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A New Hybrid Resin for Stepwise Screening of Peptide Libraries Combined with Single Bead Edman Sequencing

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Abstract: A library system was developed for the discovery of bioactive peptides. Library synthesis and peptide sequencing was performed on a solid support while the screening for bioactivity was done with peptides in solution. The peptides were synthesized by split and mix, one-bead–one-peptide library synthesis, using a Tentagel S-NH_2 solid support with a loading of approximately 100 pmol/bead. The major part of the peptide was connected to the support by a single acid-labile linker and a minor part of the peptide was acid-stabile attached to the polymer. The percentage of acid-stabile attached peptides could easily be controlled during modification of the amino functionalities of the resin at the start of the process. The cleavage rate of the acid-labile attached peptide from the resin depends on the composition of the cleavage mixture. When cleavage conditions were carefully controlled, a three-step partial cleavage protocol allowed for convergent bioactivity screening on peptide libraries using only one type of acid-labile linker. The partial cleavage and convergent screening procedure was repeated three times, after which the bead containing the bioactive peptide was sequenced. As such a bead still contained acid-stabile attached peptide, the Edman sequencing was straightforward and repetitive yields were excellent because the immobilized peptide was not washed out. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Synthetic peptide library; one-bead–one-compound; partial cleavage; soluble phase screening

INTRODUCTION

Since the introduction of the first random peptide library in 1986 [1], a number of variants have been developed and used successfully for the detection of ligand binding peptides [2–9]. Approaches in which the peptides are attached to the solid support during screening are useful for obtaining information on binding to biomolecules that are in solution. However, most cellular bioassays are hard to perform with resin-bound peptides. Synthetic library approaches that use peptides in solution need several rounds of converging synthesis and are therefore labour-intensive and have, to our knowledge, so far only been successfully applied with relatively short peptides [5]. Approaches that combine the handling of resin-bound peptides with screening of peptides in solution use resins with orthogonal cleavable linkers [10]. These methods are not widely applied, probably because of the complex chemistry needed to obtain solid supports with different linkers. Recently hybrid resins were used to synthesize a peptide library on beads that already contained a fluorogenic enzyme substrate in order to identify potential enzyme inhibitors [11].

Abbreviations: ACH, α-cyano-hydroxycinnamic acid; DMA, *N*,*N*-dimethylacetamide; Gaba, gamma-aminobutyric acid; NMM, *N*-methylmorpholine; NMP, *N*-methylpyrrolidone; PyBop, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; DNP, 2,4-dinitrophenyl.

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Here we present a novel system that applies resin-bound selection combined with solubilized peptide screening. In our newly developed system only one type of acid-labile linker is required for stepwise cleavage. A gamma-aminobutyric acid (Gaba) residue is inserted at the C-terminus of the peptide to make the cleavage rate independent of the C-terminal amino acid. After screening of peptide pools in solution, selection is done at bead level. Application of this method allows for at least three subsequent screening rounds. Since a hybrid-resin (a resin that contains acid-labile and acid-stabile attachment sites) is used for synthesis, identification of the selected bioactive peptides can be done by Edman sequencing of bead-bound peptides. Owing to the basic character of our approach it is generally applicable. No complex chemistry is needed, and the application of the system is independent of peptide length and allows for a variety of bioassays as long as the sensitivity of the assay is in the nanomolar range.

We show how the ratio cleavable versus non-cleavable peptide can be controlled in the first step of the synthesis and how the cleavage of the peptide pools can be controlled by reaction conditions. This library approach was successfully applied to identify peptides that stimulate CD4 + T cells.

