Selection of New Sequence-Selective Unnatural Peptides Binding to Double-Stranded Deoxyribonucleic Acids (dsDNA) by Means of a Gel-Retardation Experiment for Library Analysis

by **Patrick Chaltin**[a]), **Eveline Lescrinier**[a]), **Theo Lescrinier**[a]), **Jef Rozenski**[a]), **Chris Hendrix**[a]), **Helmut Rosemeyer**[b]), **Roger Busson**[a]), **Arthur Van Aerschot**[a]), and **Piet Herdewijn***[a])

[a]) Laboratory of Medicinal Chemistry, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven

[b]) Laboratory of Organic Chemistry, Osnabrück University, Barbarastrasse 7, D-49069 Osnabrück

Dedicated to Prof. *W. Pfleiderer* on the occasion of his 75th birthday

Previously, we developed a methodology for the solid-phase screening of peptide libraries for interaction with double-stranded deoxyribonucleic acids (dsDNA). In the search for new and more-potent DNA ligands, we investigated the strategy of solution-phase screening of chemical libraries consisting of unnatural oligopeptides. After synthesis of the selected amino acid building blocks, libraries were constructed with the general structure Ac-Arg-Ual-Sar-X^1-X^2-X^3-Arg-NH_2, where X represents each of twelve unnatural or natural amino acids. Optimization of the sequence of binding peptides was performed with an iterative deconvolution procedure. Selection of interacting peptides was carried out in solution by means of gel-retardation experiments, starting with libraries of 144 compounds. A 14-base-pair double-stranded DNA fragment was chosen as the target. After several cycles of synthesis and screening of libraries and individual peptides, an oligopeptide was selected with an apparent dissociation constant of $9 \cdot 10^{-5}$ M, as determined by gel-retardation experiments. This peptide was studied by NMR spectroscopy. A certain degree of conformational pre-organization of the peptides was shown by temperature-dependent circular-dichroism experiments. Finally, DNase-I-footprinting studies indicated a preferential interaction with a 6-base-pair mixed sequence 5′-CTGCAT-3′. This study demonstrates that gel-shift experiments can be used for the solution-phase screening of library mixtures of peptides against dsDNA. In general, this technique allows the selection of new sequence-selective dsDNA-interacting molecules. Furthermore, novel dsDNA-binding unnatural oligopeptides were developed with affinities in the 0.1 mM range.

1. Introduction. – The field of deoxyribonucleic acid (DNA) recognition has been sparked by studies of the huge variety of natural ligands like netropsin and the closely related compound distamycin. The basic chemical unit of these minor-groove-binding polyamides, which show a high preference for AT-rich sequences, is a carboxamide followed by a pyrrole ring [1]. Their synthetic analogues, the lexitropsins, have been developed with the aim to design sequence-specific minor-groove binders by substituting an imidazole or hydroxypyrrole for a pyrrole moiety. Recently, hairpin lexitropsins that achieve highly sensitive recognition of the four *Watson-Crick* base pairs in the minor groove have been synthesized [2–4]. Together with the lexitropsins and derivatives, peptide nucleic acids (PNA) are the most successful approaches so far in the DNA-recognition field. Because of the high enzymatic stability and the favorable RNA- and DNA-hybridization properties of PNAs, they are expected to become one of the most useful tools in molecular biology [5–7]. Next to the lexitropsin and the PNA approaches, many more molecules are being used and/or investigated because of their DNA-interaction capacities.

All of these approaches, however, encounter problems such as restriction of recognition, low ability to penetrate cells, and/or low enzymatic stability. Virtually all drugs currently available that interact with nucleic acids have limited specificity and, therefore, can not be used without the possibility of disrupting a normal function of the cell. Therapies utilizing these agents are usually reserved for life-threatening diseases such as cancer [8]. Therefore, the discovery of new sequence-specific DNA ligands is very appealing. This research may also contribute to the understanding of the mechanisms involved in the recognition of dsDNA (ds = double-stranded) by proteins and small molecules. With this in mind, we investigated a solution-phase screening assay for the identification of dsDNA ligands out of libraries of unnatural peptides. In this way, new sequence-specific dsDNA ligands with a K_d value of *ca.* 10^{-4} M were isolated (*Fig. 1*).

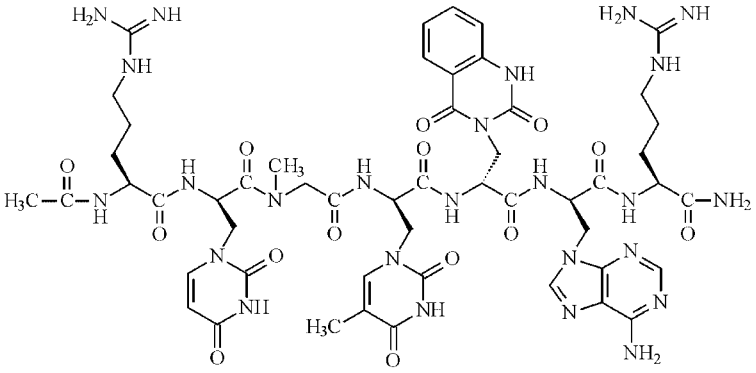


Fig. 1. *Structural representation of the isolated dsDNA-interacting oligopeptide Ac-Arg-Ual-Sar-Tal-Chi-Aal-Arg-NH$_2$, possessing an apparent dissociation constant of $2 \cdot 10^{-4}$* M

A variety of solution-screening methods have already been applied to discover DNA-interacting molecules. In most of these investigations, however, individual compounds were used in the activity tests. Therefore, the possibility of solution screening of library mixtures for their dsDNA-interaction capacities by gel mobility-shift assays was investigated. Recently, FID (fluorescent-intercalator displacement) [9] and DNase-I-footprinting experiments [10][11] have been used for the same purpose. Gel mobility-shift screening has, furthermore, already been applied to select RNA-interacting molecules [12–15].

2. Results. – 2.1. *Selection of the Amino Acid Building Blocks.* The amino acid building blocks of the library were selected on the basis of their potential to interact with DNA by means of general rules of noncovalent-bond formation: H-bonding, hydrophobic and charge interactions, and intercalation. Also, the possibility to induce conformational restrictions in the oligopeptide backbone (*e.g.*, hydroxyproline, isonipecotic acid (= piperidine-4-carboxylic acid)) and to increase the stability of the peptides against enzymatic degradation (D-amino acids) was taken into account [16].

Some library members were retained from a first selection round in our search for dsDNA-binding ligands by means of screening peptide libraries on the solid support

[17]. From this approach, we saved the amino acids depicted in *Fig. 2,a*, together with the sequence Arg-Ual-Sar, which occurred as starting sequence in all selected peptides. Other, new building blocks (*Fig. 2,b*) were then added to increase the interaction capacity of the peptides with dsDNA.

