Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 2351–2356

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

"CUT AND COMBINE": AN EASY MEMBRANE-SUPPORTED COMBINATORIAL SYNTHESIS TECHNIQUE

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Received 26 May 1998; accepted 16 July 1998

Abstract: A combinatorial synthesis process involving sequential cycles of cutting a membrane support into pieces and combining these into groups and subjecting the groups to simultaneous solid-phase chemical reactions is demonstrated by the rapid assembly of four hundred N-terminally biotinylated, soluble, octameric peptide pools. Index patterns printed onto the synthesis membrane allowed a direct identification of the compounds. These were used to study protein kinase substrate selection in a parallel microplate adapted ^{32}P-phosphorylation assay with subsequent spotting on a biotin-capture membrane.

Introduction

Methods for the simultaneous chemical synthesis and biological screening of large numbers of different compounds have attained considerable interest as novel approaches to the selection of biologically active molecules from molecular diversity. We have utilized membrane-type support materials for the simultaneous solid-phase synthesis of oligonucleotides and peptides for many years.[1-4] The basic concept of our approach is a segmentation of the support and the physical combination of segments into simultaneous chemical reactions for each assembly cycle.[5] Membrane segments that have been used so far are circular discs combined into reaction vessels/columns ("filter disc method") or spots on a membrane sheet ("spot synthesis"). We have expanded the panel of methods as described in the previous symposium-in-print edition[5] by a variation of the "filter disc method" that allows the rapid assembly of complete, combinatorial, compound libraries[6] and a direct identification of their components.

Results and Discussion

The principle of the method is demonstrated in Figure 1. We start with a larger sheet of paper that is cut into as many pieces as different monomers or pools of monomers (n1) are required in assembly cycle 1. Each piece is reacted individually with one of these monomer solutions. For the next synthesis cycle, each of the n1 pieces is cut again into n2 smaller parts according to the number n2 of different monomers in cycle 2. One part from each n1 piece is combined to give n2 new groups, each of which is then reacted with one of the n2 monomer solutions. This is repeated until a complete combinatorial library of hundreds to thousands of individual compounds or pools has been assembled. Chemical reactions are carried out with the paper segments placed in appropriately sized reaction troughs. For more delicate reaction conditions such as heating or cooling, exclusion of moisture and air, the segments are sealed in plastic bags; solutions of activated monomers are injected with a syringe and needle. All other common reaction steps are carried out in washing baths.

0960-894X/98/$ - see front matter
PII: S0960-894X(98)00425-9

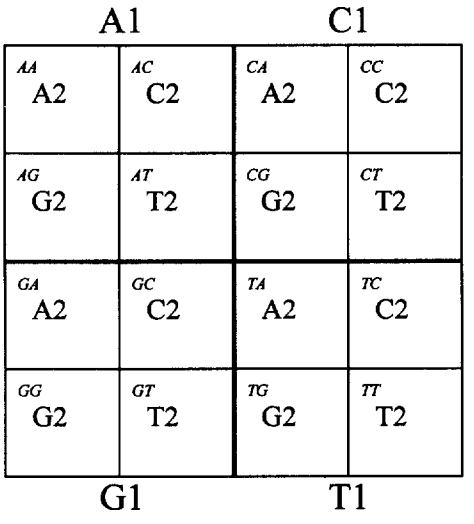

Figure 1. Example for a "cut and combine" combinatorial synthesis strategy using four monomers (e.g., nucleotides) per synthesis cycle. The parent sheet (=) is cut into four pieces (-). Each one is reacted separately with either A, C, G, or T monomer (reaction A1, C1, G1, and T1). Next, each of the pieces is cut again into four (---) and the upper left squares are combined into reaction A2, the upper right ones into C2, the lower left ones into G2 and the lower right ones into T2. This results in 16 pieces carrying one of the 16 possible dimers (*AA* to *TT*) each.
In the following cycles, this scheme of "cut and combine" is maintained: cutting of each segment into four pieces and combining the resulting upper left squares into a reaction with A monomer, the upper right ones into a reaction with C monomer etc. At the end of the synthesis all segments are rearranged to their original position in the parent sheet and the identity of the compound can be deduced.

