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Synthesis and screening of peptoid arrays on cellulose membranes

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Abstract—Rapid synthesis and screening of compound libraries enables the accelerated identification of novel protein ligands in order to support processes like analysis of protein interactions, drug target discovery or lead structure discovery. SPOT synthesis—a well established method for the rapid preparation of peptide arrays—has recently been extended to the field of nonpeptides. In this contribution we report on the systematic evaluation of the SPOT technique for the assembly of *N*-alkylglycine (peptoid) library arrays. In the course of this investigation bromoacetic acid 2,4-dinitrophenylester (**1a**) was identified to be the most suited agent for bromoacetylation in terms of yield and *N*-selectivity enabling straightforward submonomer synthesis on hydroxy-group rich cellulose membranes. The potential of this method for the rapid identification of novel nonpeptidic protein ligands was demonstrated by synthesis and screening of a library consisting of 8000 peptoids and peptomers (i.e. their hybrids with α-substituted amino acids) allowing the identification of micromolar ligands for the monoclonal antibody Tab-2.

1. Introduction

The tremendous developments in the identification of novel drug targets as well as the growing interest in understanding protein–protein and protein–ligand interactions as central biological processes has created an increased demand for peptidic and peptidomimetic[1] libraries for target validation and drug discovery. Besides recent developments of biological library techniques such as phage display, ribosome display, and retroviral intracellular libraries, rapid and effective parallel chemical synthesis of peptide and furthermore peptidomimetic libraries is still a subject of intense research and technology development. The majority of approaches dealing with the chemical synthesis of large numbers of peptides and related oligomeric compounds is based on solid phase methods.[2,3] Two general techniques have been established to generate complex libraries which use either pooling strategies resulting in compound mixtures[4–7] or highly paralleled synthesis procedures to yield discrete compounds.[8] A spatially addressed synthetic methodology for single compounds offers the advantage that the sequence of an oligomer can easily be identified by its position and laborious tagging or deconvolution procedures are not needed.

A technically simple and economic method was developed according to the SPOT synthesis concept by Frank.[9] This method displays a high flexibility compared to other multiple solid phase procedures especially with regard to miniaturization and array geometries.[10–13] The basic principle involved the positionally addressed delivery of small volumes of reagent solutions directly onto a coherent membrane sheet. The resulting droplets can be considered as micro-reactors provided that a non-volatile solvent system is used. The functional groups fixed on the membrane surface react with the pipetted reagents and conventional solid phase syntheses (SPS) occur. The volumes dispensed as well as the physical properties of the membrane surface and the solvent system define the size of the resulting SPOTs and together with the minimum distance between the SPOTs the number of oligomers which can be synthesized per membrane area. The methodology has recently been reviewed.[14–20]

The general strategy for parallel assembly of oligomers on cellulose membranes using the SPOT technique comprises

Keywords: combinatorial chemistry; epitope mimic; peptoid array; *N*-selective acylation; solid-phase synthesis; SPOT synthesis.

Abbreviations: Ac, acetyl; ar, aromatic; Bu, butyl; Cy, cyclohexyl; DBU, 1,8-diazabicyclo[5,4,0]undec-7-ene; DIC, diisopropylcarbo-diimide; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Dnp, 2,4-dinitrophenyl; EDTA, ethylendiaminetetraacetic acid; eq., equivalents; Et, ethyl; Fmoc, 9-fluorenyl-methyloxycarbonyl; HBS-EP, HEPES-buffered saline with EDTA- and polysorbate added; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; IgG, immune globuline G; Me, methyl; NMP, *N*-methylpyrrolidone; Ph, phenyl; RU, resonance units; SPR, surface plasmon resonance; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; TRIS, tris-(hydroxymethyl)-aminomethane.

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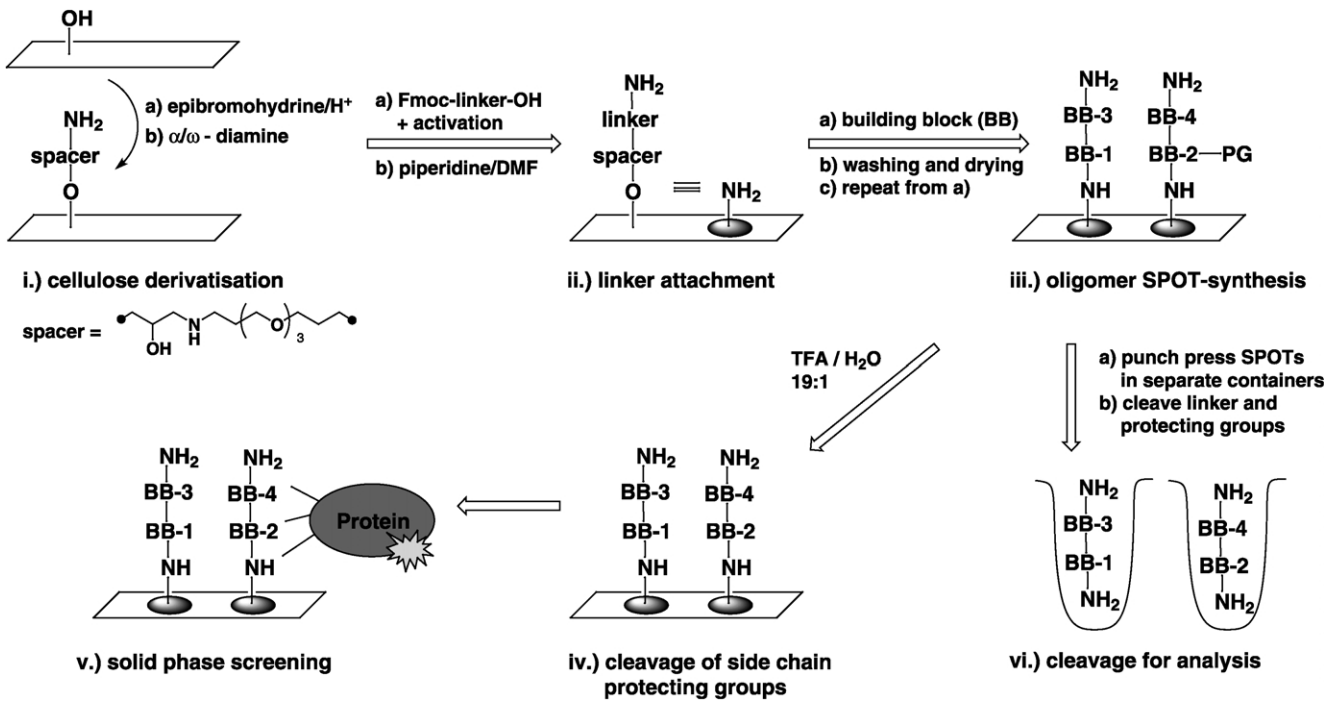
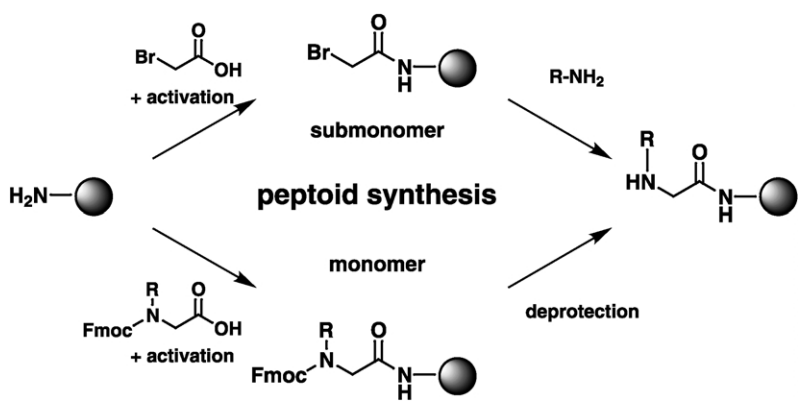


Scheme 1. Key steps outlining the SPOT synthesis technique (PG=side chain protecting group).

the following steps (Scheme 1): (i) cellulose derivatisation enabling selective attachment of activated building blocks; (ii) attachment of linkers in cases where cleavage of products is required for analysis or solution phase assays; (iii) SPOT synthesis of the oligomer; (iv) cleavage of side chain protecting groups while retaining the covalent membrane linkage; and (v) solid phase screening directly on the membrane bound oligomers or (vi) cleavage from the membrane for analysis.

