Combinatorial Synthesis of Cholesterol Ester Transfer Protein-mRNA Ligands and Screening by Nondenaturating Gel-Electrophoresis

Michael Baumann,[†,§] Hilmar Bischoff,[#] Delf Schmidt,[#] and Christian Griesinger*[,†,$]

Institut für Organische Chemie, Johann Wolfgang Goethe-Universität Frankfurt/Main, Frankfurt, Germany, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Goettingen, Germany, Massachusetts Institute of Technology, Department of Chemistry, 77 Massachusetts Avenue, Cambridge, MA 02139, USA, and Bayer AG, Wuppertal, Germany.

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RNA, as one of the biomolecules with the most structural and functional diversity, is an attractive therapeutic target.[1] Employing combinatorial chemistry methods, small peptide ligands were found, which bind to a short RNA with important biological functions. A 23-nt RNA oligonucleotide from the cholesterol ester transfer protein mRNA was chosen as a molecular target.[2] A 27-nt RNA oligonucleotide from the human immunodeficiency virus type-1 (HIV-1) TAR RNA was used to control the binding specificity.[3] Tetrapeptide libraries, composed of the amino acids Lys, Tyr, Leu, Ile, and Arg, with and without C- and N-terminal lysines, were synthesized by a combination of combinatorial and divergent solid-phase synthesis. Gel-shift affinity screening was used to extract the peptides with the best RNA binding properties. The peptide Lys−Tyr−Lys−Leu−Tyr−Lys−Cys-NH_2 (**1**) showing micromolar affinity to its RNA target was characterized with circular dichroism (CD), ultra violet (UV) measurement, and ^{1}H NMR spectroscopy.

Introduction

A number of RNA oligonucleotide/ligand interactions have been characterized and described. A 17-amino acid peptide containing the arginine-rich region of the human immunodeficiency virus (HIV) Rev protein binds to Rev response element (RRE) RNA.[4] Also basic peptides from the carboxy terminus of the human immunodeficiency virus type 1 (HIV-1) Tat protein bind to the stem-loop region of transactivation response region TAR RNA.[3] The conformations of TAR RNA and of TAR RNA with the arginine-rich peptide Tat were characterized by NMR spectroscopy.[5] This peptide that binds to the TAR RNA has proven its biological activity by inhibition of HIV-1 replication in vivo.[6] Tripeptides that bind to the TAR RNA and inhibit gene expression could be isolated from randomized pools of combinatorial libraries.[7]For our library, the following design criteria were applied: RNA as a polyanionic molecule requires a minimum of basic residues to be present in peptide ligands, such as Lys or Arg, to achieve high affinity.[3] To enhance the binding specifity of the RNA ligands, amino acids with hydrophobic side chains such as Tyr, Leu, or Ile were added to the peptide libraries. This was prompted by the structure known so far between RNA and proteins in which hydrophobic interactions between bases and amino acid side chains are observed.[8] Tetrapeptide libraries constructed out of the mentioned hydrophobic amino acids were synthesized with combinatorial chemistry methods. As the RNA target, we

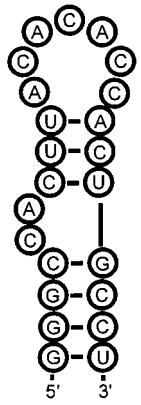

Figure 1. Nucleotide sequence of the 23-nt CETP RNA constituting an untranslated segment of the complete mRNA of this protein.[2]

chose the 23-nt RNA oligonucleotide from the 5′-untranslated region of the cholesterol ester transfer protein (CETP) mRNA that should be an ideal molecular target to inhibit translation. We screened the peptides for high affinity to this target molecule. Each library contains 625 different peptides. The libraries were synthesized as 25 mixtures of 25 different peptides and cleaved from the resin. They were then attached to a PEG-linker via a Cys.[9] The PEG-derivation was necessary to obtain a larger gel-retardation of the RNA oligonucleotides that were used for selection of the strongest binders. At first, 25 mixtures of 25 peptides ($25 \times 25 = 625$ peptides) were run in 25 lanes of a nondenaturating polyacrylamide gel with the target RNA. Afterward, the mixture containing individual peptides exhibiting the largest gel-shift and the highest content of hydrophobic amino acid residues were synthesized individually and run through the gel shift assay to obtain the best binding sequences. With this strategy, it is possible to screen the 625 peptide library by gel