MATERIALS AND METHODS

Synthesis of Beads Containing Cleavable and Non-cleavable Peptides

Tentagel S-NH_2 synthesis beads were used. These beads consist of a copolymer of polystyrene and polyethyleneglycol, particle size 90 μm, loading about 100 pmol/bead. Tentagel S-NH_2 (9.0 g, 0.26 meq/g) containing 2.34 mmol amine groups was washed with 3 × 50 ml 20 vol% piperidine in DMA and with 6 × 50 ml NMP. The initial coupling reaction was performed with a mixture of 5(4-Fmoc-aminomethyl)-3′,5′-dimethoxyphenoxy)valeric acid linker (yielding a peptide–amide after cleavage) [12] and Fmoc-Nle-OH for the permanent attachment of the peptide. Thus, for a 10:1 molar mixture, a solution of 3.220 g (6.380 mmol) linker in 16.5 ml NMP was mixed with a solution of 0.225 g (0.638 mmol) Fmoc-Nle-OH in 2.3 ml NMP (total 7.02 mmol acylating material, 3-fold molar excess). The mixed reagents were added to the resin and shaking was applied to form a homogeneous suspension. A solution of 3.74 g (7.02 mmol) PyBop and 1.42 g NMM (14.04 mmol) were added successively. The reaction mixture was shaken to obtain a homogeneous suspension and was allowed to react for 3 h. The resin was filtered over a glass filter and washed six times with 50 ml NMP.

Analysis of the Hybrid Resin

Part of the resin (approximately 200 mg) was washed with 3 × 3 ml CH_2Cl_2 and 3 × 3 ml ether, respectively, and dried under vacuum (16 h). Of the resin thus obtained, 92 mg was suspended in NMP and divided in two equal portions on two separate small filter units. In order to determine the total peptide loading, the total Fmoc content of portion 1 was measured. Portion 1 was treated 2 × 3 min with 1 ml 20 vol% piperidine in DMA and washed with 10 × 1 ml NMP. The combined washings were diluted to 50 ml with 96% ethanol and quantified by UV at 300 nm. Portion 2 was washed with 3 × 1 ml CH_2Cl_2 and 3 × 1 ml ether and dried on air (1 h). The resin was treated with 1 ml TFA/water (19/1, v/v) for 3 h, washed with 5 × 1 ml CH_2Cl_2, 3 × 1 ml CH_3CN, 3 × 1 ml water, 3 × 1 ml CH_3CN, 5 × 1 ml ether and dried. The remaining Fmoc was removed and quantified as described for portion 1.

To verify the findings by Edman sequencing Ile-Phe-Gaba was synthesized on the hybrid resin obtained. After peptide synthesis the resin was treated for 3 h with 95% TFA and washed thoroughly to remove acid-labile bound peptide. A known number of beads containing the remaining acid-stabile bound peptide were applied to the sequencer.

Peptide and Library Synthesis

Peptides and synthetic peptides libraries were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422) [13]. Synthetic peptides and libraries were synthesized as peptide amides as described before [14] with a C-terminal gamma-aminobutyric acid (Gaba) residue to make the cleavage rate of the peptides independent of the C-terminal amino acid. The purity of the peptides was determined by analytical reversed-phase HPLC using a water–acetonitrile gradient containing 0.1% TFA, and proved to be at least 80% (UV 214 nm). The integrity of the peptides was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (TOF-MALDI) on a Lasermat mass spectrometer (Finnigan MAT, UK). About 5 pmol of the peptide in 0.5 μl water/acetonitrile containing 0.1% TFA was mixed with 0.5 μl of matrix solution (ACH, 10 mg/ml in

acetonitrile/water (60/40, v/v) containing 0.1% TFA) and allowed to dry after which the target was applied to the instrument. Internal calibration was performed with peptides of molecular mass 1422.7 and 1896.1 Da.

One-bead–one-peptide library synthesis was performed using the hybrid resin described above. Repetitive couplings were performed using Fmoc amino acid/PyBop/NMM chemistry. Deprotection was done with 20 vol% piperidine in DMA. After each synthetic cycle the resin was suspended in 1,2-dichloroethane/acetonitrile (82/18, v/v), vortexed for 5 min and subsequently divided again over the various reactors (mix and split). After synthesis the peptidylresin was washed extensively with DMA, dichloromethane, dichloromethane/ether (1/1, v/v) and ether respectively, and dried.

Determination of Suitable Conditions for Partial Cleavage of Peptides

Two peptide–amides containing an N-terminal dinitrophenyl-glycine (DNP-Glycine) residue were synthesized as described above: DNP-GFKALGKVA-Gaba and DNP-GILGFVFTL-Gaba. DNP-labelled peptides in solution were quantified by UV at 300 nm, after cleavage of the peptide from the hybrid resin.