After numerous applications of the SPOT synthesis concept for peptide synthesis,[14,15,17] the method was recently extended to the synthesis of other chemical entities such as PNAs,[21,22] glycoconjugates,[23] whole protein domains,[24] as well as small heterocyclic compounds.[25–27]

In extension of preliminary communications[27,28] the present contribution reports on the systematic evaluation of the SPOT technique for the assembly of *N*-alkylglycine (peptoid[29]) library arrays. Reaction conditions needed to be adapted for SPOT synthesis protocols on cellulose membranes to circumvent problems related to the different kinetic situation on continuous surfaces and the large excess of free hydroxy-functions on the cellulose. In the course of this investigation bromoacetic acid 2,4-dinitrophenyl-ester (**1a**) was found to be best suited for *N*-selective bromoacetylations being a central part in the submonomer synthesis concept.[30] Most effective reaction conditions were applied to the synthesis of an array consisting of 8000 hexapeptoids as well as -peptomers, their hybrids with α-substituted amino acids.[31] This library was screened for binding of the anti-transforming growth factor α (TGFα) monoclonal antibody (mab) Tab-2[32] in order to identify bioactive compounds de novo. Mab Tab-2 is a well established model system used to evaluate a variety of structurally different types of ligand arrays prepared by SPOT synthesis.[11,12,25,33–36]



Scheme 2. Monomer vs submonomer peptoid synthesis according to Simon et al.[29] and Zuckermann et al.,[30] respectively.

2. Results and discussion

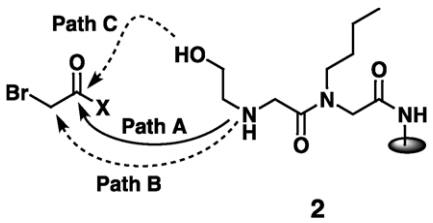
2.1. Monomer and submonomer peptoid synthesis

Since peptoids were introduced to combinatorial chemistry, both monomer[29] and submonomer[30] approaches have been successfully applied for their synthesis (Scheme 2). Whereas the monomer strategy allows a direct application of common Fmoc based peptide synthesis protocols due to the use of preformed building blocks, the latter approach makes use of a two step procedure comprising coupling of bromoacetic acid and subsequent displacement of the halogen by primary amines. Although the application of Fmoc-*N*-alkylglycine building blocks appears to be useful in some cases, e.g. to introduce functional groups inaccessible by submonomer procedures, the monomer strategy displays some major drawbacks such as limited availability of building blocks and coupling difficulties related to the high sterical hindrance at the alkylamino function. Accordingly, adaptation of the submonomer approach to the conditions of SPOT synthesis was attempted to utilize the enormous number of readily available primary amines as source of diversity and to avoid acylation problems at the *N*-alkylglycine moiety. However, despite of the advantages of the submonomer method for the assembly of oligomers consisting exclusively of *N*-substituted glycines a combination with the monomer strategy is useful in cases were peptoids and peptides are mixed in one sequence (peptomers) or when Fmoc protected *N*-alkylglycines are used to increase the diversity. In order to perform both strategies in parallel within a single library a special Fmoc synthesis protocol is necessary as described.[28]

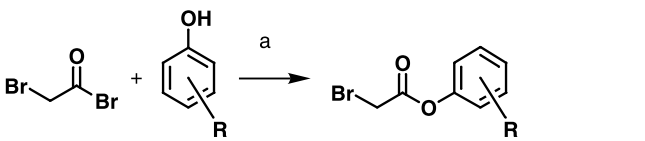
2.2. *N*-Selective bromoacetylation

The effective bromoacetylation of solid support bound amines or *N*-alkylamines represents a critical reaction step of the submonomer strategy. Several accounts reported on the successful application of the carbodiimide activation of bromoacetic acid.[30,31,37–40] While this procedure is the method of choice in SPS on resin beads the situation on cellulose membranes is different due to the presence of a large excess of cellulose OH-groups competing with the N-terminal peptoid amino groups. In order to avoid undesired acylation of the membrane support as well as unprotected OH-groups of the growing oligomer chain an *N*-selective acylation procedure would be favorable.

Since active esters of carboxylic acids have been demonstrated to show a significant *N*-selectivity in the presence of free OH-groups,[41,42] several of such esters were prepared for bromoacetic acid and tested for reactivity and selectivity using SPOT synthesis conditions. Interestingly, it was found that commonly used esters of lower reactivity such as bromoacetic acid 4-nitrophenyl[43,44] or *N*-succinimidyl[45,46] ester gave significant amounts of side products resulting from alkylation of the N-terminal secondary amino function (Path B vs. A in Scheme 3). Accordingly, it was assumed that reactivity of active esters needs to exceed a certain level



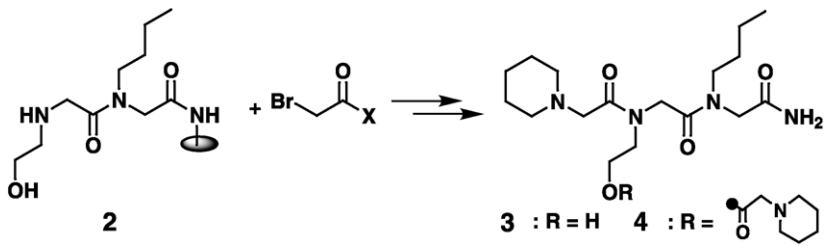
Scheme 3. Possible reaction pathways on the bromoacetylation of membrane bound peptoid **2** with activated bromoacetic acid (X=leaving group).



1a: R = 2,4-di-nitro
yield: 78%
1b: R = 2,3,4,5,6-pentafluoro
yield: 92%

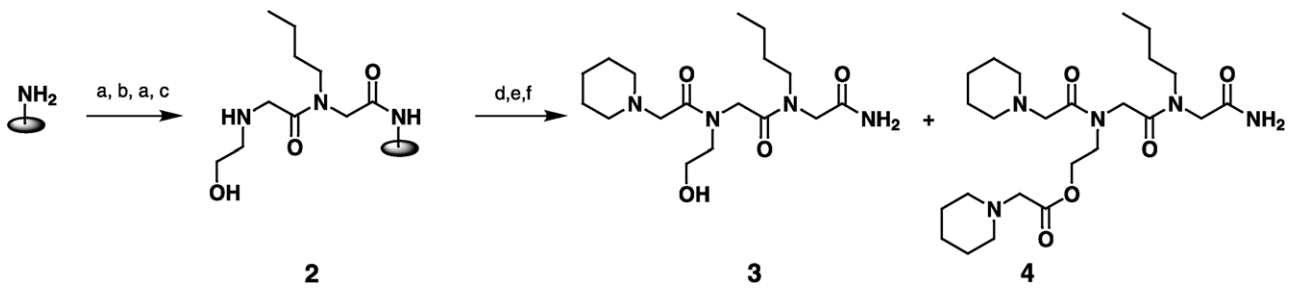
Scheme 4. Synthesis of bromoacetic acid 2,4-dinitrophenylester (**1a**) and the corresponding pentafluorophenylester **1b**. (a) pyridine/CH_2Cl_2 for **1a**; DIEA/CH_2Cl_2 for **1b**.

Table 1. Tables 1 SPOT synthesis of tripeptoid **3** using different conditions for bromoacetylation of the hydroxy dipeptoid **2**



Entry	–X	Yield **3** [%][a]	Purity **3** [%][b]	*N*-Selectivity (**3**: **4**)[c]
1	–ODnp (**1a**)	81	90[d]	93:7
2	–OH/DIC[e]	73	79	88:12
3	–OPfp (**1b**)	67	75	88:12

[a] Membrane derivatisation with linker≡100%, product was quantified by HPLC (peak area at 220 nm).
[b] HPLC, 220 nm.
[c] Ratio of yields of compounds **3** and **4**.
[d] See Figure 1 for corresponding HPLC-MS.
[e] 2 M Br-CH_2COOH, 1 M DIC.



Scheme 5. SPOT synthesis of the hydroxytripeptoid **3** and the corresponding side chain *O*-acylated side product. (a) **1a** (2×15 min); (b) *n*-butylamine (3×15 min); (c) ethanolamine (3×15 min); (d) Br-CH_2COX (for X see Table 1, 2×15 min); (e) piperidine (3×15 min); (f) TFA (95% in H_2O, 45 min) then evaporation.