* To whom correspondence should be addressed. Johann Wolfgang Goethe-Universität Frankfurt/Main, Institut für Organische Chemie, Marie-Curie-Str. 11, 60439 Frankfurt, Germany, Tel: (+49) 69-798-29131, Fax: (+49) 69-798-29128, E-mail: cigr@org.chemie.uni-frankfurt.de.

† Johann Wolfgang Goethe-Universitaet Frankfurt/Main.

$ Max Planck Institute for Biophysical Chemistry.

§ Present address: Massachusetts Institute of Technology.

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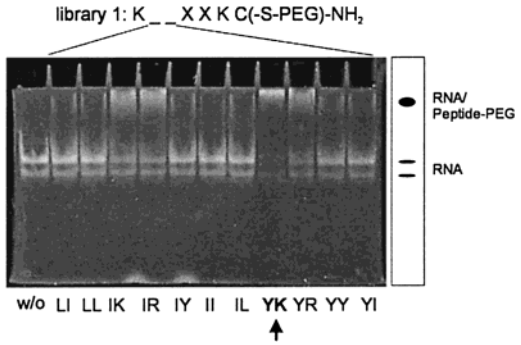

Figure 2. Gel-shift analysis of the heptapeptide library KXXXXKC-NH_2 with X = R, L, I, K, Y. The synthesis yields a product whose amino acids are known in positions 2 and 3. Position 4 an 5 are made by a split, mix and combine synthesis protocol. It turns out that the amino acids in the second and third position, X_2^{opt} and X_3^{opt}, are Y and K.

electrophoresis without the need for decoding the library by more sophisticated analytical means. The peptides identified in the gel-shift screening show a clear effect on the CD spectra of the target RNA by decreasing the CD signal and inducing bathochromic shift. As detected by NMR, the ^{1}H-signals of the amino acids in the peptide ligands were shifted upon binding of RNA. The peptide Lys−Tyr−Lys−Leu−Tyr−Lys−Cys-NH_2 (**1**) shows the largest effect with a $K_d = 32 \pm 2$ μM (from CD titration). In 2D NOESY experiments, the peptide ligand (**1**) exhibits cross-peaks to imino signals of the 23mer RNA.

Results and Discussion

Synthesis and Screening Assay. In the first screening step, each of the 25 lanes contained the target RNA and a mixture of 25 peptides to cover all 625 peptides of library 1.The peptide mixture of library 2 containing the dipeptide ^{2}Tyr−Lys was synthesized and screened again. The 23-nt RNA oligonucleotide shows two bands in the nondenaturating polyacrylamide gel, which reflects the conformational inhomogeneity of this sequence (probably dimerization). The polymer molar mass distribution (∼6500−8000 g/mol) of the PEG-linker is visible in the broad band of the RNA/peptide−PEG complex. The peptide−PEG conjugates bind to both conformations of the 23-nt RNA. From peptide library 1, only the peptide Lys−Lys−Leu−Arg−Cys-NH_2 (**2**) shows a visible affinity to the 23-nt RNA and with poor sequence specifity. Specifity was checked by running gel shift experiments with 27-nt ΔTAR RNA. We attribute the low specifity of **2** to the high content of basic amino acid residues. The peptides Lys−Tyr−Lys−Leu−Tyr−Lys−Cys-NH_2 (**1**) and Lys−Tyr−Lys−Ile−Tyr−Lys−Cys-NH_2 (**3**) extracted from the peptide library 2 show good binding affinities to the 23-nt RNA. These peptides have the same or better affinities as full basic peptides, the hydrophobic side chains of Leu and Tyr enhance the sequence specifity. As shown in Figure 3 the peptide ligands (**1**) and (**3**) have a strong binding affinity to the 23-nt CETP RNA, whereas an affinity to the 28-nt ΔTAR RNA cannot be detected in the gel shift assay.

