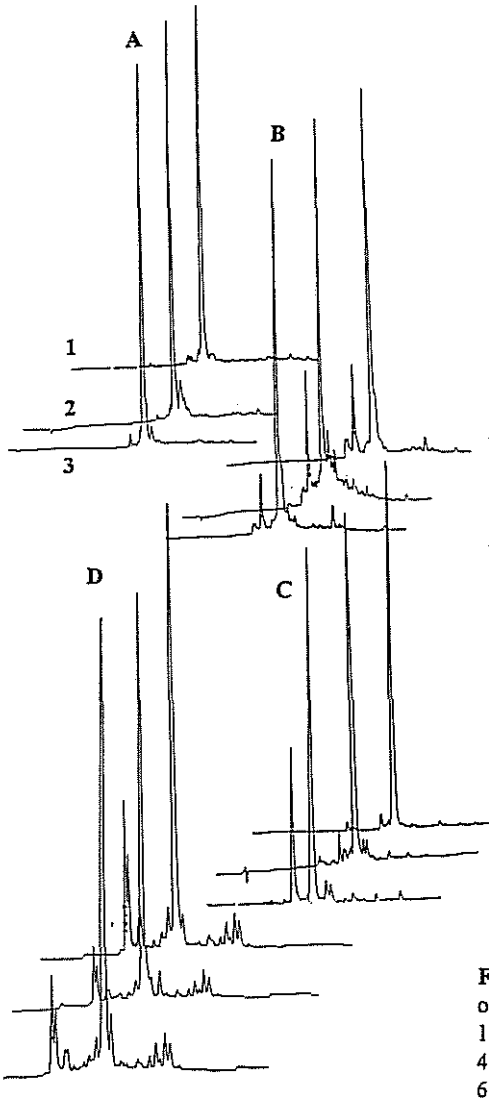

Figure 3: HPLC traces of crude peptides synthesized on various carriers.
A - Allostatin; B - Bombesin; C - ACP (65-74); D - LHRH; 1 - TentaGel; 2 - Cotton; 3 - NH_2CH_2-Polystyrene. Vydac C18 (25x0.4 cm), 1ml/min, 5-65% CH_3CN in 0.1% TFA in 30 min.

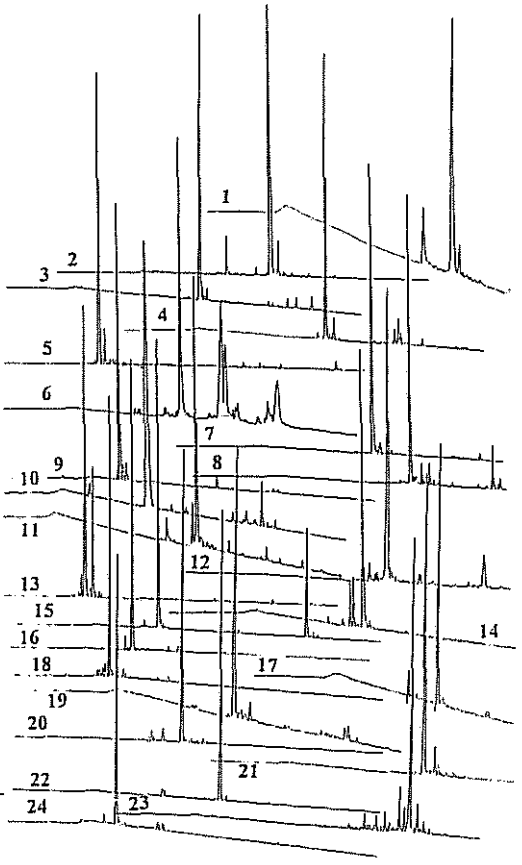

Figure 4: HPLC traces of peptides synthesized in one run on COMPAS 242 using cotton as carrier.
1 - RPPQQFFGLM; 2 - WLHID; 3 - YGGFLRRIR; 4 - YGGFLRRIRPKLK; 5 - EYRKD; 6 - VRGDKGNPGWPGAPY; 7 - YGGFLRRIRPK; 8 - YGGFLRRQFKVVT; 9 - FSQDH; 10 - GVPDI; 11 - DVPKSDQFVGLM; 12 - YPYDVPDYASLRS; 13 - QERYD; 14 - VQAAIDYING; 15 - NLYGQ; 16 - SEQVY; 17 - FLAVERKING; 18 - SNMDQ; 19 - RPPGFSPFR; 20 - FYSDM; 21 - YFLFRPRN; 22 - YAFDVVG; 23 - YYAYLASAVLEAIKN; 24 - SDDHM (all amides). Vydac C18 (25x0.4 cm), 1ml/min, 0-60% CH_3CN in 0.1% TFA in 30 min.

peptides of a different complexity were prepared, and only two or three of them did not meet our expectations.

CONCLUSIONS

COMPAS 242 has been shown to be a mechanically simple, yet powerful instrument for the automated multiple synthesis of up to 24 different peptides in yields sufficient for most screening programs, and in purities comparable to other synthesis methods. According to the length and "synthetic difficulty" of the peptides to be synthesized, either more sophisticated, but also more expensive, solid supports, such as TentaGel, or more simple and inexpensive carrier materials, such as cotton, can be chosen. COMPAS technology was patented (16) and COMPAS 242 is available from SPYDER Instruments, Inc.

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