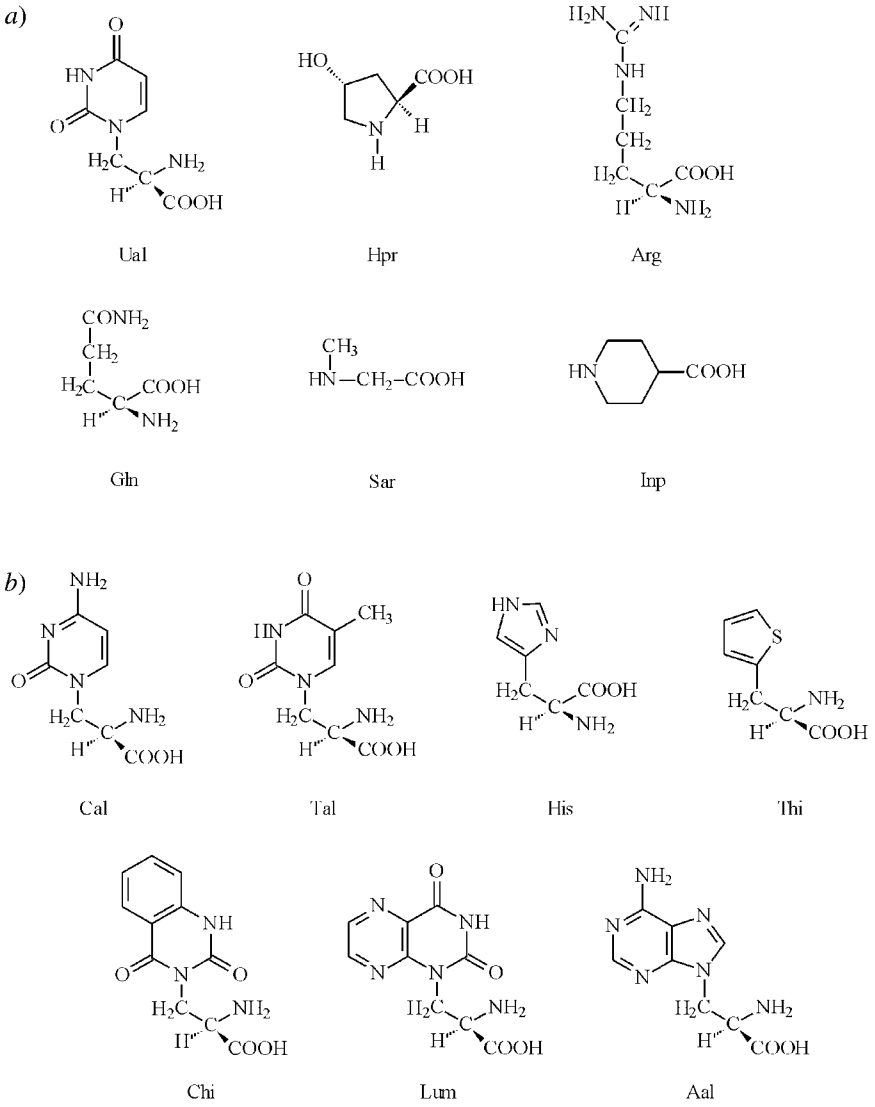


Fig. 2. a) *Amino acid building blocks retained from previously synthesized libraries: β-(uracil-1-yl)-*D*-alanine* (Ual), trans-*4-hydroxy-*L*-*proline* (Hpr), L-*arginine* (Arg), L-*glutamine* (Gln), *sarcosine* (Sar), *and isonipecotic acid* (Inp). b) *Newly selected amino acid building blocks: β-(cytosin-1-yl)-*D*-alanine* (Cal), *β-(thymin-1-yl)-*D*-alanine* (Tal), L-*histidine* (His), *β-(2-thienyl)-*D*-alanine* (Thi), *β-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-*D*-alanine* (Chi), *β-(lumazin-1-yl)-*D*-alanine* (Lum), and *β-(adenin-9-yl)-*D*-alanine* (Aal)

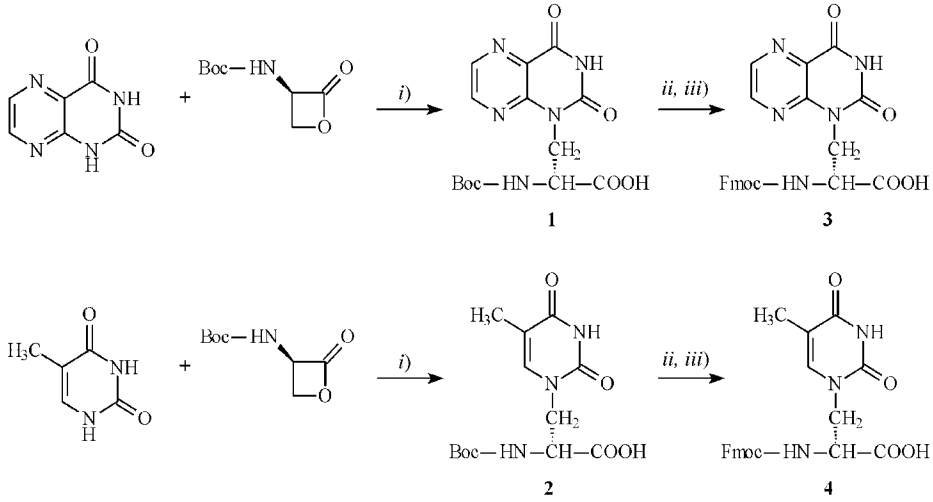
We selected Aal as a purine analogue with H-donor (d) and H-acceptor (a) properties. The selection of Cal and Tal is based on the frequent occurrence of the Ual amino acid in the previous library [17]. The Me group of thymine is known to stabilize nucleic acid interactions by increasing hydrophobic interactions. In contrast to Tal and Ual, which are ada systems, Cal represents a daa recognition system. The Cal, Tal, and Aal amino acid building blocks were already used before for the construction of alanyl-PNAs [18][19]. His was selected because of the presence of a substituted imidazole moiety in the polyamides, where it is important for minor-groove recognition of G·C base pairs. The Chi and Lum amino acids can potentially stabilize the peptide·dsDNA complex by stacking interactions. Finally, we substituted the thienyl-ornithine by Thi, the thienyl-alanine, to decrease flexibility.

2.2. *Synthesis of the Amino Acid Building Blocks.* For the assembly of the libraries, the following amino acid structures were used (Fmoc = (9*H*-fluoren-9-ylmethoxy)carbonyl, Boc = (*tert*-butoxy)carbonyl MeOTr = monomethoxytrityl = (4-methoxyphenyl)diphenylmethyl): *N*-Fmoc-isonipecotic acid, *N*-Fmoc-sarcosine, *trans*-*N*-Fmoc-4-(*tert*-butoxy)-L-proline, N^{α}-Fmoc-β-(uracil-1-yl)-D-alanine, N^{α}-Fmoc-β-(thymin-1-yl)-D-alanine (**4**), N^{α}-Fmoc-N^{G}-(2,2,5,7,8-pentamethylchromane-6-sulfonyl)-L-arginine (*G* = guanidine moiety), N^{α}-Fmoc-β-(2-thienyl)-D-alanine, N^{α}-Fmoc-β-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-D-alanine (**7**), N^{α}-Fmoc-β-(lumazin-1-yl)-D-alanine (**3**) (lumazine = pteridine-2,4-(1*H*,3*H*)-dione), N^{α}-Fmoc-β-[N^{4}-(MeOTr)-cytosin-1-yl]-D-alanine (**10**), N^{α}-Fmoc-β-(N^{6}-Boc-adenin-9-yl)-D-alanine (**13**), N^{α}-Fmoc-N^{im}-trityl-L-histidine (*im* = imidazole moiety), and N^{α}-Fmoc-N^{γ}-trityl-L-glutamine. The lumazine, thymine, quinazoline-2,4-(1*H*,3*H*)-dione, cytosine, and adenine derivatives were synthesized by means of protected serine β-lactones [20][21]. The exocyclic amino groups of cytosine and adenine were protected with a MeOTr and Boc group, respectively, to avoid side reactions, and this in contrast to the use of unprotected bases described in [19][22]. The remaining building blocks were prepared according to known procedures [17], while N^{α}-Fmoc-N^{G}-(2,2,5,7,8-pentamethylchroman-6-sulfonyl)-L-arginine and N^{α}-Fmoc-β-(2-thienyl)-D-alanine were obtained from *Novabiochem* and N^{α}-Fmoc-N^{im}-trityl-L-histidine and N^{α}-Fmoc-N^{γ}-trityl-L-glutamine from *Advanced Chemtech.*

Since lumazine and thymine contain no side-chain functionalities reactive in peptide synthesis, protection was not necessary. The nucleophilic character of the heterocycles was enhanced by a 1-h treatment with NaH in DMF. For thymine, similar reactions with DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) instead of NaH have been described [18]. The first step in the synthesis of the lumazine and thymine derivatives **3** and **4** consisted of a nucleophilic attack of the sodium salts of lumazine or thymine at C(β) of *N*-Boc-D-serine β-lactone in DMF, which led to N^{α}-Boc-(lumazin-1-yl)-D-alanine (**1**) or N^{α}-Boc-(thymin-1-yl)-D-alanine (**2**), respectively, besides minor quantities of by-products (see *Scheme 1*). After purification by extraction and column chromatography (silica gel), the Boc protecting group was cleaved with CF_3COOH and substituted by a Fmoc protecting group. The synthesis of *N*-Boc-D-serine β-lactone was performed as described [20][21].