In addition to that, the basic peptide sequence Tat10 (RKKKRRQRRRC-NH_2),[9] a natural ΔTAR RNA ligand, shows much poorer sequence specificity comparing CETP RNA and ΔTAR RNA sequences than **1**.

Biophysical Experiments. The peptide ligand has a strong influence on the CD spectrum of the target RNA. As shown in Figure 5 the maximum of the 23-nt RNA spectra at 270 nm is shifted toward longer wavelengths and attenuated after addition of peptide solution. The secondary structure of the RNA oligonucleotide is weakened by the peptide, as inferred from the decrease of the CD maximum at 270 nm.

The binding constant K_d of **1** was evaluated by CD titration with 32 ± 2 μM. The CD signal at 270 nm was used to get the 50% value bound/unbound RNA oligonucleotide (Figure 6).

By addition of the peptide ligand **1** with a concentration of 2 μM to the 23-nt RNA, the melting point of the secondary structure was decreased by 8 °C (Figure 7). This is consistent with the results of the CD experiments that indicate a weakening of the secondary structure observed by the decrease of ellipticity of the maximum of the RNA at 270 nm.

In the NMR spectra, peptide **1** shows variably strong shifts of the signals and line broadening. The aliphatic and aromatic proton signals of **1** are shifted to low field (Figure 8 a,b). This indicates that the involved protons have contact with the RNA and the aromatic π-systems of the bases. In a 2D NOESY experiment (with jump return water suppression),[10] the Leu (δ) protons (δ = 0.6 ppm) of ligand **1** show cross-peaks with the imino signals (δ = 12.9 ppm) of the 23-nt RNA (data not shown). These are unequivocally intermolecular NOEs due to the low field shift of the imino protons of nucleobases. Since imino protons are normally only observed for bases involved in stem regions and the 23-nt RNA in a hairpin,[2] this peptide should have contact to the stem region.

Biological Tests. Peptide **1** was tested in transgenic CETP-expressing mice for its physiological activity. These mice express the human CETP and are a good model for the CETP expression and activity. As a ligand of the 5′-untranslated region (UTR) of the CETP mRNA, the peptide may inhibit the translation to the CETP protein. CETP transfers cholesterol esters from the HDL to the VLDL (both lipoproteins). As a result of that, the VLDL level will be lowered, which has a negative effect on the progression of arthereosclerosis. The CETP activity (cholesterol ester transfer) can be measured by a fluorescence test (CETP activity kit, Roar Biomedical, Inc., New York) by observation of the cholesterol ester transfer between HDL and VLDL (pmol/h). A lower CETP activity will be detected by a reduced cholesterol ester transfer. Groups of three mice were injected (i.v.) with **1** (100 mg/kg) or 0.9% NaCl (control group), and a blood sample was taken. Because it was possible to take blood only once, for each time point, a different group of mice was used. Each value in Figure 9 corresponds to the median of three mice (the 15 min group of **1** corresponds to only two mice). In case of a stronger lowering of the cholesterol ester transfer, as in the control group, the CETP activity should be lowered. The stronger reduction of the CETP activity as in the control group after 15 min is unclear. Only two mice were used for this experiment. After 90 min, the mice treated with **1** show a clear reduction of the cholesterol ester transfer. To check the stability of the peptide **1** under physiological conditions, **1** was incubated with mouse serum (10%)