Such type of combinatorial synthesis procedure has been described for "divisible carriers" made of cotton tissue or thread as well as for polymer membranes.[7] Here, we combine this approach with the special features of cellulose membranes (paper). In particular, we describe an easy way of indexing individual compounds by marking the corresponding membrane segments. Cellulose paper can be readily labelled by a variety of well known and

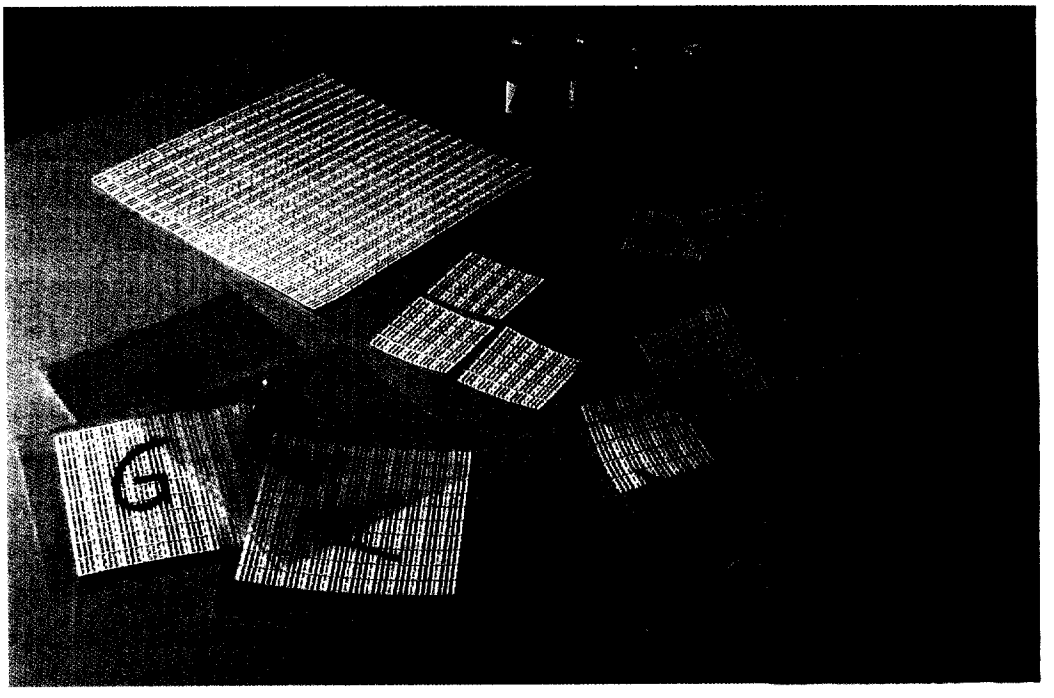

Figure 2. Demonstration of an experimental arrangement during the "cut and combine" combinatorial chemical synthesis showing also a typical index pattern printed onto a synthesis sheet.

easily available methods such as writing with a pencil or by utilizing more delicate printing technologies such as laser printers (Figure 2). The black carbon deposite is stable under most chemical reaction conditions. Text or graph editing software can then be used to generate an intelligent index pattern and this is directly printed onto the synthesis sheet. The information content per segment of this index pattern should allow the immediate perception of the relative orientation of each segment to the parent sheet. This could be achieved by, for example, an ordinary text file. A uniform procedure for cutting, combining, and reacting the segments (Figure 1) finally results in a known order of different structures, which is retrieved after the pattern has been reconstituted from all segments like a puzzle. A more elaborate information content per segment index (Figure 2) would even allow a direct identification of its position on the parent sheet and, thus, the structure of the respective compound attached.

To prove this concept we constructed a library of 400 soluble octamer peptide pools with the general format biotin-X-X-X-2-1-X-X-X (with X representing an equimolar mixture of all 20 genetically coded amino acids and 1, 2 only one individual amino acid), which can be subjected to a variety of biological assays exploiting the biotin tag. A corresponding 20 × 20 cm^2 sheet of Whatman 1Chr paper was chosen as the synthesis membrane so that each of the 400 product pools with a defined combination of amino acids at positions 1 and 2 will be obtained on a final 1 cm^2 piece of support. We first tested a number of different printer models and photocopying machines for the generation of an index pattern that is stable under the standard reaction conditions used in Fmoc-peptide synthesis. The model HP LaserJet III™ gave the best results probably because this older device works at a higher printing temperature than newer ones. A pattern of 20 × 20 squares, each one containing a well distinct index script for the respective pool composition (e.g., T03 for threonine at position 1 followed by aspartate, the third amino acid in a one letter code list, at position 2) was designed with a text editing software and printed directly onto the synthesis sheet. To increase safety and clearness, the code was printed in multiple copies on every square: 