Originally, the amino acid β-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-1-yl)-D-alanine, consisting of quinazolinedione alkylated at N(1) with alanine, was selected to be used in the oligopeptide library. But addition of *N*-Boc-D-serine β-lactone to the

Scheme 1. *Synthesis of* N$^{\alpha}$*-Fmoc-β-(lumazin-1-yl)-*D*-alanine* (**3**) *and* N$^{\alpha}$*-Fmoc-β-(thymin-1-yl)-*D*-alanine* (**4**)



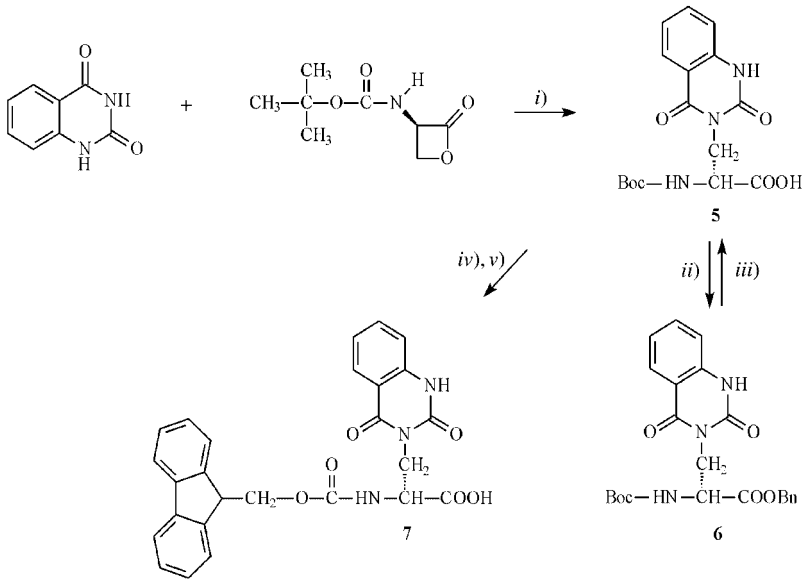
i) NaH, DMF. *ii*) CF_3COOH, thioanisole, CH_2Cl_2. *iii*) FmocCl, Na_2CO_3, dioxane, H_2O.

sodium salt of quinazoline-2,4(1*H*,3*H*)-dione under the same conditions as for lumazine (1.1 equiv. in rel. to NaH, DMF) yielded practically no tractable compound. By replacing NaH with DBU, three reaction products were obtained, of which the major one (60%) was the N^3-substituted Boc derivative **5** of *β*-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-D-alanine, as established by NMR data (*Scheme 2*). This prompted us to evaluate this molecule as a building block for the unnatural oligopeptides. The N^3-alkylated quinazolinedione is still able to stack and presents amide functionalities capable of forming H-bonds. In addition, the structural diversity of the amino acid building blocks increases by introduction of *β*-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-D-alanine. Compound **5** was purified *via* its benzyl ester **6**, and after chromatography and debenzylation of the latter by hydrogenolysis, the desired pure Fmoc product **7** was obtained in the usual way (*Scheme 2*).

The exocyclic amino group of the cytosine base was protected by a monomethoxytrityl group, before reacting with the *β*-lactone (*Scheme 3*). The reaction was performed as described by *Breipohl* and *Uhlmann* [23]. A mixture of monomethoxytrityl chloride and *N*-methylmorpholine was added to cytosine in pyridine and left to react overnight after a short temperature raise to 40°. The desired product **8** was obtained by precipitation from the reaction mixture.

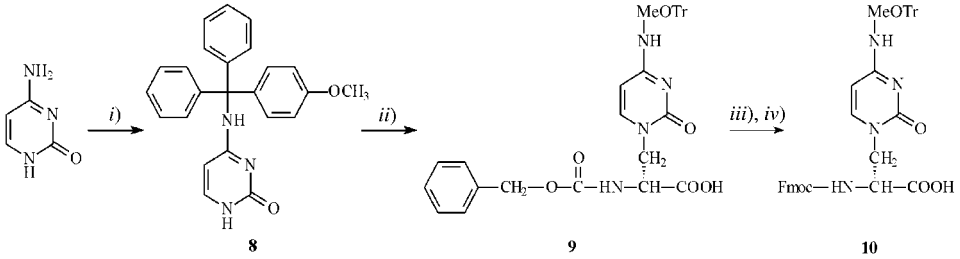
Adenine was protected by reaction with di(*tert*-butyl)dicarbonate (Boc_2O) since the conjugate base of N^6-(MeOTr)-adenine reacted too slowly with the *β*-lactone (*Scheme 4*). Reaction of adenine with Boc_2O afforded several by products, probably corresponding to the N^6-, N^7-, and N^9-mono-Boc-substituted isomers, besides two di-Boc-protected adenine derivatives, *i.e.*, the N^7- and N^9-substituted N^6-Boc-adenine, or the N^6-di-Boc-substituted product. Although reactions with the N^6-di-Boc-adenine are described [24], hydrolysis of the di-Boc derivatives converted these compounds mainly to the N^6-monosubstituted adenine **11**.

Scheme 2. *Synthesis of* N$^{\alpha}$*-Fmoc-β-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-*D*-alanine*



i) DBU, DMF. *ii*) BnBr, Et_3N, DMF. *iii*) H_2/Pd, MeOH. *iv*) CF_3COOH, thioanisole, CH_2Cl_2. *v*) FmocCl, Na_2CO_3, dioxane, H_2O.

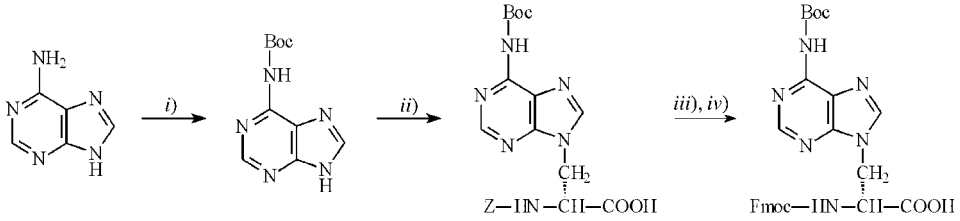
Scheme 3. *Synthesis of* N$^{\alpha}$*-Fmoc-β-[*N^4*-(MeOTr)-cytosin-1-yl]-*D*-alanine*



i) MeOTrCl, *N*-methylmorpholine, pyridine, 40°. *ii*) NaH, *N*-Z-D-serine β-lactone, DMF. *iii*) H_2/Pd, MeOH. *iv*) FmocCl, Na_2CO_3, dioxane, H_2O.

The protected bases **8** and **11** were used in a reaction with *N*-Z-D-serine β-lactone (Z = (benzyloxy)carbonyl) since the acid lability of the protecting groups at the cytosine and adenine is not compatible with the acidic conditions necessary for the removal of the Boc group of the *N*-Boc-substituted β-lactone. The Z group can be removed by hydrogenolysis. Thus, following nucleophilic attack of N^4-(MeOTr)-cytosine (**8**) or N^6-Boc-adenine (**11**) at *N*-Z-D-serine β-lactone (→ **9** or **12**, resp.), hydrogenolysis of the Z group and reaction with FmocCl furnished the desired final compound **10** or **13**, respectively (*Schemes 3* and *4*). Since hydrogenolysis never went to completion, an intermediate extraction procedure was included to purify the free amino compound before conversion to the desired Fmoc derivative.

Scheme 4. *Synthesis of* N^{α}*-Fmoc-β-(*N^{6}*-Boc-adenin-9-yl)-*D*-alanine*



11 **12** **13**

i) $(Boc)_2O$, DMF, pyridine, 80°, 1 h. *ii*) DBU, *N*-Z-D-serine β-lactone, DMF. *iii*) H_2/Pd, MeOH. *iv*) FmocCl, Na_2CO_3, dioxane, H_2O.

2.3. *Evaluation of Peptide Synthesis.* Before starting with the synthesis of libraries, the feasibility of using the new amino acids in a library assay was verified by synthesizing two test peptides on solid support with the randomly chosen sequences Ac-Arg-Tal-Lum-Chi-Aal-Arg-NH_2 and Ac-Arg-Chi-Tal-Cal-Cal-Hpr-Sar-Arg-NH_2. The synthesis of these peptides was necessary to optimize the conditions for the coupling reactions and to trace side reactions. Therefore, Fmoc determination was performed after each coupling to investigate the yield, while HPLC and mass spectrometry of the final product served to reveal the presence of possible by-products. The ideal coupling conditions (yield > 95%) were achieved for all amino acid building blocks with 4 equiv. of DIC (diisopropylcarbodiimide), 4 equiv. of HOBt/HOAt (1-hydroxybenzotriazol/1-hydroxy-7-azabenzotriazol), 4 equiv. of $^{i}Pr_2EtN$, and 2 to 4 equiv. of *PyBOP*™. After cleavage from the solid phase, the peptides were purified by HPLC. For both peptides, the chromatogram showed one major peak (> 80%), corresponding to the mass of the desired peptide sequence (*Table 1*).