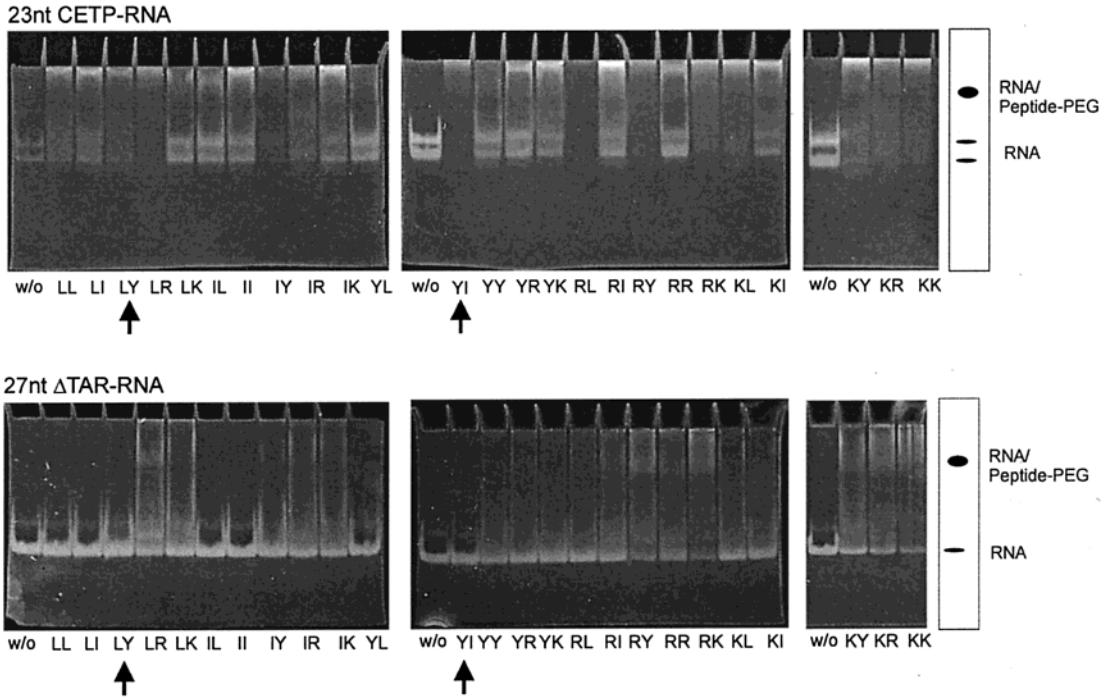

Figure 3. Top: Gel-shift analysis of the optimized heptapeptide library KYKXXKC-NH_2 with X = R, L, I, K, Y after the synthesis protocol containig X_2^{opt} = K and X_3^{opt} = Y. The most significant gel shift of the 23-nt CETP RNA induced by peptides with the least basic amino acids are observed for the heptapeptides with amino acids 4 and 5 pointed to by an arrow. Bottom: Gel shifts of the same peptides with ΔTAR RNA: The arrowed sequences show no gel shift with this RNA and are the most specific. More basic sequences bind less specifically to both RNAs.

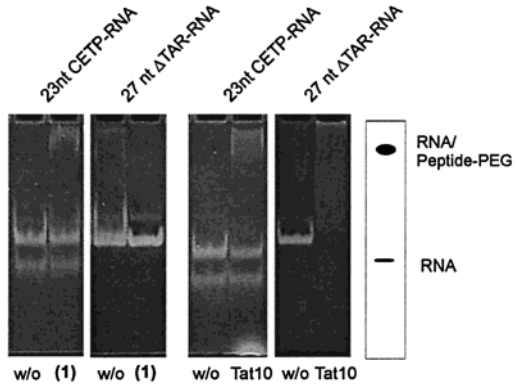

Figure 4. Gel shifts induced by peptide **1** and Tat10 on the 23nt CETP RNA and the 27-nt ΔTAR RNA, respectively. While Tat10 induces the most significant gel shift for ΔTAR RNA, it also induces a shift of the 23nt CETP RNA. Tat10 is less specific than peptide **1**.

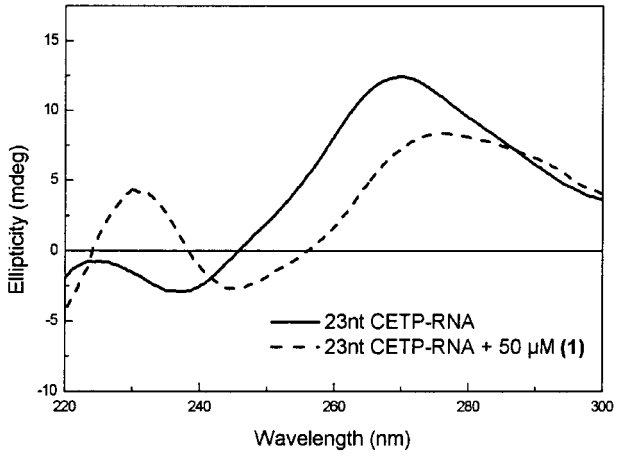

Figure 5. Change in the CD spectrum of CETP RNA when 50 μM of **1** are bound. There is a distinct bathochromic shift of the ellipticity maximum at long wavelengths.

for 24 h and analyzed by HPLC. After 24 h, more than 90% of the peptide was still unchanged.