Peptide Cleavage and Screening

Synthesis of the hybrid resin and partial cleavage of peptides from the hybrid resin, which makes library screening feasible, are schematically shown in Figure 1. Screening was performed with some minor alterations compared with the protocol described in earlier studies [15]. Library beads were suspended in 1,2-dichloroethane/acetonitrile (82/18, v/v) and dispensed in multiscreen-PFI 96-wells PP-plates with hydrophillic PTFE membranes (pore size 1 μm) (Millipore) to 20000 beads per well. Beads were washed on a filtration assembly (Millipore) with 2 × 200 μl CH_2Cl_2 and 3 × 200 μl ether per well and dried at the air. Cleavage of the first quarter of the peptides was performed with 150 μl TFA/CH_3CN/water (10/9/1, v/v/v) per well. After 90 min the solution was collected in 96-wells serocluster PP U-bottom plates (Costar). Beads were washed with 75 μl of the same mixture and combined filtrates were dried under vacuum. Peptide pools were treated for 3 h with TFA/water/ethanethiol (18/1/1, v/v/v) to remove all side-chain protecting groups and dried with an N_2-flush system. Each peptide pool was dissolved in 5μl DMSO of which aliquots were used for screen-

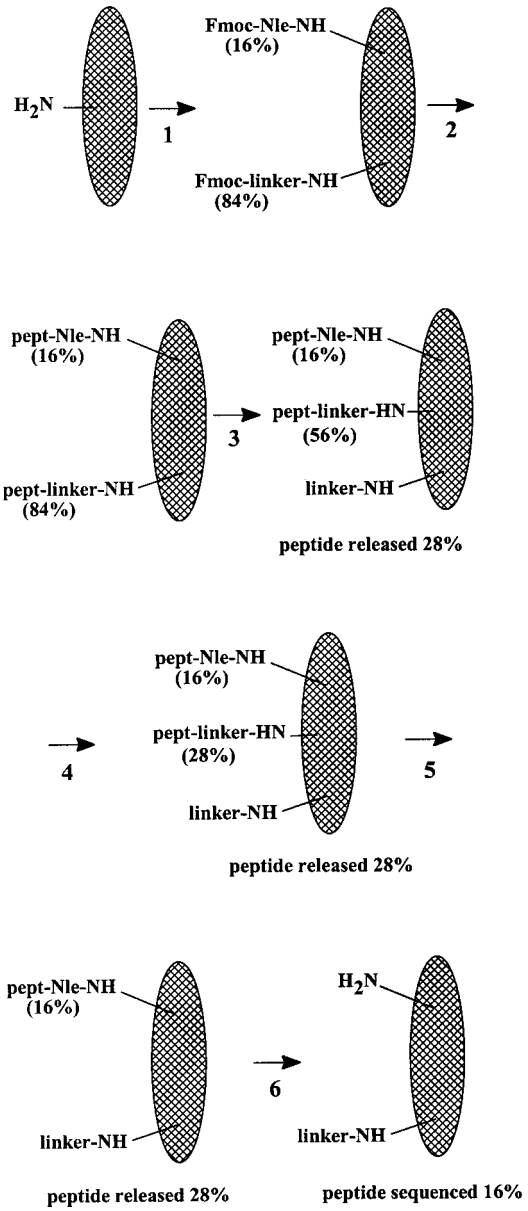

Figure 1 Schematic representation of one bead during: hybrid resin synthesis, peptide library synthesis (one-bead–one-peptide), stepwise library screening and single peptide identification. Percentages shown indicate the amount of peptide. Total peptide quantity of one bead immediately after library synthesis = 100%. **1**: A mixture of Fmoc-linker and Fmoc-Nle-OH is coupled to TentaGel S-NH_2, yielding a hybrid resin. **2**: Peptide is synthesized on the hybrid resin. **3**: A first part of acid-labile attached peptide is cleaved off, to be used in the first cycle of solution screening of the library. **4**: A second part of acid-labile attached peptide is cleaved off, to be used in the second cycle of solution screening of the library. **5**: The remaining part of the acid-labile attached peptide is cleaved off, to be used in the last cycle of solution screening of the library. **6**: The acid-stabile attached peptide is used for identification of the peptide by Edman bead sequencing.