Table 1. *Calculated and Observed Molecular Mass of the Test Peptides as Determined by LSI-MS*

	M_r, calc.	M_r, found
Ac-Arg-Chi-Tal-Cal-Cal-Hpr-Sar-Arg-NH_2	1341	1342
Ac-Arg-Tal-Lum-Chi-Aal-Arg-NH_2	1234	1234

2.4. *Synthesis of Oligopeptide Libraries, Solution-Phase Screening, and Identification of Binding Ligands by a Deconvolution Procedure.* The oligopeptide libraries were constructed by means of solid-phase synthesis and the mix-and-split method. Screening was performed in solution rather than on solid phase, since, for the latter, the polymer on which the peptides are bound can influence the results of the experiments. Therefore, an acid-labile benzhydrylamine (=(diphenylmethyl)amine) linker was placed between the solid phase and the peptides. The general structure of the peptides in the libraries was as follows: Ac-Arg-Ual-Sar-X^1-X^2-X^3-Arg-NH_2, where X^n stands for each of the 13 amino acid building blocks depicted in *Fig. 2*, *a* and *b*, except arginine. To determine (for each position X) the amino acids with the highest interaction capacities, an iterative deconvolution procedure was used [25]. The solution-phase screening was performed with a 14-base-pair-long dsDNA sequence as target, representing an important recognition site of the NF-IL6, *i.e.*, 5′-(AGATTGTG-

CAATGT)-3′ · 5′-(ACATTGCACAATCT)-3′, and by gel-retardation experiments. The different peptide mixtures were separately added to the ^{32}P-labeled dsDNA target in PBS buffer (see *Exper. Part*). These reaction mixtures were incubated for 2 h and applied to a 15% native polyacrylamide gel. After electrophoresis and imaging, the obtained signals were compared. Since, in most cases of library screening, the band of the DNA · oligopeptide complex was not observed (either a smear, DNA precipitation, or a shift was obtained), the degree of complex formation was quantified by measuring the residual amount of free DNA. This is, in any case, the most rigorous way to work, since the possibility exists that complexes dissociate during electrophoresis [26]. Experiments with a ssDNA target (ss = single-stranded) yielded no disappearance of the target, establishing that interaction with dsDNA is necessary to obtain either a shift, a smear, or even precipitation. Furthermore, gel-mobility assays with modified dsDNA targets provided different results depending on the sequence, which leads to the same conclusion that unspecific aggregation of the peptides is not the cause of the observed smear or precipitation. In addition, the gel-retardation method proved to be reliable for the selection of dsDNA-binding ligands, as we could demonstrate that it gives the same results as the ethidium bromide displacement assay (*vide infra*).

After the synthesis of the first library in which position X^1 was fixed, and following deprotection and cleavage of the peptides from the solid phase, gel-retardation assays were performed. Twelve different samples, each containing a mixture of 144 synthetic peptides, were evaluated in one screening assay (*Fig. 3*). These experiments showed that libraries with the general structure Ac-Arg-Ual-Sar-**Chi**-X^2-X^3-Arg-NH_2 and Ac-Arg-Ual-Sar-**Tal**-X^2-X^3-Arg-NH_2 possessed the highest interaction capacities with the dsDNA target, together with Hpr at position X^1. Subsequently, two new libraries were synthesized with the quinazolinedione and thymine derivatives at the X^1 position.

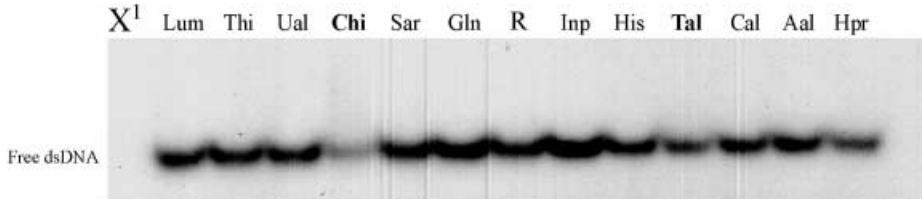


Fig. 3. *Gel shift experiments of the oligopeptides of the first library with fixed position X*^{1}. General structure of the peptides is Ac-Arg-Ual-Sar-**X^1**-X^2-X^3-Arg-NH_2. R represents the blank. In each run, a mixture of 144 peptides is present at a total concentration of 2 mM.

In the second cycle of screening, two times twelve samples, each containing twelve different synthetic peptides were screened. The screening experiments of these peptide mixtures showed that, for both series, the compounds with the quinazoline derivative at position X^2 possessed high interaction capacities (*Fig. 4*).

In the case where X^1 was already fixed as Chi, similarly strongly binding peptides could be observed with His, Cal, Aal, and Lum at position X^2. To facilitate the selection procedure for the next round of synthesis, an additional assay was performed, based on the binding selectivity of the small libraries. Therefore, besides the dsDNA target used in the previous gel shift assays, four modified dsDNA strands were used as targets for

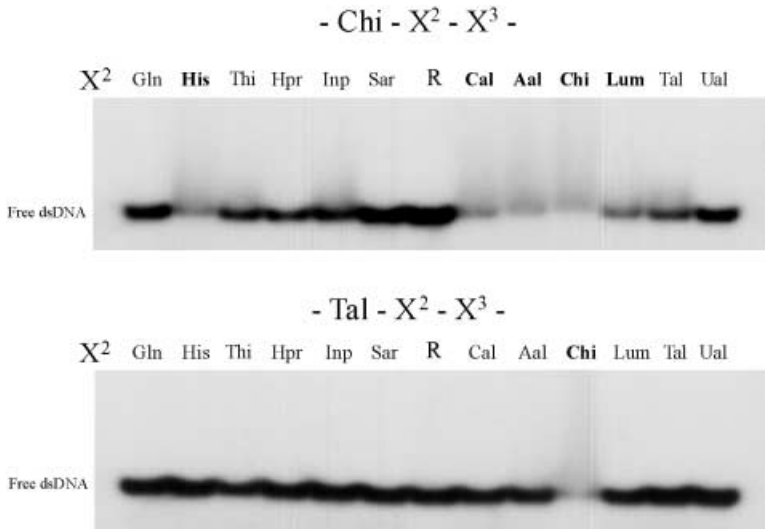


Fig. 4. *Gel-retardation experiments of the oligopeptides of the second library.* The general structure of the peptides is Ac-Arg-Ual-Sar-Chi-X^2-X^3-Arg-NH_2 (-Chi-X^2-X^3-) and Ac-Arg-Ual-Sar-Tal-X^2-X^3-Arg-NH_2 (-Tal-X^2-X^3-). Each run represents a mixture of twelve peptides that are screened at a total concentration of 1 mM. R represents the blank.

the selectivity assays. These dsDNA targets were selected so that additional information about the binding site of the peptides could be obtained. The original dsDNA target (T^0) was modified either at the left side (T^1), the right side (T^2), in the middle (T^3), or randomly (T^4) (*Fig. 5,a*). In all modified sequences, adenine was replaced by guanine and thymine by cytosine (and *vice versa*). The ratio of purine/pyrimidine bases remained, as such, the same in all targets ($T^0 - T^4$). As the original sequence is partially palindromic, we realize that the selectivity obtained could not be very high. These tests, however, showed that there was a distinct difference in specificity between the peptides and that the presence of the quinazolinedione derivative did not lead to a lack of selectivity *per se* (*Fig. 5, b*). The peptides with Cal at the X^2 position, however, showed low specificity. The sequences with the Chi-Chi pair at positions X^1-X^2 showed stronger binding to the original and the T^2 sequences than to the T^1, T^3, and T^4 sequences. This suggests that the peptides are preferentially bound to the 5′-AGATTGT-3′ terminal of the dsDNA. The sequences with the Tal-Chi pair at positions X^1-X^2 are more selective for binding to T^3 than to T^0. This demonstrates that introducing a selectivity test at this level of screening (mixtures of twelve compounds) is of relative value in the optimization. Based on both screening results, the following structures were selected for synthesis of individual peptides: Ac-Arg-Ual-Sar-**Chi-Chi**-X^3-Arg-NH_2 and Ac-Arg-Ual-Sar-**Tal-Chi**-X^3-Arg-NH_2.

The last step in the deconvolution procedure is the synthesis and screening of the 24 individual peptides (*Fig. 6*). All peptides were purified by reversed-phase HPLC and analyzed by mass spectrometry. The HPLC profiles showed one dominant peak (> 80%), and the calculated masses corresponded to the measured ones. The purified individual peptides were compared for their binding to the target by means of gel-

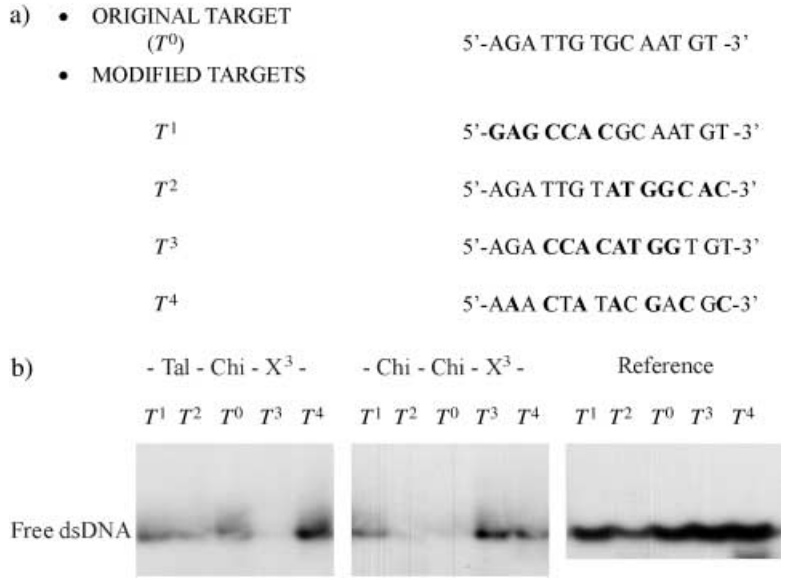


Fig. 5. a) *Modified targets used in specificity assays* (as double strands, in combination with the respective complementary sequence). b) *Specificity tests: Gel-retardation experiments with the original target* (T^0) *and four modified targets* (T^1, T^2, T^3, and T^4). The general structure of the peptides is Ac-Arg-Ual-Sar-Chi-X^2-X^3-Arg-NH_2 and Ac-Arg-Ual-Sar-Tal-X^2-X^3-Arg-NH_2. In the reference lanes, no peptide mixture was added. The total concentration of the mixture of twelve peptides was 1 mM in each case.

retardation experiments with the normal dsDNA target. It should be mentioned that, in every selection round, the concentration of the peptides used for screening could be decreased. The concentration of all peptides or peptide mixtures in a comparative screening assay was always the same. The strongest-binding peptides were also analyzed for their selectivity of binding with the modified targets.