Summary

This work has shown that it is possible to obtain peptide ligands for different RNA targets by the described gel-shift assay. Because of the anionic character of RNA, a minimum of basic amino acids such as Lys and Arg is required to obtain sufficient binding affinities. To enhance the sequence specificity of the ligands, hydrophobic amino acid residues are essential. The peptides found contain all Tyr or other hydrophobic amino acid residues in a basic environment and meet this goal. Tyr has a much stronger effect to enhance the affinity of peptide ligands to the chosen RNA target, as Phe. The peptides with Lys attached to the C- and N-terminus show also a higher affinity. Peptides with hydrophobic amino acid residues in a basic environment have good RNA binding properties. Peptide **1** meets this goal and has micromolar binding affinity to the 23-nt RNA and has a visible influence on the RNA's secondary structure as proved with CD, UV, and NMR techniques. Injected in transgenic mice, **1** induces reduced cholesterol ester transfer.

Experimental Section

General. Fmoc-amino acids and 1-Hydroxy-benzotriazol (HOBt) used were purchased from NovaBiochem (Switzerland). Chiral amino acids used were all of L-configuration. Side chain protecting groups used were Arg(Pmc), Cys(Trt), Lys(Boc), and Tyr(*t*Bu). Synthesis was performed using Rink Amide MBHA resin NovaBiochem (Switzerland). Diisopropylcarbodiimid (DIC) and the solvent dimethylformamid (DMF)

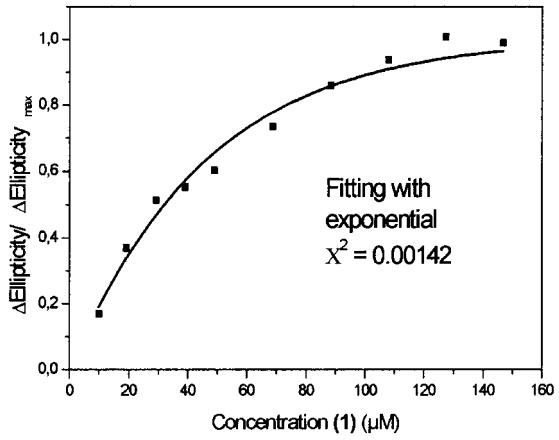

Figure 6. Concentration-dependent ellipticity measured at 270 nm relative to the maximum ellipticity when titrating (**1**). This curve serves to determine the K_d of the binding of **1** to the CETP RNA.

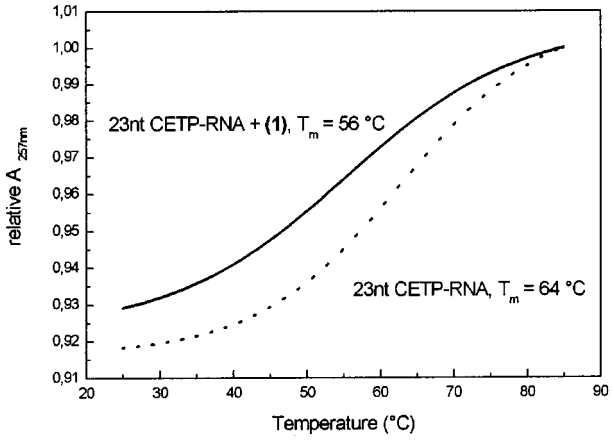

Figure 7. UV melting curves of the 23-nt CETP RNA with and without **1**. The melting of the RNA is less cooperative and at lower temperature in the presence of the peptide.