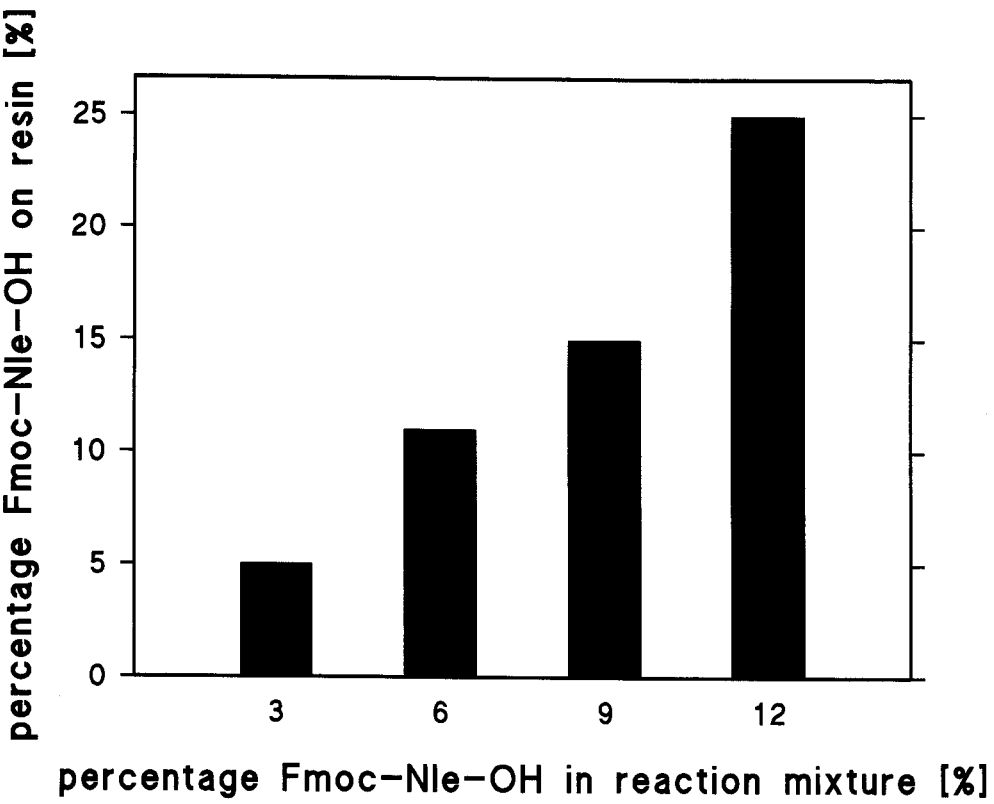

Figure 2 Correlation between the molar percentage Fmoc-Nle-OH in the coupling mix (Fmoc-Nle-OH) and Fmoc-linker) during hybrid resin synthesis and the molar percentage Fmoc-Nle-OH on the resin after synthesis.

ing. The resin pools were washed twice immediately after the TFA treatment with 200 μl 0.5 M Tris pH 7.5/CH_3CN (1/1, v/v) and incubated for 1 h in 100 μl of the same buffer. After washing with CH_3CN/water (1/1, v/v), CH_3CN, CH_2Cl_2 and ether (all 200 μl/well) and drying, the beads were stored at −20°C. For the cleavage of a second quarter of the peptide from each bead, beads were incubated in TFA/CH_3CN/water (10/9/1, v/v/v) for 180 min, after which the same work-up procedure was used for solubilized peptides and resin beads. In the third step of the screening, beads were only incubated for 150 min with TFA/water/ethanethiol (18/1/1, v/v/v).

Sequencing of Peptide Linked to Resin Beads

Sequencing analysis of the peptide material bounds to the beads was carried out on a Hewlett Packard G1005A protein sequencer. One bead was pipetted into a sequencing cartridge which was applied to the sequencer. The use of ethyl acetate instead of chlorobutane in the extraction was found to improve the transfer (and thus the yield) of the anilinothiazolinone from the bead, especially in the case of the arginine derivative. Furthermore, it turned out to be essential to increase the delivery of the solvent from three to five portions of 10 s duration, each time followed by a pause of 10 s before transfer to the conversion flask. Apparently, the diffusion of solvents through the rather hydrophillic bead matrix is a relatively slow process. All peptides could be sequenced completely and repetitive yields were high owing to acid-stabile attachment of the peptide to the bead.