These experiments led to the selection of seven oligopeptides that seemed to possess the strongest and most specific binding capacities. For the peptides with the Tal-Chi (X^1-X^2) sequence, the compounds with Aal and Chi at X^3 displayed the strongest binding (*Fig. 6*). However, only the Tal-Chi-Aal compound was selected for further studies as the Tal-Chi-Chi compound showed less selectivity. More versatility of binding peptides was found in the Chi-Chi (X^1-X^2) series, but here the sequence with Lum at position X^3 was not selected because of low selectivity. Six peptides emerged from this series, *i.e.*, those with Ual, Tal, Chi, Cal, Aal, and Thi at position X^3. All the selected peptides are shown in *Table 2* with their apparent dissociation constants (see below).

To verify the gel-retardation experiment, the selection procedure was compared to results obtained with a high-throughput ethidium bromide displacement test [27]. In this experiment, the same relative strength of binding of the oligopeptides was observed, demonstrating the utility of the described gel-retardation assay for screening of library mixtures and the high similarity with the described FID assay.

2.5. *Determination of Apparent Dissociation Constants* (K_d). To evaluate the strength of binding in comparison with other DNA-interacting molecules, apparent

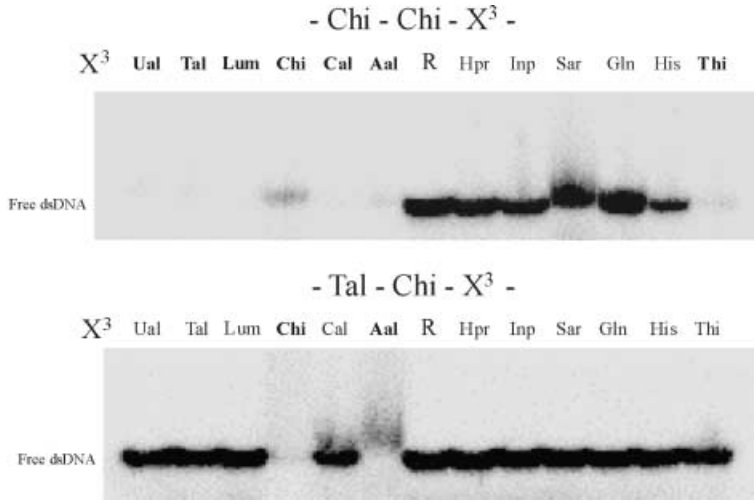


Fig. 6. *Gel shift experiments with 24 individual oligopeptides* (1 mM). The general structure of the peptides is Ac-Arg-Ual-Sar-Chi-Chi-X^3-Arg-NH_2 (-Chi-Chi-X^3-) and Ac-Arg-Ual-Sar-Tal-Chi-X^3-Arg-NH_2 (-Tal-Chi-X^3-). R represents the blank.

Table 2. *The Seven Selected Peptides with Their Apparent* K_d. The K_d values are averaged from 3–4 experiments.

	Apparent K_d [M]
Ac-Arg-Ual-Sar-**Chi-Chi-Cal**-Arg-NH_2	$6.3 \cdot 10^{-4} \pm 0.7$
Ac-Arg-Ual-Sar-**Chi-Chi-Ual**-Arg-NH_2	$6.1 \cdot 10^{-4} \pm 0.8$
Ac-Arg-Ual-Sar-**Chi-Chi-Tal**-Arg-NH_2	$4.9 \cdot 10^{-4} \pm 0.6$
Ac-Arg-Ual-Sar-**Chi-Chi-Aal**-Arg-NH_2	$3.9 \cdot 10^{-4} \pm 0.5$
Ac-Arg-Ual-Sar-**Chi-Chi-Thi**-Arg-NH_2	$2.3 \cdot 10^{-4} \pm 0.3$
Ac-Arg-Ual-Sar-**Tal-Chi-Aal**-Arg-NH_2	$2.2 \cdot 10^{-4} \pm 0.3$
Ac-Arg-Ual-Sar-**Chi-Chi-Chi**-Arg-NH_2	$\mathbf{9.1 \cdot 10^{-5}} \pm 0.1$

dissociation constants were determined for the seven selected peptides (see *Table 2*). Gel shift experiments with different peptide concentrations were used for this purpose. The concentration of the peptides was increased from 0.02 mM to 1 mM (*Fig. 7*). The apparent K_d was determined as the peptide concentration at which 50% of the target is mobility shifted.

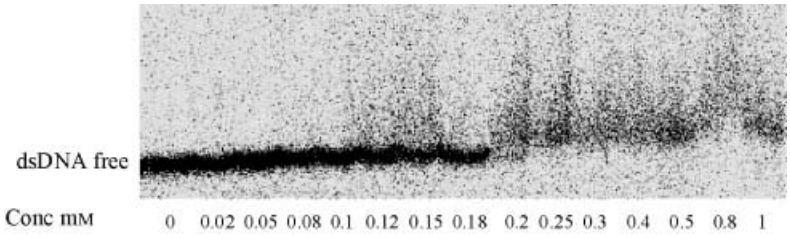


Fig. 7. *Gel shift assay with different concentrations of the peptide Ac-Arg-Ual-Sar-Tal-Chi-Aal-Arg-*NH_2*, in order to determine the apparent* K_d

The binding affinities of the seven peptides for the dsDNA target are very similar, although the values vary by a factor of *ca.* 8. All the apparent dissociation constants, however, are still higher than the dissociation constants of known DNA-interacting molecules, such as netropsin and distamycin with a K_d between $2 \cdot 10^{-6}$ and $2 \cdot 10^{-9}$ M [1]. The peptide Ac-Arg-Ual-Sar-Chi-Chi-Chi-Arg-NH_2 possesses the highest binding capacity ($K_d = 9.1 \cdot 10^{-5}$ M).

2.6. *CD Experiments.* Binding interactions between molecules are favored by low entropy changes. Therefore, temperature-dependent circular-dichroism (CD) experiments were performed to determine whether a certain pre-organization or preferred peptide conformation could be recognized. Characteristic CD bands for protein secondary structures (α-helix, β-sheet, mixed, unordered) can be found in the far-UV range (<250 nm), while the near-UV region reflects the contributions of aromatic side chains, disulfide bonds, and induced CD bands of prosthetic groups. Together, these measurements provide information about the overall structure of a protein molecule as well as about its local conformation around the aromatic and prosthetic groups and disulfide linkages [28]. The far-UV CD spectra of the investigated oligopeptides clearly show that all of them possess a certain degree of pre-organization and, thus, can be excluded from the group of unordered proteins, which usually show CD spectra with a strong negative band near 200 nm, together with some weak positive or negative bands between 220 and 230 nm.

According to their CD spectra, the seven investigated oligopeptides can be classified into four categories. The CD spectrum of Ac-Arg-Ual-Sar-Chi-Chi-Chi-Arg-NH_2 seems to possess the characteristics of an α-helical-like structure (or mixed α/β secondary structure). Indeed, the double minimum (at *ca.* 222 and 208–210 nm) and the strong maximum (at 191–193 nm) typical for α-helix structures, are visible in its spectrum at 242 and 231 nm and at 212 nm, respectively (*Fig. 8,a*). Four other peptides can be classified as peptides with probably β-sheet-like secondary structures. Regular all-β-proteins usually have a single negative minimum between 210 and 225 nm and a stronger positive band between 190 and 200 nm. The peptides Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2, Ac-Arg-Ual-Sar-Chi-Chi-Ual-Arg-NH_2, Ac-Arg-Ual-Sar-Chi-Chi-Cal-Arg-NH_2, and Ac-Arg-Ual-Sar-Chi-Chi-Aal-Arg-NH_2 each shows a similarly red-shifted single negative band at *ca.* 229–230 nm and a single positive maximum (a double for Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2) between 202 and 210 nm (*Fig. 8,b*). The intensities are, however, higher than those of the α-helical peptide Ac-Arg-Ual-Sar-Chi-Chi-Chi-Arg-NH_2.