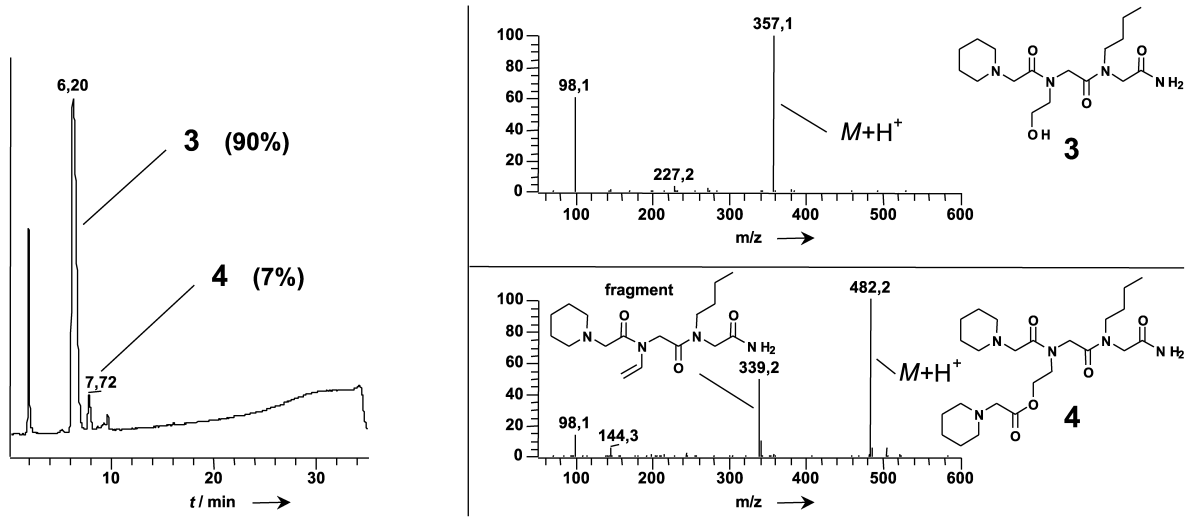


Figure 1. HPLC-chromatogram (220 nm) and MS-spectra corresponding to the annotated signals obtained from the SPOT synthesis of tripeptoid **3** using bromoacetic acid 2,4-dinitrophenylester (**1a**) as acylating reagent (the signal at t=2 min is the injection-peak).

to provide predominant formation of the acylation products. Thus, the more reactive, stable 2,4-dinitro- and pentafluorophenyl esters (**1a** and **1b**)[‡] were synthesized in high yields starting from bromoacetic acid bromide and the corresponding electron deficient phenols (Scheme 4).[§,¶] The reactivities and *N*-selectivities of these esters were evaluated under conditions of the SPOT synthesis and compared to carbodiimide activation using the synthesis of a tripeptoid as a simple model system. The key step involved acylation of the membrane bound dipeptoid **2** (Scheme 5) with the corresponding active esters of bromoacetic acid. Whereas the intended exclusive acylation of the *N*-alkylamine followed by bromine substitution with piperidine yields the tripeptoid **3**; varying proportions of tripeptoid **4** result from partial *O*-acylation at the side chain and subsequent nucleophilic substitutions of both bromines with piperidine (see path C in Scheme 3). The ratios of both products were determined by HPLC and the *N*-selectivities of the acylating species applied were subsequently deduced (Table 1).[‖] It was found that all three reagents gave rise to the desired *N*-acylated product **3** in purities between 75 and 90% (by HPLC, 220 nm) and yields ranging from 67 to 81% (by quantitative evaluation of the UV-absorbtion). Bromoacetic acid 2,4-dinitrophenylester (**1a**) showed the best results (see Fig. 1) and was superior to the other reagents applied in terms of *N*-selectivity as well (93:7) therefore being the reagent of choice for submonomer peptoid synthesis on cellulose membranes.

2.3. Suitability of submonomer building blocks

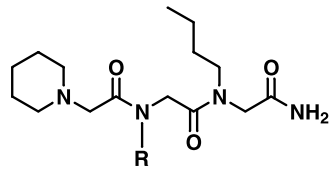
In order to examine the applicability of different amines for SPOT synthesis of peptoids, an array consisting of tripeptoids **5** was synthesized on a Rink-linker[50] modified cellulose membrane based on a variety of amines as building blocks for the central position. The amines were applied in large excess compared to the membrane loading:

[‡] Application of **1a** was previously published.[47] However, no synthetic or analytical data were provided. The isolated ester **1b** has—to our knowledge—not been described yet, although it has been applied generated in situ.[48]

[§] Ester **1a** is crystalline while ester **1b** was isolated as an oil. No decomposition was observed (^{1}H NMR) upon storage for at least 4 months at 20°C under exclusion of light (**1a**) or at 4°C in a sealed bottle with some 4 Å molecular sieve added (**1b**), respectively.

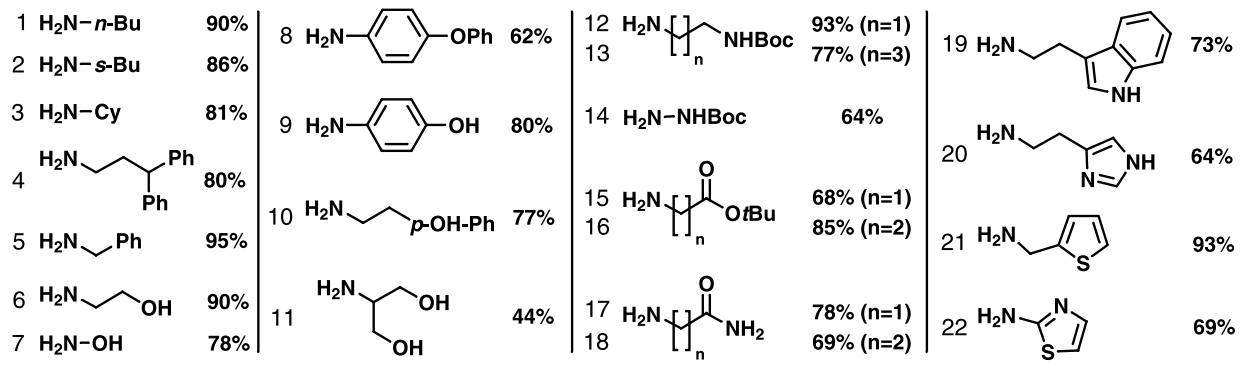
[¶] Attempts to synthesize bromoacetic acid esters with hydroxybenzotriazole or 7-aza-hydroxy-benzotriazole using carbodiimides gave insoluble products (NMP, DMSO, DMF). The observed problems are in accordance with described unsatisfactory results attempting to use bromoacetic acid and HOBt-based in situ activation.[30,49]

[‖] Compounds **3** and **4** were independently synthesized on Tentagel-S-RAM-resin as HPLC-standards using the submonomer procedure.[37] Determination of the molar absorptions of both trimers at 220 nm enabled the calculation of absolute yields from HPLC analyses. Comparison of both absorption coefficients revealed a neglectable contribution of the side chain ester group of **4** to the total absorption, enabling the deduction of the molar ratios of both compounds directly from their ratios in absorption at 220 nm.