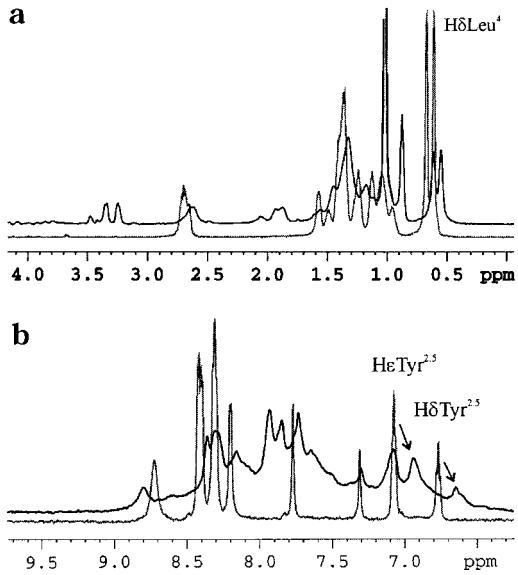

Figure 8. Comparison of the peptide NMR spectra with (black) and without the RNA (gray). Note the significant broadening of the peptide resonances upon binding of the RNA in the aliphatic (a) and aromatic (b) part of the resonances.

was purchased from Merck-Schuchard (Germany). *O*-(2-Maleimidoethyl)-*O*′-methyl-polyethylenglycol 5000 (M-Mal-PEG) was purchased from Fluka (Switzerland). All other reagents

Table 1. NMR Data of Selected Peptide Protons[a]

proton	δ (ppm) free	Δδ (ppm) free/complex	line width (Hz) free	line width (Hz) complex
HδLeu⁴	0.64	0.06	12	24
HδTyr²·⁵	6.54	0.14	22	29
HεTyr²·⁵	6.85	0.13	9	18
Cys⁷-NH₂[b]	7.08	>0.01	9	18
Cys⁷-NH₂[b]	7.54	0.04	9	20
NHLeu⁴	7.97	0.04	14	34
NHLys³·⁶	8.08	0.01	23	overlapped
NHTyr²·⁵	8.20/8.17	0.05	29	overlapped
NHCys⁷	8.50	0.07	45	50

[a] δ (ppm) are the chemical shifts of selected peptide protons without the RNA, and Δδ is the difference of the chemical shifts of selected protons in the absence versus the presence of RNA. The line width data are listed without and with RNA. [b] Amidated N-terminus.

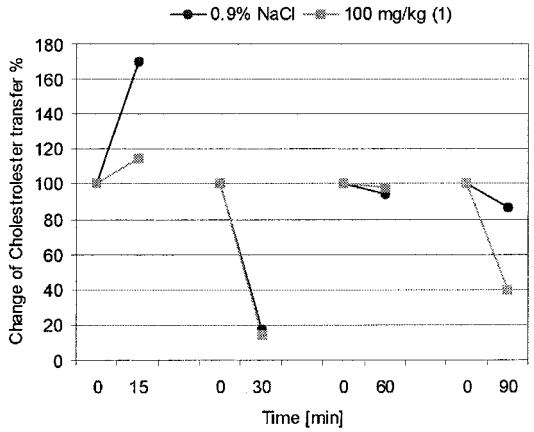

Figure 9. Graphical representation of the biological test summarized in Table 2. For each of the measured cholesterol ester transfer rates after a given time after application of either physiological salt solution or **1** the value of the rate was set to 100% and served as reference for the rate after 15, 30, 60, or 90 min had elapsed. The biggest effects due to the peptide are observed after 15 min and after 90 min. For the 15 min values, only two mice were used as opposed to the three mice used in the other three measurements.

Table 2. Averaged Rate of Cholesterol Ester Transfer between HDL and VLDL in pmol/h in Mice Measured after the Given Time after Injection of Physiological Salt Solution and 100 mg/kg of **1**

	0 min	15 min	0 min	30 min	0 min	60 min	0 min	90 min
0.9% NaCl	75.83	128.67	85.02	15.13	115.87	108.9	105.8	91.33
100 mg/kg (**1**)	85.4	98.05	107.83	15.53	122.77	120.73	161.7	64.3

used were of the highest quality commercially available. NMR experiments were done with a BRUKER Avance DRX 600 NMR spectrometer. CD measurements were done with a Jasco J-701 CD spectropolarimeter. The gel-shift experiments were performed with a Biometra multigel (8 × 11 × 0.1 mm).