The peptide Ac-Arg-Ual-Sar-Tal-Chi-Aal-Arg-NH_2 also shows a CD spectrum with two positive maxima at 195 and 210 nm, but the two minima at 240 and 250 nm are not negative as should be expected for β-sheet-type peptides. The consecutive intense maxima of Ac-Arg-Ual-Sar-Chi-Chi-Thi-Arg-NH_2 make it impossible to make any suggestions about its secondary structure by means of reference spectra, although it certainly differs from the spectrum of unordered oligopeptides. Further, it was observed that temperature changes, in a range from 10° to 70°, did not substantially influence the shape of the CD spectra, but did alter the intensities of the signals. For Ac-Arg-Ual-Sar-Tal-Chi-Aal-Arg-NH_2 and Ac-Arg-Ual-Sar-Chi-Chi-Thi-Arg-NH_2, a clear flattening of the curve shape is observed at higher temperatures. This points, at first sight, to stable secondary structures, which gradually loose their pre-organization upon heating.

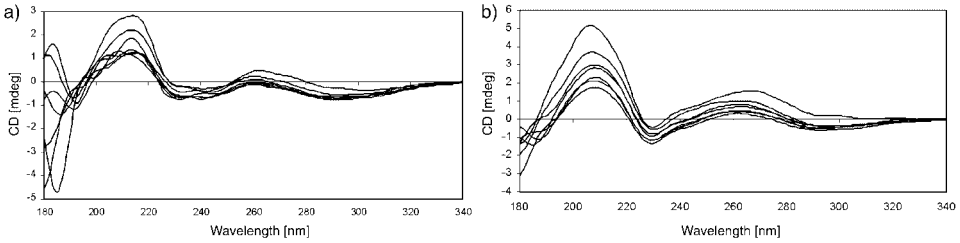


Fig. 8. *CD Spectra of the peptides* a) *Ac-Arg-Ual-Sar-Chi-Chi-Arg-NH$_2$ and* b) *Ac-Arg-Ual-Sar-Chi-Chi-Cal-Arg-NH$_2$ in function of the temperature.* Curves were measured from 10° (upper curve) to 70°, with increments of 10°.

2.7. *Footprinting Experiments.* To investigate whether the selected oligopeptides bound dsDNA in a sequence-specific manner, DNase-I-footprinting experiments were performed [29]. The 271-base-pair fragment used in these assays was obtained from the Pvu II – Asn I digest of the plasmid pUC 19 which contained the 14-mer target site used for screening (nucleotide positions 139 – 152). Patterns of DNase-I digestion in absence and in presence of 100 μM of the oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Chi-Arg-NH$_2$ are shown in *Fig. 9*.

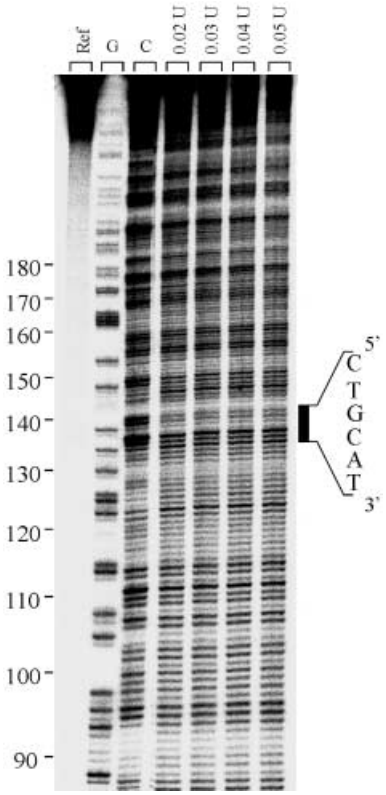


Fig. 9. *DNase-I footprinting on the 271-base-pair Asn I-Pvu II restriction fragment, cut out from the cloned plasmid pUC 19, in the presence of 100* μM *oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Chi-Arg-NH$_2$.* The duplex DNA was 3′-end labeled at the Asn I site with [α-^{32}P]dTTP in the presence of AMV reverse transcriptase. The products of the DNase-I digestion were resolved on an 8% polyacrylamide gel containing 8M urea. DNase-I concentrations used for digestion are indicated at the top of each lane (U/10 μl reaction mixture). The track labeled reference (Ref) contained dsDNA that was not digested with DNase I, the track labeled G represents dimethyl sulfate/piperidine markers specific for guanine and the track labeled control (C) contained no drug. Numbers at the side of the gel refer to the numbering scheme of the fragment. The sequence and position of the most important oligopeptide binding site is indicated.

With the peptide bound, the DNase-I cleavage pattern differs clearly from that seen in the control lane. Differential cleavage plots (*Fig. 10*) revealed one site of pronounced inhibited cleavage located around nucleotide positions 135–140. This 6 base-pair-long mixed sequence 5′-CTGCAT-3′ contains a part of the original target namely 5′-CT-3′ and shows an interesting similarity with 5′-TTGCAC-3′, present in the original target 14-mer. Part of this last sequence, namely 5′-TTG-3′, was also inhibited for DNase-I cleavage, together with two other sites at nucleotide positions 107–112 (5′-CATAGC-3′) and 96–100 (5′-CTGTG-3′), but less pronounced. Further investigation of the exact binding mode of the peptide to dsDNA remains to be done in the future.

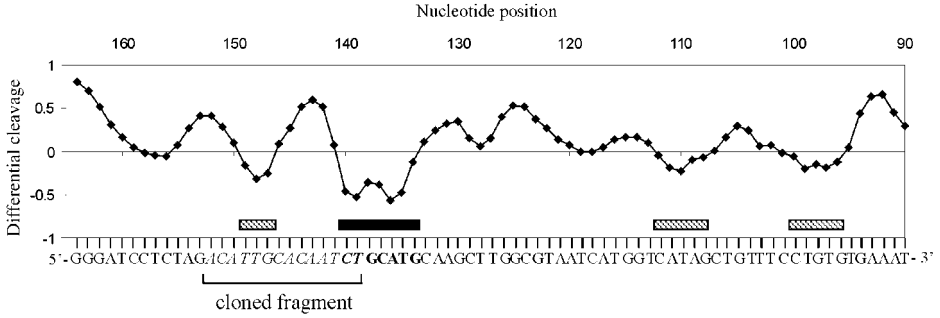


Fig. 10. *Differential cleavage plot comparing the susceptibility of the 3′-labeled 271-base-pair fragment to DNase-I cutting in the absence and in the presence of 100* μM *of the oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Chi-Arg-*NH_2. Negative values correspond to a ligand-protected site and positive values represent enhanced cleavage. Vertical scale: units of $\ln(f_a) - \ln(f_c)$, where f_a is the fractional cleavage at any bond in the presence of the oligopeptide and f_c is the fractional cleavage of the same bond in the control. The filled box indicates the most efficient binding site, while hatched boxes indicate less-pronounced interaction sites of the oligopeptide. Data are compiled from quantitative analysis of several sequencing gels like the one shown in *Fig. 9* and must be considered a set of average values.

2.8. *NMR Experiments.* ^{13}C- and ^{1}H-NMR Spectroscopy was used to determine the correct structures of the peptides and the possible presence of different conformers. The peptide Ac-Arg^1-Ual^2-Sar^3-Chi^4-Chi^5-Chi^6-Arg^7-NH_2 showed the lowest apparent K_d (*Table 2*) and was, therefore, selected for further structural study by NMR spectroscopy. The peptide was found present in solution as an equilibrium mixture of two structures. They were identified as the *cis* and *trans* conformers (*i.e.* (*E*) and (*Z*) configuration, resp.) at the peptide bond between Ual^2 and Sar^3 by their characteristic NOE interactions (*Fig. 11*).