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For a cleavage and isolation of the final products with a minimum of purification effort, we functionalized the paper with the DKP forming anchor of Bray et al.[8] by first esterifying Fmoc-proline to the cellulose followed by the coupling of Boc-Lys(Fmoc)OH.[9,10] The resulting support capacity of 1.5 μmol/cm^2 yields about 1 mg quantities of the final products. The peptides were then assembled on the ε-amino function of the lysine.[11] Random X-positions were introduced by double couplings with 0.8 equivalents each of an equimolar solution of all amino acid derivatives.[12] After the third synthesis cycle the sheet was cut into 20 stripes following the imprinted code. Each of these stripes was reacted individually with one amino acid derivative according to its signature ("A" with Fmoc-Ala, "C" with Fmoc-Cys, etc). For the next cycle, each of these 20 stripes was cut into 20 squares and all the "01"-indexed ones were collected in one reaction bag, all "02"-indexed in another bag, etc. For the last four coupling steps, including the final biotinylation, all 400 squares were combined again. After the joint removal of the side chain protecting groups, the squares were distributed individually into 400 labelled tubes and the peptide-diketopiperazides were released into an aqueous phosphate buffer (pH 7.5) to a final concentration of 250 μM.

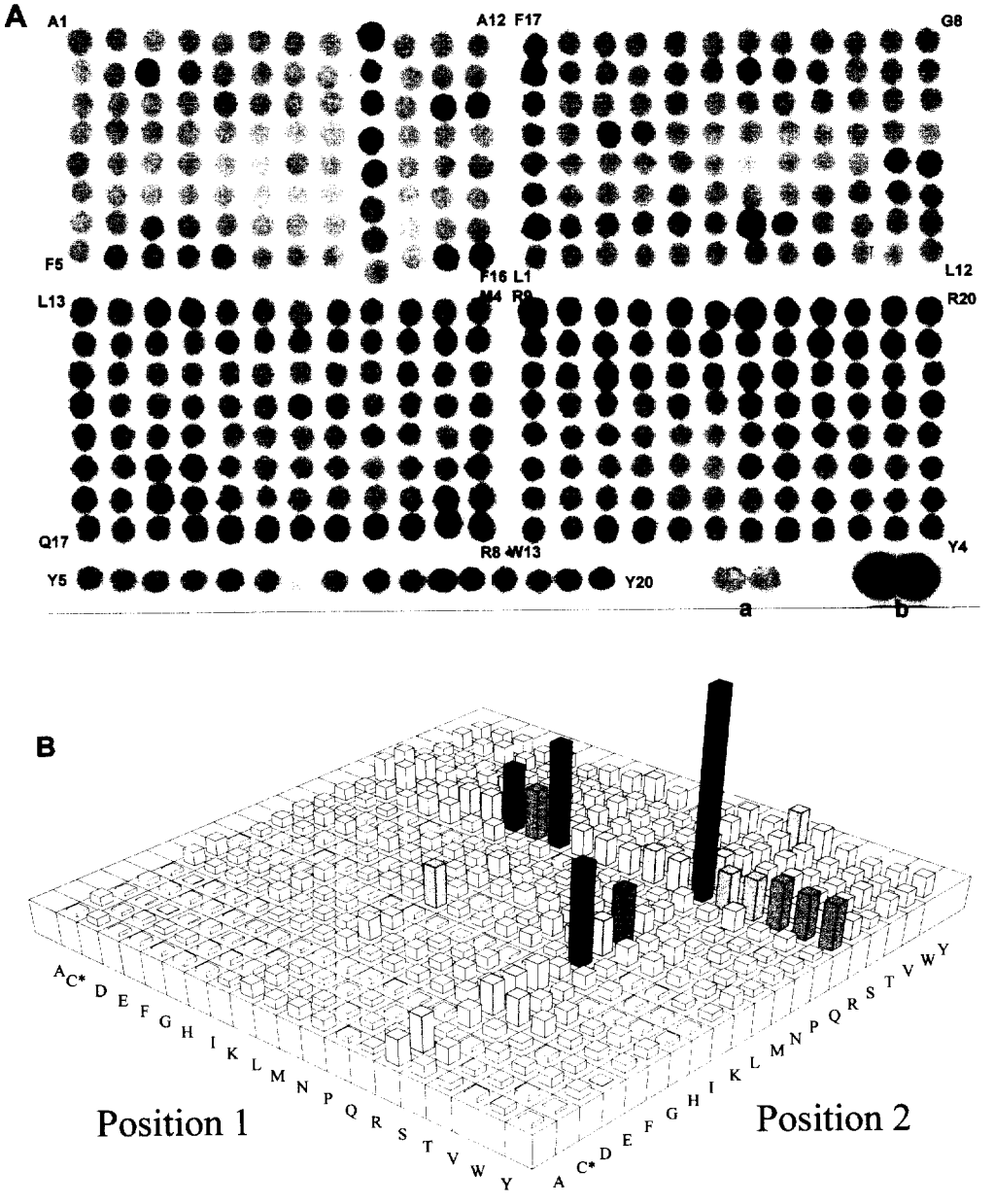

Figure 3. (A) PhosphorImager printout obtained from the streptavidin-membrane spotted with the 400 aliquots of the phosphorylation reactions. The direct transfer from the microtiter plates onto the membrane gave the 4 x 96 arrays; the remaining 16 reactions (to complete the 400) are the spots below. a and b are control reactions (a: Ac-RALRRASL-DKP; b: Biotin-RALRRASL-DKP, reduced in intensity by a factor of 60). (B) 2-D bar diagram showing the correlation of the integrated spot pixel values to the amino acid combination at positions 1 and 2 in the peptide pools [C* = Cys(Acm)]. The height of the columns represents the intensities of the signals in a linear dependence.

The biological screening of this peptide library and, thus, the practicability of the synthesis concept was demonstrated by a peptide phosphorylation assay to select for the best substrates of the catalytic subunit of protein kinase A.[13] The assay of the 400 peptide pools was adapted to the parallel performance in 96-well microtiter plates[14] by modifying a published procedure.