Combinatorial Peptide Synthesis. All peptides and peptide libraries were synthesized manually. A pentapeptide library with diversity in the first four amino acid, library 1: ^{1}X−X−X−X−Cys(-S-PEG)-NH_2, X = Arg, Ile, Leu, Lys, and Phe, and a heptapeptide library with diversity in the amino acids 2−5 was constructed, library 2: ^{1}Lys−X−X−X−X−Lys−Cys(-S-PEG)-NH_2 with X = Arg, Ile, Leu, Lys, and Tyr. After cleavage of the peptides from the resin (92.5% TFA, 2.5% water, 2.5% ethandithiole, 2.5% triisopropylsilane for 3 h), the peptide libraries were precipitated with *t*-butylmethyl ether. A total of 0.5 mg of each peptide mixture was shaken with 1.5 eq. *O*-(2-Maleimidoethyl)-*O*′-methyl-polyethylenglycol 5000 (M-

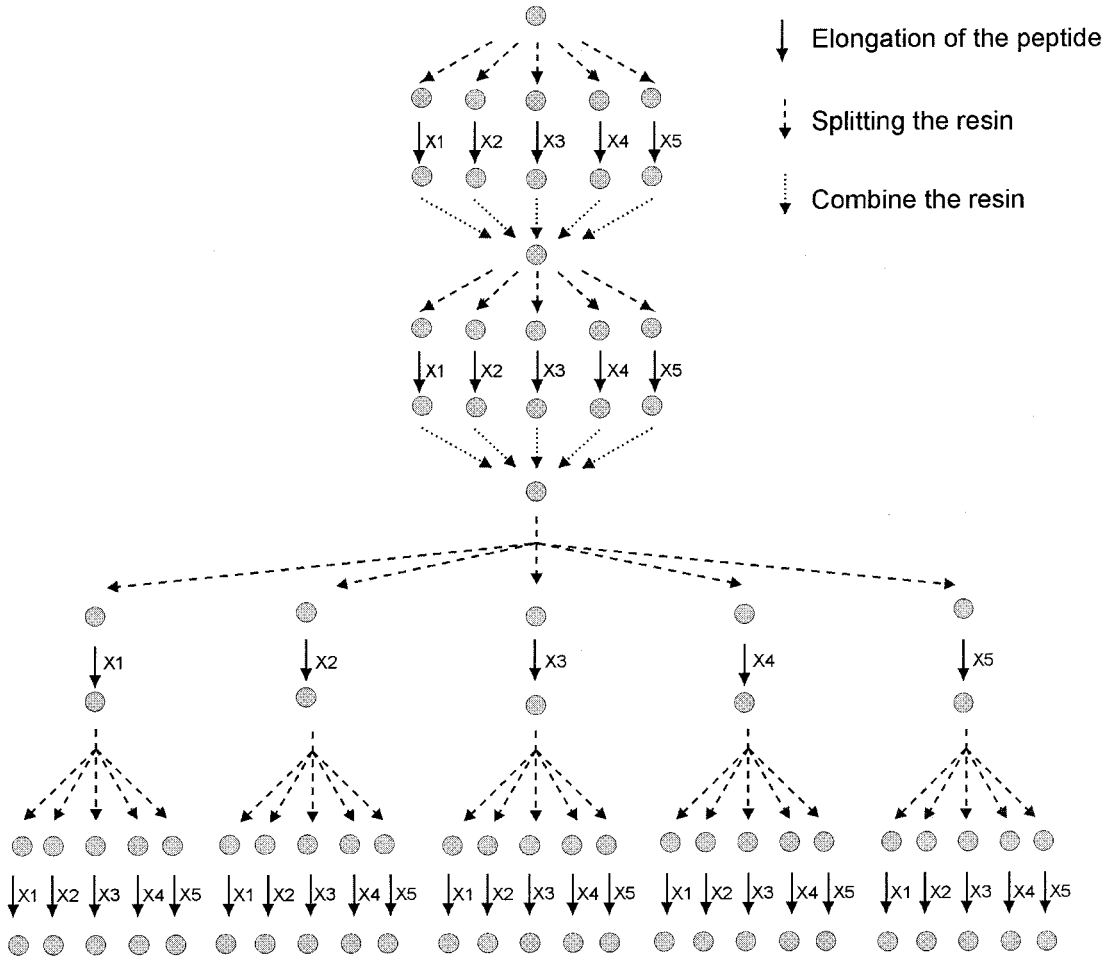

Figure 10. Protocol for the synthesis of the first type of peptide library. The synthesis proceeds from the C- to the N-terminus. The figure refers to amino acids 1−4 for the pentapeptide library 1 and 2−5 for the heptapeptide library 2. X1 to X5 are the amino acids Arg, Ile, Leu, Lys, and Phe for library 1. In library 2, Phe has been replaced by Tyr. The synthesis yields 25 product mixtures with known amino acids in positions 1 and 2 for library 1 and in positions 2 and 3 for library 2.

Mal-PEG) in 100 mM Na_2HPO_4, pH 7, for 2 h. The first two variable amino acid positions were synthesized with the split, mix and combine method, the last two positions were synthesized linear (by only splitting the resin without mixing and combining) (Figure 10).After screening libraries 1 and 2, the mixtures with the highest affinities were synthesized in parallel (by only splitting the resin without mixing and combining) to obtain all 25 peptides as individual compounds. Selected peptides were synthesized again for further investigation on a larger scale. They were purified by reverse-phase HPLC and characterized by electrospray ionization mass spectrometry. All peptides were quantified with amino acid analysis for the gel-shift, CD, UV, and NMR experiments.