Due to the spacious Me group at the N-atom of Sar, sterical hindrance of Ual^{2}C(α) with Sar^{3}C(α) in the *cis* conformer or Sar^{3}Me in the *trans* conformer are comparable. This effect makes the *trans* form only slightly favored, which allows for the detection of a nearly one-to-one ratio of *cis* and *trans* conformers that interconvert only slowly on the NMR time scale (ms). A complete assignment of non-exchangeable protons and C-atoms (except quaternary C-atoms) in both conformers was obtained by two-dimensional homonuclear and heteronuclear correlation experiments. *Table 3* shows the chemical-shift data. Strongest differences in chemical shift were observed for

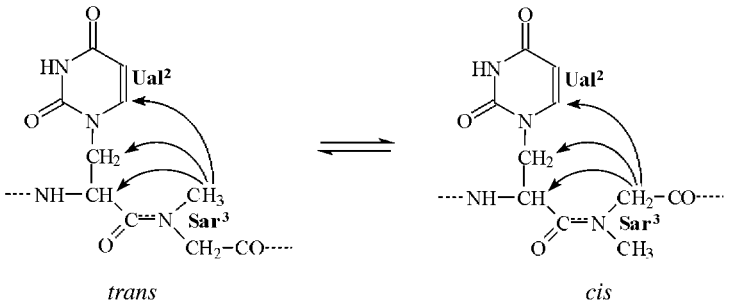


Fig. 11. *Schematic representation of the* cis *and* trans *conformers that were observed at the peptide bond between Ual²* and *Sar³*. Inter-residue NOE interactions typical for each of the conformers are depicted by arrows.

residues closest to the Ual²-Sar³ bond. Interconversion of the two sets of signals was confirmed in a ROESY experiment at room temperature with mixing time 250 ms.

3. Discussion. – In our effort to find new DNA ligands, we pursued the strategy of creating and investigating chemical libraries of unnatural oligopeptides. In a first part of this research, amino acid building blocks for assembling the dsDNA recognition libraries were selected. After the synthesis of the unnatural amino acids, two test peptides with random sequences were synthesized to optimize the coupling conditions and to trace side reactions. Starting with 13 building blocks, a first library was constructed with the following general structure: Ac-Arg-Ual-Sar-X^1-X^2-X^3-Arg-NH_2, where X stands for each of twelve amino acids. The peptides were prepared by solid-phase synthesis and the mix-and-split method. Optimization of the peptide sequence was carried out by an iterative deconvolution procedure. Since screening on solid phase has several limitations, the peptides were cleaved from the solid support to allow screening in solution. The binding potential of the peptides to dsDNA was investigated by means of gel-retardation experiments. This experimental setup allows to select at least medium-binding compounds, as it is generally expected that ligands with an apparent K_d value of 10^{-4} M or higher cannot be detected in gel shift assays. The 14-base-pair-long dsDNA target used in these assays was a partially palindromic recognition sequence of NF-IL6. During the screening experiments of the resulting peptides, besides the strength of binding, a supplementary parameter for selection, namely the binding specificity, was used.

After two rounds of synthesis and screening of libraries (=the deconvolution process), 24 individual oligopeptides with apparently the highest binding capacities and some degree of specificity were synthesized, purified, analyzed, and applied to gel shift experiments. From this pool, seven strong-binding oligopeptides were selected for further investigations. Dissociation constants were determined by performing gel shift assays at different peptide concentrations, and CD showed the presence of a certain degree of preorganization of the oligopeptides binding to dsDNA. DNase-I foot-printing experiments showed that the oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Chiv-Arg-NH_2 binds selectively to the sequence 5′-C<u>TGCA</u>T-3′, which was not explicitly present in the original fragment, but which shows similarity to the sequence 5′-T<u>TGCA</u>C-3′

Table 3. *Chemical-Shift Assignments at 22° in D_2O for* cis *and* trans *Conformers of Ac-Arg[1]-Ual[2]-Sar[3]-Chi[4]-Chi[5]-Chi[6]-Arg[7]-NH_2.* δ(H) Internally referenced to the residual H_2O signal and δ(C) externally referenced to DSSA (= 2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt (= sodium 3-(trimethylsilyl)propane-1-sulfonate)).

	cis	*trans*		*cis*	*trans*
Arg[1]:H–C(α)	4.25	4.27	Chi[5]:H–C(α)	4.97	4.75
H–C(β)	1.76	1.77	CH_2(β)	4.42	4.45
H′–C(β)	1.65	1.63	H–C(5)	7.77	7.84
CH_2(γ)	1.59	1.60	H–C(6)	7.04	7.12
CH_2(δ)	3.17	3.17	H–C(7)	7.36	7.42
C(α)	58.42	58.17	H–C(8)	6.87	6.92
C(β)	32.98	33.11	C(α)	57.72	58.73
C(γ)	29.35	29.35	C(β)	46.67	45.90
C(δ)	45.39	45.39	C(5)	129.77	129.96
			C(6)	126.24	126.35
Ual[2]:H–C(α)	4.97	5.37	C(7)	138.40	138.64
H–C(β)	3.76	4.11	C(8)	117.53	117.74
H′–C(β)	3.48	3.69			
H–C(5)	5.57	5.75	Chi[6]:H–C(α)	4.97	4.75
H–C(6)	7.25	7.46	H–C(β)	4.36	4.38
C(α)	53.30	52.84	H′–C(β)	4.36	4.34
C(β)	54.46	54.89	H–C(6)	7.20	7.23
C(5)	103.85	103.99	H–C(5)	7.83	7.82
C(6)	149.18	149.85	H–C(7)	7.61	7.64
			H–C(8)	7.03	7.05
Sar[3]:H–C(α)	4.60	4.05	C(α)	55.88	57.44
H′–C(α)	3.89	3.92	C(β)	46.54	46.24
MeN	2.50	3.08	C(5)	129.96	129.96
C(α)	57.15	56.26	C(6)	126.52	126.52
MeN	39.74	41.28	C(7)	138.81	138.92
			C(8)	117.94	118.04
Chi[4]:H–C(α)	4.94	4.76			
H–C(β)	4.47	4.47	Arg[7]:H–C(α)	4.44	4.37
H′–C(β)	4.39	4.38	H–C(β)	1.94	1.87
H–C(5)	7.83	7.89	H′–C(β)	1.80	1.71
H–C(6)	7.24	7.29	CH_2(γ)	1.68	1.58
H–C(7)	7.62	7.69	CH_2(δ)	3.23	3.16
H–C(8)	6.99	7.10	C(α)	58.03	57.98
C(α)	56.64	57.58	C(β)	33.32	33.28
C(β)	46.50	46.16	C(γ)	29.35	29.35
C(5)	129.96	129.96	C(δ)	45.47	45.39
C(6)	126.52	126.65			
C(7)	138.81	138.92	Ac:MeCO	2.30	2.35
C(8)	117.83	118.04	*Me*CO	24.83	24.83

of the 14-mer target. The exact way the peptides are bound to dsDNA is not known yet, although several explanations of the observed cleavage pattern are possible. The sequence 5′-TGCA-3′ plays, most likely, an important role in the recognition of the dsDNA by the peptides. The flanking nucleotides in the 6-base-pair inhibited site (C and T) lead to a higher stability of the DNA · peptide complex, compared to the sequence present in the 14-base-pair-long target (5′-TTGCAC-3′). Alternatively, it is possible that only shorter sequences like 5′-TTG-3′ or 5′-TGC-3′ are recognized by the

oligopeptides, or even that only part of the peptide is bound at 3-base-pair sites, while the whole peptide is bound at the 6-base-pair-long inhibited sequence 5′-CTGCAT-3′. More research to determine the exact binding mode is, therefore, needed.

Results of the deconvolution procedure with the oligopeptide libraries reveal that *β*-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-D-alanine (Chi) possesses properties that are especially suited for interaction with dsDNA. Stacking of the heterocyclic ring systems inside or outside the duplex together with H-bonding interactions may contribute to this effect. Stacking between the quinazolinedione rings of the amino acid outside the duplex may induce some pre-organization of the peptides, reducing the entropy cost of binding to dsDNA. In this respect, it is interesting to observe that the lumazine-substituted amino acid does not display a similar effect. The different orientation of the two ring systems, lumazine being attached *via* N(1) and quinazolinedione *via* N(3), may account for this observation.

During NMR characterization of the peptide Ac-Arg-Ual-Sar-Chi-Chi-Chi-Arg-NH_2, the presence of two conformers at the peptide bond between Ual and Sar was observed. This hampers initial modeling experiments of the peptide – DNA interaction. Further research and freezing of the peptide structures in one of the conformers by synthesizing conformationally restricted analogues will be necessary to facilitate interaction studies and to obtain better-binding compounds.

This study demonstrates the utility of the gel-retardation assay for selection of dsDNA ligands out of library mixtures. Comparison of the gel-retardation experiment with other described methods reveals that each approach has its limitations. The higher throughput and technically easy FID consumes more product and is not generally applicable to all dsDNA-interacting molecules, since the presence of fluorophores or fluorophore quenching could be troublesome. Although a lot of information can be gathered in footprinting assays to screen mixtures of dsDNA ligands, the technique is time-consuming, demands relatively high technical proficiency, and the selection of active compounds is not straightforward due to sensitivity problems [10]. Therefore, the gel shift experiment has certainly its place in the discovery process of new dsDNA-binding molecules out of library mixtures.