RESULTS AND DISCUSSION

Synthesis of the hybrid resin was performed as described above, using mixtures of different molar ratios of linker and Fmoc-Nle-OH. The percentage of acid-stabile peptide was determined based on the Fmoc-content of the resin before and after acid treatment. The coupling rate of Fmoc-Nle-OH was shown to be higher than the coupling rate of the linker, since the percentage of Fmoc-Nle-OH in the coupling mixture was always lower than on the resin after synthesis. The data of a representative

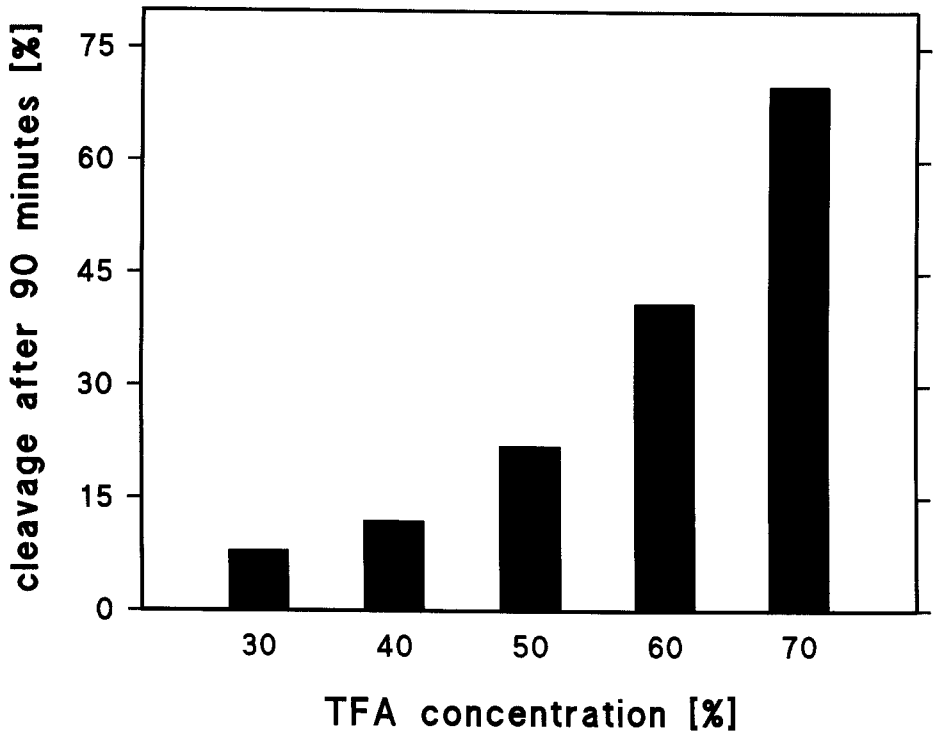

Figure 3 Correlation between cleavage rate of peptides from the hybrid resin (16% Fmoc-Nle-OH) and TFA concentration. The percentage cleavage of acid-labile attached peptides after 90 min is shown. The water concentration is constant for all incubations: 5% (v/v). Together the TFA concentration and the CH_3CN concentration are 95% (v/v) for all incubations.

experiment are shown in Figure 2. For further experiments and library screenings a coupling mix resulting in approximately 16% Fmoc-Nle-OH on the hybrid resin was chosen. This 16 pmol per bead guaranteed proper Edman sequencing of the attached peptide in all cases. The quantity of Fmoc-Nle-OH sites per bead was verified by sequencing a sample of 11 beads containing the peptide Ile-Phe-Gaba, after 3 h incubation in 95% TFA to remove all acid-labile attached peptide material. This yield 165 pmol Ile and 150 pmol Phe. Thus on average 11 beads still contain about 150–160 pmol. This is about 14 pmol per bead. Taking into account some material losses in the sequencer, this quantification is in good agreement with the numbers obtained from the spectrophotometric determination described above.