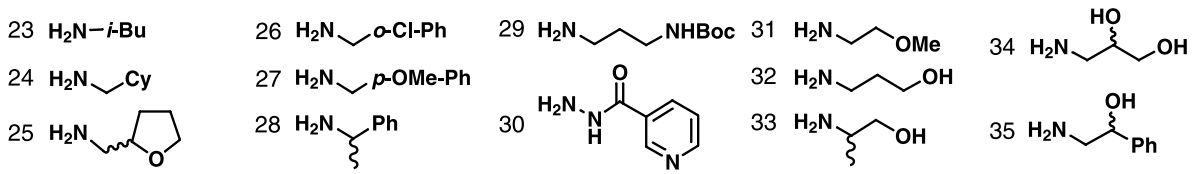


5

A.) submonomer building blocks and HPLC-purities of corresponding model trimer **5**



B.) additional submonomer building blocks



C.) monomer building blocks

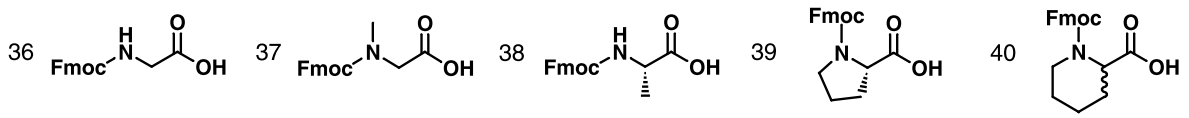


Figure 2. Building blocks applied for the synthesis of a library consisting of 8000 hexapeptoids and -peptomers. The purities annotated in (A) are HPLC-purities (220 nm) of tripeptoids **5** derived from the corresponding amines after cleavage from the Rink-linker by TFA (therefore Boc and *t*-Bu-protecting groups are cleaved in **5**).

when possible as 50% solutions in NMP** or in cases of lower solubility near saturation, at least 0.8 M (for details see Section 4). The resulting products were analyzed by HPLC-MS after cleavage. While most of the amines gave rise to the desired tripeptoid **5** in purities above 75% (220 nm), some building blocks were less suited (Fig. 2(A)). We observed, that simple alkylamines generally gave good results, as expected (entries 1–5). Anilines have to be more electron rich than aniline itself to give reasonable results (entries 8–9). Aminoalkohols and -phenols as well as hydroxylamine were successfully introduced without protection (entries 6–7 and 9–11). Boc or *tert*-butyl protection was applied for amino or carboxylic acid side chains, respectively (entries 12–16). Highly polar building blocks like glycine or β-alanine derivatives could be successfully introduced into **5** using water instead of NMP as solvent (0.05% Tween 20 was added in this case to decrease the surface tension; entries 7 and 15–18). Selected heterocyclic amines gave good results (entries 19–22). Some amines such as those containing nucleophilic nitrogens in their side chains, electron deficient anilines or α,α-dialkylhydrazines turned out to be unsuited (data not shown).

2.4. Synthesis and screening of a peptoid library

The most widely used application of the SPOT synthesis concept is parallel synthesis of peptide arrays for subsequent evaluation of bioactivity especially affinity to a binding partner[17–20] using solid phase screening directly with the membrane linked compounds. Two successful examples involved the de novo identification of the immunogenic TGFα epitope recognized by the monoclonal anti-TGFα antibody Tab-2[32] using synthetic cellulose-bound hexamer[12,33] or 15-mer[36] peptide libraries. As an extension of these results we synthesized a library consisting of 8000

** Most amine solutions were prepared v/v rather than in defined molarity for ease of preparation. In a typical synthesis (membrane loading: 1.0 μmol cm^{-1}, reagent volume: 3.6 μl cm^{-1} per cycle, three cycles) using a representative amine (M=100, *d*=1.0) this corresponds to a 5.0 M solution and results in 54 equiv. amine compared to the membrane bound bromide.

hexapeptoids and -peptomers aiming on the identification of novel non-peptide epitope mimics by using a de novo approach.

A set of 40 building blocks was selected for library synthesis consisting of 35 amines (alkyl- and arylamines, acylhydrazines and hydroxylamine) and five Fmoc-amino acids chosen to introduce functional groups inaccessible by submonomer procedures (Fig. 2). The selection of amines was based on those which yielded the model trimer **5** in purities above 60% (see above). In one case (2-amino-1,3-propandiole, entry 11) we decided to tolerate a lower purity in order to increase the diversity of the building block set. In addition to these 22 amines 13 building blocks with similar molecular structures and anticipated comparable synthesis efficiencies were selected (Fig. 2(B)).

While it was not possible to synthesize all hexapeptoids and -peptomers based on the selected building block set ($40^6 \approx 4 \times 10^9$) we randomly chose 8000 members—a set that can be synthesized efficiently on a single cellulose membrane (19×28 cm). The unbiased, random sequences and the corresponding sequence data file required to operate the SPOT synthesizer were generated by LISA, a software capable of handling monomer and submonomer procedures simultaneously. Whereas the hexapeptomers meant for screening were directly attached to the cellulose without a cleavable linker, eight hexapeptomers were synthesized in parallel at the same membrane on a cleavable Rink-linker to monitor the quality of synthesis. Cleavage yielded the desired hexamers in an average HPLC-purity of 44% (220 nm) with side products being truncated sequences as expected. However the target product was the predominant compound in six of the eight cases. Thus, the purity of the library was considered to be sufficient for screening purposes. This assumption was driven by the fact that truncated sequences of low molecular weight are less probable antibody ligands.

The cellulose-bound hexapeptoids and -peptomers were incubated with a buffered aqueous solution of the antibody Tab-2. Specifically bound antibody was detected after washing by a second antibody labeled with horseradish peroxidase and subsequent monitoring of chemoluminiscence on treatment with a suitable substrate (Fig. 3). Several signals (i.e. black SPOTs in Fig. 3) indicated binding of the antibody Tab-2 to specific hexamers. Undesired binding with the second antibody could be excluded in a control experiment without Tab-2.

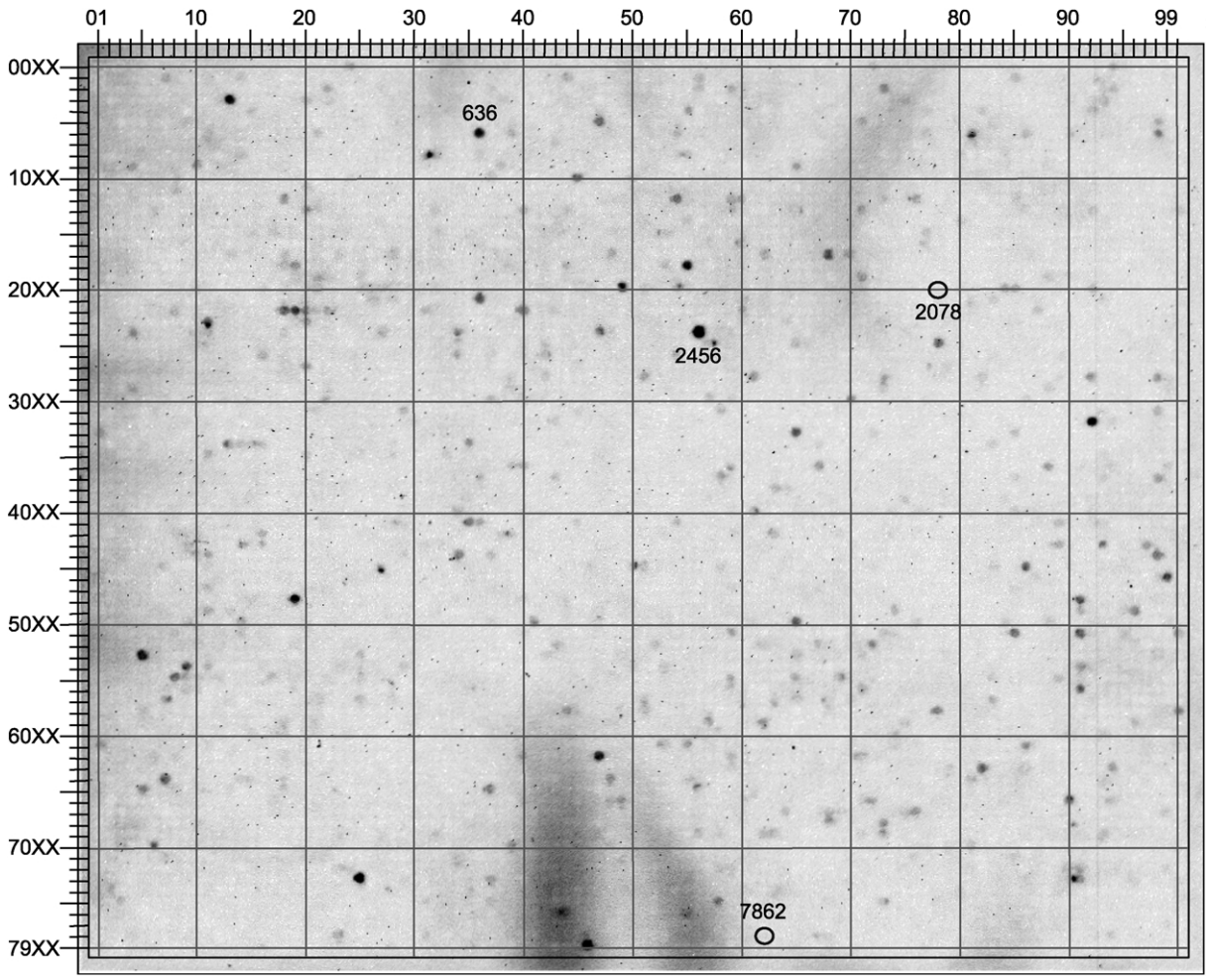


Figure 3. Binding of the antibody Tab-2 to a library consisting of 8000 cellulose bound hexapeptoids and -peptomers. Every SPOT determined by the grid contains a compound with a specific sequence. Dark SPOTs indicate binding of the antibody—marked compounds were subjected to resynthesis and quantification of binding (circles indicate resynthesized inactive compounds chosen as negative controls).