[15] For a corresponding parallel determination of the ^{32}P-label incorpo-rated into the peptide substrates, the biotin tag was exploited by the use of a specially manufactured streptavidin-membrane[16] which allows first to spot small aliquots of the reaction mixtures next to each other, second to remove excess [γ-^{32}P]-ATP simultaneously by a simple but effective washing of the membrane and third an easy quantification of adsorbed radiolabel with the PhosphorImager.[17] The result of such a screening is shown in Figure 3. As expected, basic residues at positions 1 and 2 gave the strongest signals with -R-R- being the best. This is in perfect agreement with results obtained from a similar peptide library immobilized as spots on a paper membrane.[15] The reliability and efficiency of this spotting assay was proven with the two reference substrates, acetyl- or biotinyl-RALRRASL-DKP. Both are efficiently phosphorylated (phosphocellulose assay). The biotinylated one gives a strong signal on the membrane, whereas the acetylated one shows only background. A different and equally efficient type of biotin-capture membrane is commercially available from Promega Corp., Madison, USA.

In conclusion, we have demonstrated an easy way of utilizing a segmentable cellulose membrane support for the generation of combinatorial compound libraries exploiting features of the "filter method",[1–3] which are the simple indexing, handling, and grouping of support compartments. However, the tedious and time consuming track-keeping of the many individual segments through the synthesis cycles by manual sorting and redistribution is avoided. Only a simple scheme of cutting segment by segment into sub-segments and directly distributing these into the new groups for the next monomer additions is required (Figure 2). This scheme is either uniform through all cycles if always the same number of monomers is used or it is varied from cycle to cycle and then only needs to be recorded for the final decoding of the compounds. The imprinted index pattern easily allows to identify the orientation of each segment at any synthesis step and guides the distribution of the sub-segments to the separate reaction vessels. Finally, the identity of each compound can be deduced from the position of its segment in the parent sheet or directly from the index script on the segment. The smooth surface of some commercially available paper qualities and the high resolution of the laser printer technology should allow a size of the final segments of about only 2×2 mm^2. Respectively, 10,000 compounds at a scale of about 50 nmol can be synthesized starting with one single 20×20 cm^2 sheet.