RNA Synthesis. All RNA oligonucleotides were enzymatically synthesized in vitro using T7 RNA polymerase and chemically synthesized DNA templates.[11] The usage of the full length dsDNA template was nesessary to obtain the 23-nt RNA oligonucleotide. RNAs for gel-shift analysis were purified by using denaturating PAGE. We separated the desired 23-nt RNA species from the N+1 and N−1 species by denaturating gel electrophoresis to obtain clear gel-shift with the peptides. After gel purification, the RNA samples were precipitated by ethanol and dissolved in water. The RNA NMR samples were purified by using ion exchange HPLC and desalting with Sephadex G-10.

Gel-Shift Analysis. 10% nondenaturating PAGE (10 × 16 cm) was used for the gel-shift screening. The binding reaction mixtures (20 μL) contained 7.5 μM RNA, 50 μM peptide mixture/peptide. The solution contained 50 mM Tris·HCL (pH 7.5), 70 mM NaCl, 1 mM EDTA, 0.01% NP-40 (nonylphenoxy polyethoxy ethanol), and 10% (w/w) glycerol. The reaction mixtures were incubated at 37 °C for 15 min prior to electrophoresis in nondenaturating 10% polyacrylamide gels (40:1 w/w acrylamide/bisacrylamide). The gels were pre-run at 100 V for 1 h and run at 100 V for 40 min at RT in TBE buffer (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.3). The gels were stained with ethidium bromide.

CD Measurements. The experiments were performed at 22 °C. The sample RNA concentration was 2 μM in 10 mM K_2HPO_4, pH 7. Peptide ligand were added from 2 μM up to 144 μM.

UV Melting Curves. The sample RNA concentration was 2 μM in 10 mM K_2HPO_4, pH 7.

NMR Spectroscopy. All NMR-measurements were recorded on a Bruker DRX-600 NMR spectrometer. The homonuclear 1D and 2D NMR spectra were recorded at ~0.5 to 1 mM 23-nt RNA concentration in 10 mM K_2HPO_4, pH 6.6, with 100 mM NaCl. Peptide **1** was added up to 1.5 excess over RNA. As reference TSPA (trimethylsilylpropionic acid) was used. The jump return (JR)[11] pulse sequence was used for water suppression.

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Supporting Information Available: Analytical data, Gel-shift screening data. This material is available free of charge via the Internet at http://pubs.acs.org.

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