2.5. Quantitative determination of binding

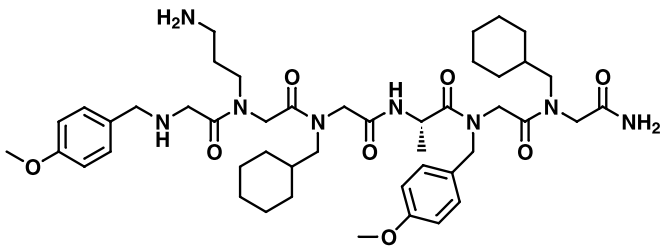
The sequences of the most active ligands were extracted from the synthesis file and two of them (**6** and **7**) as well as two inactive compounds (**8** and **9**) as negative controls were resynthesized on resin beads applying published procedures (Fig. 4).[30,37] Binding with the antibody Tab-2 was quantified in a series of surface plasmon resonance experiments using a two channel setup (Biacore X). Determination of paratope specific binding was ensured by coupling of mab TE33,[51] an antibody with the same subtype but different specificity (anti-cholera toxin) in the reference cell.

Both selected ligands have dissociation constants (K_D) in the micromolar range (K_D=2.7 μM for **6** and 408 μM for **7**) whereas for the negative controls **8** and **9** no binding was detected (Fig. 4). On comparison of both active compounds **6** shows better binding in the SPR experiment whereas **7** gives a stronger signal in the cellulose assay. A possible explanation to this observation is the influence of the solid phase on array bound compounds compared to the same entities in solution as occurring in all heterogenous assay systems. Depending on the degree and mode of conformational restriction induced upon binding to the antibody the fraction of the entropic term of the binding free energy varies for the different assay systems. Another explanation is based on the different C-termini of cellulose bound and corresponding isolated compounds: if the C-terminal amide binds to the antibody specifically the linked membrane would disturb this interaction in the cellulose assay, whereas if the linker is partly contributing to antibody binding the affinity should be lower with the free compound in the SPR assay. Nevertheless activity on cellulose led to compounds binding the antibody in solution as well. In order to compare the potency of the epitope mimics **6** and **7** to the native ligand as well as to complement the quantification method, we additionally included a hexapeptide covering the TGFα epitope (VVSHFND-NH_2) in the surface plasmon resonance experiments. Its dissociation constant was determined to be 20 nM in accordance to previous ELISA[34] and isothermal titration calorimetry[35] experiments. Thus we could identifiy non-peptidic antibody ligands considerably differing from the wild-type peptide epitope within a single experiment starting from a naïve library.

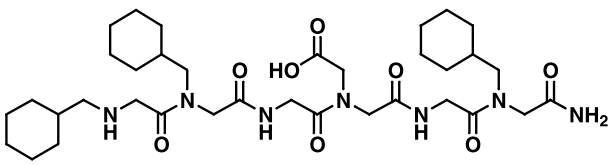
3. Conclusion

The SPS of peptoids—previously established using standard polystyrene-based resins—could be successfully adapted to cellulose membranes using the monomer as well as the submonomer approach. The procedures had to be adjusted to the special requirements related to the different kinetic situation on continuous surfaces and the large number of free hydroxy-functions on the cellulose. Within the submonomer approach bromoacetic acid 2,4-dinitrophenyl-ester (**1a**) was found to be ideally suited for efficient acylation of the secondary amine of the growing peptoid chain. In contrast to other applied reagents this ester neither showed any *N*-alkylation nor significant *OH*-acylation.

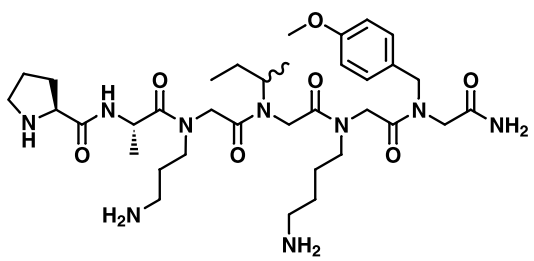
A library consisting of 8000 hexapeptoids and -peptomers was synthesized under optimized conditions. By screening for binding with the monoclonal anti-TGFα-antibody Tab-2 ligands with micromolar affinity were identified. The approach enabled the identification of non-peptide epitope mimics de novo in a single step, i.e. without any input of structural information derived from the epitope originally recognized by the antibody. The dissociation constant of the best compound from the library was only two orders of magnitude worse compared to the wild-type peptide epitope. These findings shows peptoid synthesis and screening on cellulose membranes to be a valuable and rapid tool for lead identification processes.



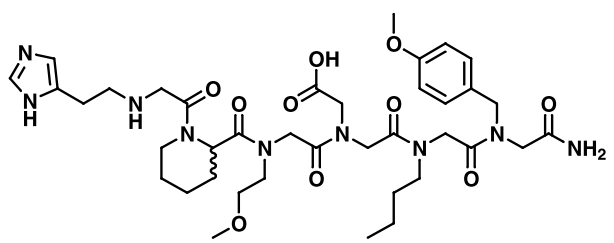
6 (= SPOT 636) K_D = 2.7 μM



7 (= SPOT 2456) K_D = 408 μM



8 (= SPOT 2078) K_D > 2000 μM



9 (= SPOT 7862) K_D > 2000 μM

Figure 4. Hexapeptoids selected for quantification of binding to the antibody tab-2 (two ligands and two non-binders as indicated in Figure 3). The dissociation constants shown were determined by SPR-experiments.

4. Experimental

4.1. General

NMR spectra were recorded on a Varian Unity-plus 300 spectrometer in $CDCl_3$ at 25°C (^1H at 300 MHz, ^{13}C at 75.45 MHz and ^{19}F at 284 MHz, respectively). Chemical shifts are given in parts per million (ppm) relative to internal standards: $Si(CH_3)_4$ (^1H and ^{13}C) or $CFCl_3$ (^{19}F), respectively; coupling constants (*J*) are given in Hertz (Hz). FT-IR spectra were performed on a Bruker IFS-66. HRMS-FAB was performed on a Finnigan MAT 95ST (Trap) equipped with a 'Cs-gun' using a glycerol/*m*-nitrobenzylalcohol matrix and CsI/glycerol as standard. Elemental analyses (EA) were carried out on a LECO CHNS-933 microanalyzer (for C,H,N) or by titration with 0.01N $Hg(ClO_4)_2$/diphenylcarbazone (Dirscherl/Erne) after Schöninger-decomposition (for Br), respectively. Analytical HPLC-MS analysis was carried out on a Hewlett Packard 1100 HPLC system coupled to a Finnigan LCQ ion-trap ESI-mass spectrometer. In a typical analysis-run approx. 50 nmol substance was analyzed using a linear gradient (eluent A 0.05% TFA in water, eluent B 0.05% TFA in acetonitrile; gradient time: 25 min; flow rate: 0.3 ml min^{-1}) on a C18 RP-column (Vydac 218 TP5215, 2.1×150 mm). Preparative separations were carried out on a Merck/Hitachi system (L-6250, L-7400, D-7000) and a Merck column (LiChrospher 100, RP18, 10×250 mm, flow rate: 6.0 ml min^{-1}) using an optimized gradient derived from the analytical chromatogram. Melting points are uncorrected. SPOT synthesis was done on the Auto Spot Robot ASP 222-system (Abimed, Langenfeld, Germany); the software LISA was used for generation of the sequence-files (by W. -J. Wu, Inst. Med. Immunol., Charité, Humboldt University, Berlin, Germany; all rights at Jerini AG). SPR-experiments were carried out on a Biacore X-system (Biacore AB, Uppsala, Sweden).