With the hybrid resin the TFA concentration and time dependency of the C-terminal Gaba peptide cleavage was determined. This was done by spectrophotometric quantification of peptides with N-terminal DNP-Gly. Results of experiments are shown for peptide DNP-GFKALGKVA-Gaba in Figure 3 and Figure 4. Cleavage rates were found not to depend on the sequence of the peptide (data not shown). To allow for three rounds of selection and screening of the library a mixture of TFA/CH_3CN/water (10/9/1, v/v/v) was chosen. Reaction times were elongated in the second selection round compared with the first selection round in order to cleave about the same quantity of peptide in both selection rounds (20–25 pmol/bead). This might reflect diffusion-limited cleavage in the bead or the existence of cleavage sites with different stability. As the same cleavage cocktail is used (TFA/CH_3CN/water, 10/9/1, v/v/v), the work-up procedure can be identical after both cleavages. Cleavage times increase from 90 min in the first cleavage to 180 min in the second, which represents a compromise: the reactions are slow enough to perform the experiments reproducibly but are still quick enough to enable efficient laboratory practice. After liberation from the resin, the peptides are treated with TFA/water/ethanethiol (18/1/1, v/v/v) to ensure the removal of all side-chain protecting groups. The washing and storage of the resins after partial cleavage is an important aspect of the procedure. Short washings to neutralize the acid in the beads were insufficient to stop the cleavage reaction. This is probably due to the diffusion-controlled mass

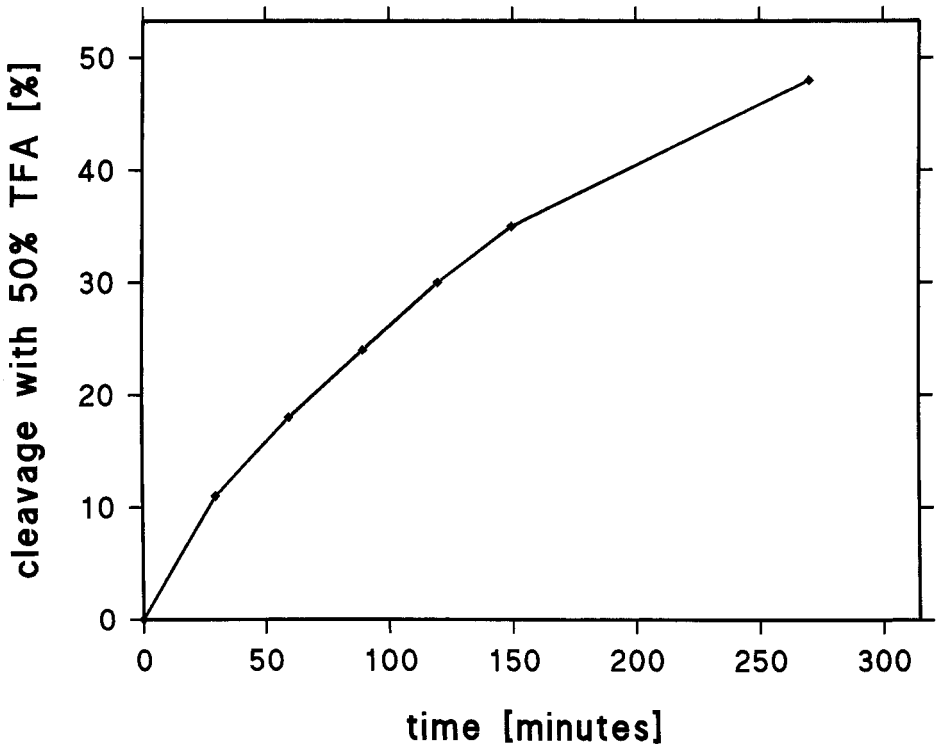

Figure 4 Time dependency of the cleavage of acid-labile attached peptides from the hybrid resin (16% Fmoc-Nle-OH). The percentage of cleaved peptide using TFA/CH_3CN/H_2O (10/9/1, v/v/v) at various time points is shown.

transport of reagents and solvents in and out of the beads. It is essential that beads are washed thoroughly in order to enable storage in stable form for prolonged periods. For the third selection round, the beads can be treated with TFA/water/ethanethiol (18/1/1, v/v/v) to cleave the peptide from the resin and remove protecting groups in one step. Various experiments were performed with three successive selection and screening rounds. However, we expect the slight modifications in the cleavage conditions might even allow for more cleavage cycles.

Screening of various libraries was performed using CD4+ T cell proliferation assays. Several new epitopes could be defined for a variety of clones. Details about these experiments will be published elsewhere.

CONCLUSIONS

It is concluded that the new hybrid resin enables stepwise screening of large synthetic peptide libraries in solution using only one type of linker and Edman bead sequencing with very high repetitive yield of the acid-stabile part of the peptide. The system introduced offers flexibility with regard to the percentage of acid-stabile attached peptides as well as to the number of possible screening rounds.

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