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9. *Preparation of the synthesis membrane*: A pattern of 400 cm^2 squares containing a respective index code was printed onto a 20 × 20 cm^2 sheet of chromatography paper (Whatman 1Chr) and excess toner was washed out with three times DMF, NMP and ethanol (250 mL for 10 min). After drying the paper in a vacuum overnight at 90 °C, Fmoc-proline was reacted as preformed symmetrical anhydride (0.3 M in 10 mL dry DMF) in the presence of 1.7 equiv of N- methylimidazole for 5 h in a sealed polyethylene bag. Excess reagents were washed out with DMF, Fmoc was cleaved with 20% piperidine in DMF (twice with 250 mL for 10 min) and the sheet was washed three times with DMF, twice with ethanol, and dryed. A loading of 1.8 μmol/cm^2 was determined by quantitative bromophenol blue staining.[10] Boc-Lys(Fmoc)OH was then coupled as described under *Peptide Synthesis*.[11]
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11. *Peptide Synthesis*: Amino acid derivatives and reaction conditions were as described.[10] 20 μL of preactivated amino acid (HOBt ester) was used per 1 cm^2 of synthesis membrane sealed in polyethylene bags. All synthetic steps were followed with the bromophenol blue stain on an extra control sheet of 30 cm^2. This was subjected to the same treatments and only small pieces were removed for the determination of coupling yields. About 0.5 μmol of final products were obtained as determined by amino acid analysis of 12 randomly taken samples.
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14. *Protein kinase A assay performance:* 1 nmol (4 μL) of each of the 400 peptide pools was pipetted in numerical order from A1 to Y20 into 96-well microtiter plates. The plates were sealed and kept at 30 °C. 40 mL of reaction buffer [45 mM MOPS (pH 6.9), 0.10 mg/mL BSA, 0.10 mM ATP (500–1000 cpm/pmol), 10 mM DTT] was passed over a filtration membrane (0.2 μm) and 40 μg of catalytic subunit of protein kinase A in 4 mL buffer [50 mM MOPS, 15 mM Mg acetate, 4 mM EGTA, 200 mM NaCl, 0.1% BSA (pH 6.9 adjusted with NaOH)] was added at 30 °C. The enzymatic reaction was started by distributing 96 μL aliquots to the peptide solutions into each well. After a 3 min incubation the reactions were stopped by the addition of 50 μL of 7.5 M guanidinium hydrochloride per well. The sealed plates were stored at +4 °C.
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16. *Manufacturing of covalently modified Streptavidine-membrane sheets*: A 12 × 12 cm^2 sheet of amino-PEG modified chromatography paper (0.5 μmol NH_2 /cm^2; AIMS Scientific Products, Braunschweig, Germany) was treated with 50 mL of 0.5 M glutardialdehyde in 50 mM phosphate buffer (pH 7.2) for 40 min at room temperature on a rocker plate. After washing (5 times for 10 min in the same buffer) and air drying it was incubated with 2 mL of streptavidin solution (1 mg per mL in 10 mM Na acetate pH 8.3) for 2 h at room temperature sealed in a plastic bag. The membrane was air dried again, then treated with 50 mL of sodium borohydride solution (30 mM in 10 mM sodium phosphate pH 7.2) for 15 min, washed with 100 mL each of 10 mM sodium phosphate pH 7.2 (3 times for 10 min) and 2 M NaCl (twice for 15 min) on a rocker plate and finally dried. An array of 400 pencil dots was drawn on the membrane to mark the positions for the application of the sample aliquots (a distance of 5 mm between 2 dots is sufficient for 1 to 1.5 μL of sample).
17. *Assay Evaluation:* Aliquots of 1.5 μL from each well were transferred to their assigned positions on the streptavidin-membrane and allowed to diffuse into the membrane structure. 15 min after the last pipetting step the sheet was intensively washed with 250 mL each of 4 M NaCl, 4 M NaCl plus 1% H_3PO_4 (3 times for 10 min on a rocker plate), 8 M guanidinium hydrochloride plus 1% SDS (twice for 30 min at 50 °C under sonification) and H_2O (5 times for 10 min). The sheet was then dried and sealed in a plastic bag. Radioactivity was determined with the PhosphorImager system (Molecular Dynamics) and quantified by integrating the resulting circular spots.