4.2. Materials

Solvents and reagents were obtained from Sigma-Aldrich (Munich, Germany), VWR International (Darmstadt, Germany) or Lancaster Synthesis (Frankfurt, Germany). Amino acid derivatives were provided by Novabiochem (Läufelfingen, Swizerland); Tentagel-S-RAM-resin (0.23 mmol g^{-1}) was purchased from Rapp-Polymere (Tübingen, Germany). SPOT synthesis was performed on Whatman 50 cellulose (Whatman, Maidstone, UK). The antibodies Tab-2 and TE-33 were kindly provided by W. Höhne (Inst. for Biochemistry, Charité, Humboldt-Universität, Berlin, Germany) and T. Scherf (Weizmann Inst. of Science, Rehovot, Israel), respectively. The assays were performed in 10 mM TRIS-buffered saline (TBS) at pH=8.0. The anti-mouse IgG antibody used for detection of bound Tab-2 was provided by Sigma (Taufkirchen, Germany). Antibodies bound to peptoids/peptomers on cellulose membranes were detected using the recommended luminescence solution (=100 equiv. of luminescence substrate solution A and 1 equiv. of the corresponding solution *B*) with a Lumi-Imager system (Roche Diagnostics, Mannheim, Germany). Blocking buffer consisted of 1 equiv. of the blocking reagent which was provided together with the Boehringer Mannheim chemiluminescence blotting substrate (POD) (Roche Diagnostics; Cat. #1 500 708) and 9 equiv. of TBS.

4.3. Methods

4.3.1. General procedures. *Washing*. If not otherwise noted, all membranes were washed in a stainless steel dish between each reaction step with DMF, MeOH and Et_2O three times each.

Amino derivatisation of cellulose. Cellulose membranes with amino-loadings of 0.6–1.4 μmol cm^{-2} were prepared according to a previous report:[28] a cellulose sheet (19×28 cm) was immersed in a solution of epibromohydrine and 15 M aq. perchloric acid in dioxane [10:1:90 (v/v/v)] for 3 h, washed with MeOH (2×) and dried in air. The membrane was incubated with neat 4,7,10-trioxa-1,13-tridecanediamine for 1 h at 80°C in a stainless steel dish on a heating plate. The membrane was washed with MeOH (2×), 5 M NaOMe in MeOH, MeOH, H_2O (4×), MeOH, and Et_2O and dried in air.

Manual SPOT synthesis[9,15–17] *of peptoids* (*submonomer protocol*[30,37]). A 2.0 μl amount of a solution of *N*-Fmoc-4-[amino-(2,4-dimethoxyphenyl)-methyl]-phenoxyacetic acid, pentafluorophenol and diisopropylcarbodiimide in NMP (0.2 M for all components) was pipetted to an amino derivatized cellulose membrane on a 1×1 cm pencil drawn grid after 30 min of preactivation. The procedure was repeated after 15 min followed by washing and drying in air. Capping of unreacted amino groups by acetylation was achieved by immersing the membrane in a solution of Ac_2O and DIEA in DMF [1:2:7 (v/v/v)] followed by washing and drying. The degree of membrane derivatisation was determined by punching a test-SPOT (0.23 cm^2) and quantifying the absorption of the dibenzofulvene-piperidine adduct at 301 nm after release by piperidine (20% in DMF).[52] The remaining membrane was deblocked by treatment with piperidine (20% in DMF; 2×20 min) followed by washing and drying in air. Submonomer synthesis was performed by alternately pipetting either 2.0 μl of a solution of **1a** (1.0 M in NMP) with one repetition after 15 min or 2.0 μl of a solution of an amine (in NMP or H_2O containing 0.05% Tween 20) with two repetitions after 15 and 30 min, respectively. Excesses of reagents were removed between each synthesis step by washing. An extended washing procedure was applied after treatment with amines: DMF (4×), MeOH (1×), 0.5 M aq. NaOH, H_2O (5×), MeOH (2×), and Et_2O. After completion of the synthesis sequence, the SPOTs were punched out and transferred into individual 2.0 ml Eppendorf tubes. The peptoids were cleaved from the linker with TFA (95% in H_2O; 70 μl; 45 min) and dissolved in acetonitrile (30% in H_2O, 50 μl) immediate after evaporation in an vacuum centrifuge (Eppendorf) at 45°C. The solution was analyzed by HPLC-MS.

4.3.2. 2,4-Dinitrophenyl-bromoacetate (1a). A solution of anhydrous 2,4-dinitrophenol (54.3 mmol) and pyridine (70.6 mmol) in CH_2Cl_2 (80 ml) was cooled to 0°C and bromoacetylbromide (59.7 mmol) in CH_2Cl_2 (25 ml) was added dropwise. After 1 h at 25°C the organic layer was extracted with H_2O and citric acid (10% in H_2O), dried over

Na_2SO_4 and evaporated under reduced pressure to give a yellow oil (16.2 g). The crude product was crystallized from Et_2O (200 ml) at −26°C to yield **1a** as clear yellow crystals (13.0 g, 42.6 mmol, 78%). Mp: 108–109°C; ^{1}H NMR: δ=4.19 (s), 7.56 (d, ^3J=8.9 Hz), 8.91 (dd, ^3J=8.9 Hz, ^4J=2.7 Hz), 8.98 (d, ^4J=2.7 Hz); ^{13}C NMR: δ=24.3, 121.8, 126.4, 129.3, 141.2, 145.5, 147.8, 164.2; IR (KBr) 3112, 3071, 2957, 1783 (C=O), 1608, 1538, 1344, 1219, 1097, 919, 836, 733 cm^{-1}; EA (in %, calcd values in parenthesis): C: 31.43 (31.50), H: 1.65 (1.65), N: 9.27 (9.18), Br: 26.32 (26.19).

4.3.3. Pentafluorophenyl-bromoacetate (1b). Bromoacetylbromide (21.7 mmol) in CH_2Cl_2 (30 ml) was cooled to 0°C. A solution of pentafluorophenol (21.7 mmol) and DIEA (21.7 mmol) in CH_2Cl_2 (30 ml) was added within 15 min. After 1 h at 25°C the organic layer was extracted with H_2O (2×), dried over Na_2SO_4 and evaporated under reduced pressure to give **1b** as a colourless oil (6.10 g, 20.0 mmol, 92%); ^{1}H NMR: δ=4.15 (s); ^{13}C NMR: δ=23.2, 124.2–125.0 (m), 135.8–143.2 (m), 163.7; ^{19}F NMR: δ=−162.16 (dd, ^3J=22, 18 Hz), −157.25 (t, ^3J=22 Hz), −152.93 (d, ^3J=18 Hz); IR (film) 2969, 2672, 2464, 1810/1790 (C=O), 1655, 1520, 1473, 1425, 1403, 1241, 1222, 1145, 1095, 1000, 599 cm^{-1}; EA (in %, calcd values in parenthesis): C: 31.36 (31.50), H: 0.77 (0.66), Br: 25.93 (26.20). Impurity pentafluorophenol:[53] <3% (^{19}F NMR).

4.3.4. *N*-Selective bromoacetylation. The tripeptoid **3** was synthesized on a cellulose membrane according to the general procedure for manual SPOT synthesis of peptoids using modified conditions during the second acylation step. The amines *n*-butylamine, ethanolamine, and piperidine were applied as building blocks (5 M in NMP each). For the second bromoacetylation, conditions were varied according to Table 1: either **1a**, **1b** (1 M in NMP each), or a solution of bromoacetic acid (2 M in NMP) treated with 0.5 equiv. DIC 30 min prior to synthesis were applied (2.0 μl and another 2.0 μl after 15 min). After completion of the synthesis cleavage yielded the crude product for analysis by HPLC-MS. The contained amounts of tripeptoid **3** and its derivative **4** were quantified by determination of the corresponding peak areas in HPLC at 220 nm and subsequent calculation of the absolute amounts using calibration curves (authentic material for calibration was synthesized on resin, see below). Chemical yields shown in Table 1 result from the comparison of the amounts of isolated products with the Rink-linker loading of the membrane. All data shown result from at least five independent experiments; the deviation of yields and purities was ≤10% for **3** and ≤4% for **4**.

4.3.5. Synthesis of tripeptoid 3 on resin. Tripeptoid **3** was synthesized on Tentagel-S-RAM-resin (190 mg) according to a published general procedure.[37] The following modifications were applied: bromoacetylations for both C-terminal positions were performed with bromoacetic acid (20 equiv.), DIC (10 equiv.), and 2,6-dimethylpyridine (10 equiv.) in DMF using a single coupling step. The third acylation yielding the N-terminus was performed using **1a** (1.5 equiv.) in DMF in a single coupling step. The pure tripeptoid **3** was isolated as a salt with TFA (1 equiv.) after cleavage and purification by prep. HPLC (15 mg, 31,9 μmol, 73%).

4.3.6. Synthesis of tripeptoid 4 on resin. Tripeptoid **4** was synthesized on Tentagel-S-RAM-resin (190 mg) according to a published general procedure.[37] The following modifications were applied: bromoacetylations for both C-terminal positions were performed with bromoacetic acid (20 equiv.), DIC (10 equiv.), and 2,6-dimethylpyridine (10 equiv.) in DMF using a single coupling step. The third acylation yielding the N-terminus was performed with bromoacetic acid (20 equiv.), DIC (10 equiv.), and 2,6-dimethylpyridine (40 equiv.) in CH_2Cl_2 in order to induce side-chain esterification. The pure tripeptoid **4** was isolated as a salt with TFA (2 equiv.) after cleavage and purification by prep. HPLC (23 mg, 32.4 μmol, 74%).

4.3.7. Suitability of submonomer building blocks. A series of 22 different tripeptoids **5** was synthesized on 22 SPOTs on a cellulose membrane according to the general procedure for manual SPOT synthesis. The first building block was incorporated using *n*-butylamine (5 M in NMP). The central position was applied by 22 different *N*-nucleophiles each of them introduced on a different SPOT (all amines 50% in NMP, if not noted differently): 1: *n*-butylamine; 2: *rac*-*s*-butylamine; 3: cyclohexylamine; 4: 3,3-diphenylpropylamine; 5: benzylamine; 6: ethanolamine; 7: hydroxylamine (50% in H_2O containing 0.05% Tween 20); 8: 4-phenoxyaniline (2.0 M in NMP); 9: 4-aminophenol (saturated in NMP=approx. 1 M); 10: tyramine (1.0 M in NMP); 11: 2-amino-1,3-propandiole (30% in NMP); 12: *N*-boc-1,2-diaminoethane; 13: *N*-boc-1,4-diaminobutane; 14: *tert*-butyl carbazate (2 M in NMP); 15: glycine *tert*-butyl ester acetate (2.5 M in H_2O containing 0.9 equiv. of NaOH and 0.05% Tween 20); 16: β-alanine *tert*-butyl ester hydrochloride (3.0 M in H_2O containing 0.9 equiv. of NaOH and 0.05% Tween 20); 17: glycine amide hydrochloride (5.0 M in H_2O containing 0.9 equiv. of NaOH and 0.05% Tween 20); 18: β-alanine amide hydrochloride (5.0 M in H_2O containing 0.9 equiv. of NaOH and 0.05% Tween 20); 19: tryptamine (1.5 M in NMP); 20: histamine (1.5 M in NMP); 21: 2-aminomethylthiophene; 22: 2-aminothiazole (1.5 M in NMP). The trimer was completed using piperidine (5 M in NMP). Each SPOT was punched out and transferred into a 2 ml Eppendorf tube. Cleavage with TFA yielded the products for analysis by HPLC-MS. The purities (220 nm) of the products are listed in Figure 2. The integrity of each compound was assured by analysis of the ESI-mass spectra corresponding to the product HPLC-signal [shown data found for M+H$^+$ (calcd values in parenthesis)]: 1: 369.2 (369.29); 2: 369.3 (369.29); 3: 395.3 (395.30); 4: 507.3 (507.33); 5: 403.2 (403.27); 6: 357.1 (357.25); 7: 329.2 (329.22); 8: 481.2 (481.28); 9: 405.1 (405.25); 10: 433.2 (433.28); 11: 387.2 (387.26); 12: 378.2 (356.27); 13: 384.2 (384.30); 14: 350.1 (328.23); 15: 371.2 (371.23); 16: 385.2 (385.25); 17: 392.1 (370.25); 18: 384.2 (384.26); 19: 456.2 (456.30); 20: 407.1 (407.28); 21: 409.2 (409.23); 22: 396.2 (396.21).

4.3.8. Automated SPOT synthesis of a library consisting of 8000 hexapeptoids and -peptomers. A cellulose membrane with an amino-loading of 0.5 μmol cm^{-2} was prepared based on our previous report:[28] A cellulose sheet

(19×28 cm) was immersed in a solution of epibromohydrine and 15 M aq. perchloric acid in dioxane [10:1:90 (v/v/v)] for 1 h, washed with MeOH (2×) and dried in air. The membrane was incubated with 4,7,10-trioxa-1,13-tridecanediamine (20% in DMF) for 2 h at 25°C in a stainless steel dish. The membrane was washed with MeOH (2×), 5 M NaOMe in MeOH, MeOH, H_2O (4×, MeOH, and Et_2O and dried in air. The synthesis of the hexapeptomers was performed semi-automatically using the ABIMED autospot system configured to address an array consisting of 100×80 SPOTs evenly spaced on the membrane ("screening array", diameter of the SPOTs: 1.2 mm, distance between the SPOTs: 2.2 mm). In addition eight hexapeptoids were synthesized at the edge of the array ("analysis array", SPOT-distance: 1.0 cm) applying the 45-fold reagent volume thus yielding SPOTs with a diameter of 9 mm enabling HPLC-MS-analysis of products after cleavage from the linker to monitor the quality of synthesis. In a first step 40 nl of Fmoc-β-alanine pentafluorophenylester (0.3 M in DMSO) for SPOTs of the screening array and 1.8 μl of a solution of *N*-Fmoc-4-[amino-(2,4-dimethoxyphenyl)-methyl]-phenoxyacetic acid, pentafluorophenol and diisopropylcarbodiimide in NMP (0.2 M for all components) for SPOTs of the analysis array were pipetted to the membrane using the Auto Spot Robot. The procedure was repeated after 15 min followed by detachment of the membrane from the synthesizer, washing in a stainless steel dish, and drying in air. Capping of unreacted amino groups by acetylation was achieved by immersing the membrane in a solution of Ac_2O and DIEA in DMF [1:2:7 (v/v/v)] followed by washing. Amino groups of the membrane bound amino acids were deblocked by treatment with piperidine (20% in DMF; 2×20 min, followed by washing) and visualized by staining with bromophenolblue (0.01% in MeOH). SPOTs at the corners of the array were subsequently marked with a pencil in order to enable a proper reposition of the membrane on the synthesizer prior to each synthesis step.

The hexamers were synthesized according to a sequence file generated by the program LISA prior to synthesis containing 8000 sequences randomly and unbiased chosen based on a set of 40 building blocks (see Fig. 2). Since monomer and submonomer syntheses were applied on the same membrane in parallel a previously published procedure comprising the following steps was used:[28] all acylations [with Fmoc-amino acids (monomers) and bromoacetic acid (submonomers)] were performed in in a first step whereas Fmoc removal (by 4% DBU in NMP, monomers) and bromosubstitution (by primary amines, submonomers) were carried out in a second step after washing. In both steps, the synthesizer was controlled by the sequence file. See Section 4.3.7 for building blocks no. 1 to 22. Additionally the following reagents were used: (all amines 50% in NMP, if not noted differently): 23: *iso*-butylamine; 24: cyclohexylmethylamine; 25: *rac*-tetrahydrofurfuryl-amine; 26: *o*-chlorbenzylamine; 27: *p*-methoxybenzylamine; 28: *rac*-1-phenylethylamine; 29: *N*-boc-1,3-diaminopropane; 30: nicotinic hydrazide (0.8 M in NMP); 31: 2-methoxyethylamine; 32: 3-amino-1-propanol; 33: *rac*-2-amino-1-propanol; 34: *rac*-3-amino-1,2-propandiol (30% in NMP); 35: *rac*-2-amino-1-phenylethanol. All Fmoc-amino acids were applied as 0.6 M solutions in NMP, activated with 0.55 equiv. DIC 30 min prior to synthesis: 36: Fmoc-glycin; 37: Fmoc-sarcosine; 38: Fmoc-L-alanine; 39: Fmoc-L-proline; 40: *rac*-Fmoc-pipecolic acid. Bromoacetylations were carried out with 2,4-dinitrophenyl-bromoacetate (**1a**, 1.0 M in NMP). All acylations were repeated once, whereas bromine displacements by amines were repeated twice. Excesses of reagents were removed between each synthesis step by washing as described above ('Manual SPOT synthesis of peptoids'). After completion of the hexamer synthesis, the 'screening array' was separated from the 'analysis array' and treated twice with TFA, and TIPS in H_2O [94:2:3 (v/v/v) with 1 g phenol/100 ml added] for 15 min and once with TFA, TIPS, and H_2O in CH_2Cl_2 [50:2:3:45 (v/v/v/v) with 1 g phenol/100 ml added] for 2 h in order to remove side chain protecting groups. The membrane was washed with CH_2Cl_2 (2×), DMF, Et_3N (2% in DMF), DMF (2×), MeOH (2×), and Et_2O and dried in air. The SPOTs on the 'analysis array' were punched out and transferred into individual 2.0 ml Eppendorf tubes. The peptoids and peptomers were cleaved from the linker with TFA (95% in H_2O; 70 μl; 20 min) and dissolved in acetonitrile (30% in H_2O; 50 μl) immediate after evaporation of TFA in a vacuum centrifuge (Eppendorf) at 45°C. The solutions were analysed by HPLC-MS. The sequences (from N- to C-terminus, numbers see Fig. 2), purities (220 nm, HPLC, mp=target is the Main Product), and ESI-MS-data ($M+H^+$, calcd values in parenthesis) are: 41*-05-01-18-05-01, 63% (mp), 791.4 (791.48); 41*-05-01-18-05-25, 76% (mp), 819.4 (819.48); 39-31-33-23-16-10, 42% (mp), 764.3 (764.42); 39-02-12-17-18-31, 44% (mp), 685.2 (685.40); 31-08-06-37-29-38, 55% (mp), 715.2 (715.38); 14-35-35-37-39-24, 47% (combined purity of 2 peaks caused by 4 diastereomers, mp), 765.2 (765.43); 20-04-33-22-19-08, 11%, 1100.3 (1100.48); 11-32-15-24-30-23, 10%, 822.3 (822.44) [*41: piperidine (50% in NMP)].

4.3.9. Screening of 8000 cellulose bound hexapeptoids and -peptomers for binding with the antibody Tab-2. The cellulose membrane with peptomers covalently bound was washed with MeOH and TBS (3×) followed by incubation with blocking buffer (1 h). The solvents were decanted and the wet membrane was incubated with mab Tab-2 (1 μg ml^{-1} in 100 ml of blocking solution; 2 h). The membrane was washed with TBS containing 0.05% Tween 20 (T-TBS; 3×). Bound antibody was detected after incubation with a second peroxidase (POD)-labeled anti-mouse IgG antibody (1 μg ml^{-1} in 100 ml of blocking solution; 2 h). After washing with T-TBS (6×) POD activity was measured and quantified on a LumiImager after incubation with the chemoluminesence substrate (40 ml; 1 min). The result is shown in Figure 3 (10 min exposure time).

4.3.10. Synthesis of hexapeptomers 6, 7, 8, and 9 on resin. The hexamers **6**, **7**, **8**, and **9** were synthesized on Tentagel-S-RAM-resin (400 mg) using modifications of known protocols for the introduction of submonomer[37] and monomer building blocks,[31] respectively. After initial removal of the Fmoc-group [(i) piperidine (20% in DMF), 4.0 ml, 1×40 min; (ii) DMF, 5.0 ml, 5×1 min] each of the 6 building blocks was introduced according to the desired sequence (Table 2) using either the submonomer or the monomer protocol. Submonomer procedure: The resin bound amine was bromo-acetylated [(i) **1a** (0.3 M in

Table 2. Analytical data for hexapeptomers **6**–**9**

	Sequence[a] (N- to C-terminus)	Yield	Purity[b] (%)	ESI-MS Calcd	(M+H$^+$) Found
6	27-29-24-38-27-24	54 mg (54%)[c]	98	863.54	863.4[d]
7	24-24-36-15-36-24	7.4 mg (10%)[e]	98	706.45	706.3
8	39-38-29-02-13-27	31 mg (32%)[f]	98	718.46	718.3
9	20-40-31-15-01-27	33 mg (35%)[c]	97	800.43	800.2

[a] Numbers correspond to building blocks listed in Figure 2.
[b] HPLC, 220 nm.
[c] Salt with 2 equiv. TFA.
[d] High resolution FAB-MS (M+H$^+$): 863.5402 (calcd: 863.53949).
[e] Salt with 1 equiv. TFA.
[f] Salt with 3 equiv. TFA.

DMF), 1.5 ml, 1×30 min; (ii) DMF, 5.0 ml, 5×1 min] and treated with a solution of the appropriate primary amine in DMF [(i) 1.5 ml amine solution, 1×45 min; (ii) DMF, 5.0 ml, 5×1 min]. The following amines were used (concentrations in parentheses): 1: *n*-butylamine (5 M); 2: *rac-s*-butylamine (5 M); 13: *N*-boc-1,4-diaminobutane (3 M); 15: glycine-*tert*-butylester acetate (3 M in H_2O containing 0.9 equiv. of NaOH and 0.05% Tween 20; the resin was additionally washed with H_2O prior to and after amine treatment); 20: histamine (1.5 M); 24: cyclohexylmethylamine (5 M); 27: 4-methoxybenzylamine (5 M); 29: *N*-boc-1,3-diamino-propane (3 M); 31: 2-methoxyethylamine (5 M). Monomer procedure: The resin bound amine was acylated with the appropriate Fmoc-amino acid after preactivation for 30 min with 0.55 equiv. of DIC [(i) Fmoc-amino acid (0.6 M in DMF), 1.5 ml, 1×30 min; (ii) DMF, 5.0 ml, 5×1 min] followed by Fmoc-removal [(i) piperidine (20% in DMF), 2.0 ml, 1×20 min; (ii) DMF, 5.0 ml, 5×1 min]. The following amino-acids were used: 36: *N*-Fmoc-glycine; 38: *N*-Fmoc-L-alanine; 39: *N*-Fmoc-L-proline; 40: *N*-Fmoc-D/L-pipecolic acid.

After completion of the sequence the resin was washed extensively [piperidine (20% in DMF); DMF; H_2O; DMF; MeOH; CH_2Cl_2; Et_2O, each 5.0 ml, 3×1 min] and dried. Cleavage from the resin as well as deprotection was achieved by treatment with TFA (95% in H_2O; 6.0 ml; 30 min). The solution was filtered, the resin washed with acetonitrile and the combined filtrates were evaporated to dryness. The crude product was purified by prep. HPLC and analyzed by HPLC-MS (results see Table 2).

4.3.11. Analysis of mab Tab-2 binding by surface plasmon resonance (SPR) spectroscopy. The K_D values were determined using an SPR protocol[54] for a double-channeled Biacore X instrument. In our binding experiments the immobilized phase was generated by coupling monoclonal mouse-IgG-antibodies (measuring cell: Tab-2, 50 μg ml^{-1} in NaOAc-buffer, pH=5.0; reference cell: TE-33, 75 μg ml^{-1} in NaOAc-buffer, pH=4.0) to a *N*-hydroxysuccinimide activated dextrane-coated sensor chip (CM5) using the Biacore amine immobilisation kit (resulting in an antibody-loading between 5000 and 6000 RU). Binding was analysed at concentrations between 200 nM and 2.0 mM in HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20, pH=7.4). The chip was regenerated with glycine/HCl-buffer (10 mM, pH=2.0) prior to each measurement. The ammount of peptomer-binding at equilibrium was determined. Equilibrium values were analyzed using the steady-state kinetics plot and K_D-values were determined with the 'BIAevaluation' Software (version 3.0.1, Biacore AB, Uppsala, Sweden). Binding of the peptide VVSHFND-NH_2 was calculated from the corresponding kinetic association- and dissociation-constants, respectively ('BIAevaluation' Software, 1:1 Langmuir-binding, concentration range: 16 nM–4.0 μM, k_a=1.10×10^5 M^{-1} s^{-1}; k_d=2.18×10^{-3} s^{-1}). Determined values for χ^2 were below 1.2. The integrity of the chip was ensured by control-measurements in the course of approx. 130 measurement cycles yielding stable response.

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