This investigation shows that solution-phase screening of libraries consisting of 1728 different peptides against dsDNA is feasible by means of gel-retardation experiments. New sequence-selective dsDNA-interacting oligopeptide sequences with an apparent dissociation constant of at least 10^{-4} M were selected with this technology. The affinity is still two to three magnitudes lower than for existing DNA ligands, but optimization of the unnatural amino acid sequences will further increase the binding potential in the future.

4. Conclusions. – Earlier work showed that it is possible to screen for dsDNA-binding ligands with an unnatural peptide-based library bound on solid support. However, screening in solution is preferred over screening on solid phase since the presence of the polymer can influence the screening results. Here, we demonstrate that screening of an unnatural peptide-based library (twelve pools of 144 compounds) for dsDNA binding is feasible in solution by means of gel-retardation experiments. Several unnatural oligopeptides showing dsDNA affinity were selected out of 1728 library compounds. Apparent dissociation constants of these peptide · DNA complexes were

determined. The compounds with the highest binding affinity were Ac-Arg-Ual-Sar-Chi-Chi-Chi-Arg-NH_2 ($K_d = 9.1 \cdot 10^{-5}$ M), Ac-Arg-Ual-Sar-Talv-Chi-Aal-Arg-NH_2 ($K_d = 2.2 \cdot 10^{-4}$ M), and Ac-Arg-Ual-Sar-Chi-Chi-Thi-Arg-NH_2 ($K_d = 2.3 \cdot 10^{-4}$ M). Thereby, the amino acid β-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-D-alanine seems to possess interesting properties for dsDNA interaction. NMR Characterization revealed the presence of two conformers around the Ual-Sar peptide bond. Temperature-dependent circular-dichroism experiments were performed and showed the presence of some degree of pre-organization of most of the selected oligopeptides binding to dsDNA. DNase-I footprinting experiments showed that the oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Chi-Arg-NH_2 bound in a sequence-specific manner to a 6-base-pair-long dsDNA fragment. This procedure establishes that it is feasible to select new sequence-selective dsDNA-binding ligands with a solution-phase screening method based on gel-retardation. The strategy lends itself to enlarge the group of molecules that can interfere with dsDNA expression. The diversity of unnatural amino acids that can be used in a library approach opens new perspectives for further increasing the binding strength and for altering the sequence selectivity. Further optimization of the structure of these peptides and investigations of their binding mode are the subject of ongoing research.

Experimental Part

1. *General*. Anh. solvents were obtained as follows: THF was refluxed over $LiAlH_4$ overnight and was distilled; CH_2Cl_2 was stored over CaH_2, refluxed, and distilled; pyridine, Et_3N, and *N,N*-diisopropylethylamine ($^iPr_2EtN$) were refluxed overnight over KOH and distilled. Dimethylformamide (DMF) was stored over activated molecular sieves (4 Å) for 3 days and was tested for absence of Me_2NH by the bromophenol test prior to use. MeCN for HPLC was purchased from *Rathburn* (grade S) and H_2O for HPLC purification was distilled twice. *Tentagel S-NH*$_2$ was obtained from *RAPP-Polymere* (Tübingen, Germany). CH_2Cl_2, DMF, Ac_2O, and pyridine were obtained from *BDH* (Poole, England). Piperidine, CF_3COOH, diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt), 1-methyl-1*H*-imidazole (NMI), di-*tert*-butyl dicarbonate ($(Boc)_2O$), benzyl carbonochloridate, *H*-fluoren-9-ylmethyl carbonochloridate (FmocCl), lumazine, thymine, adenine, cytosine, D-serine, benzyl bromide, (4-methoxyphenyl)diphenylmethyl chloride (MeOTr), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and *N*-methylmorpholine were supplied by *Acros* (Geel, Belgium). The 1-hydroxy-7-azabenzotriazole (=1-hydroxy-1*H*-1,2,3-triazolo[4,5-*b*]pyridine; HOAt) was purchased from *Perspective Biosystems* and (1*H*-benzotriazol-1-yloxy)-tri(pyrrolidin-1-yl)phosphonium hexafluorophosphate (*PyBOP*®) from *Advanced Chemtech*. Quinazoline-2,4(1*H*,3*H*)-dione, diethyl azodicarboxylate (DEAD), and NaH were purchased from *Sigma-Aldrich*. *para*-{(*RS*)-α-{[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino}-2,4-dimethoxybenzyl}phenoxyacetic acid ('Fmoc-Rink' linker) was supplied by *Novabiochem*. Oligonucleotides were purchased from *Eurogentec*. DNA Sequencing was performed by the dideoxynucleotide-chain-termination method with fluorescent universal forward and reverse M13 primers, and the sequencing reaction products were run on an automated laser fluorescent DNA sequencer (*Pharmacia Biotech*) under standard conditions. T4 Polynucleotide kinase and DNase I were purchased from *Gibco BRL* and [γ-^{32}P]dATP and [α-^{32}P]dTTP from *ICN*. *NAP-5*® columns were from *Pharmacia*. AMV Reverse transcriptase was obtained from *Amersham*. The plasmid pUC 19 and the restriction enzymes Pvu II, Asn I, Xba I, and Sph I were purchased from *Roche*. HPLC: semi-prep. *PLRP-S*^{R} column (250 × 9 mm, 100 Å, 15–20 μm). TLC: precoated *Machery-Nagel Alugram*®*-SilG/UV 254* plates; detection by UV light and H_2SO_4/anisaldehyde spray. Column chromatography (CC): *Acros* silica gel (0.06–200 nm). UV Spectra: *Philips PU-8740*-UV/VIS spectrophotometer. CD Spectra: *Jasco 600* spectropolarimeter; thermostatically controlled 1-cm cuvette connected with a *Lauda RK-20* bath. ^{1}H- and ^{13}C-NMR Spectra: unless stated differently, *Varian Gemini-200-MHz* spectrometer, (D_6)DMSO soln.s; ^{13}C-assignments were based on usual shift increments and calculus values and, where necessary, confirmed by APT experiments; assignments (mostly tentative) of quaternary C-atoms of the aromatic rings by comparison with other compounds; for exact structural determinations, NOE difference and GHMQC (gradient heteronuclear multiple quantum coherence) experiments were performed with a *Varian Unity-500-MHz* spectrometer; δ in

ppm, *J* in Hz. Liquid secondary ion (LSI) MS: Cs^+ as primary ion beam *Kratos Concept-IH* spectrometer (*Kratos*, Manchester, UK).

2. *Amino Acids Used in the Libraries.* For the assembly of the libraries, the following amino acid building blocks were used: *N*-Fmoc-isonipecotic acid (= 1-[(9*H*-fluoren-9-ylmethoxy)carbonyl]piperidin-4-carboxylic acid; Fmoc-Inp), *N*-Fmoc-sarcosin (= *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-*N*-methylglycine; Fmoc-Sar), *trans*-*N*-Fmoc-4-(*tert*-butoxy)-L-proline (= (4*R*)-4-(*tert*-butoxy)-1-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-proline; Fmoc-Hpr), N^{α}-Fmoc-*β*-(uracil-1-yl)-D-alanine (= *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-3-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-alanine; Fmoc-Ual), N^{α}-Fmoc-*β*-(thymin-1-yl)-D-alanine (= *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-3-(1,2,3,4-tetrahydro-5-methyl-2,4-dioxopyrimidin-1-yl)-D-alanine; Fmoc-Tal; **4**), N^{α}-Fmoc-N^{6}-(2,2,5,7,8-pentamethylchroman-6-sulfonyl)-L-arginine (= N^{2}-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-N^{5}-{[(3,4-dihydro-2,2,5,7,8-pentamethyl-2*H*-1-benzopyran-6-sulfonyl)amino]iminomethyl}L-ornithine), N^{α}-Fmoc-*β*-(2-thienyl)-D-alanine (= *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-3-(2-thienyl)-D-alanine; Fmoc-Thi), N^{α}-Fmoc-*β*-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-D-alanine (= *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-4-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-D-alanine; Fmoc-Chi; **7**), N^{α}-Fmoc-*β*-(lumazin-1-yl)-D-alanine (= *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-3-(1,2,3,4-tetrahydro-2,4-dioxopteridin-1-yl)-D-alanine; Fmoc-Lum; **3**), N^{α}-Fmoc-*β*-[N^{4}-(MeOTr)-cytosin-1-yl]-D-alanine (= *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-3-(1,2-dihydro-4-{[(4-methoxyphenyl)diphenylmethyl]amino}-2-oxopyrimidin-1-yl)-D-alanine; Fmoc-Cal; **10**), N^{α}-Fmoc-*β*-(N^{6}-Boc-adenin-9-yl)-D-alanine (= 3-(6-{[(*tert*-butoxy)carbonyl]amino}-9*H*-purin-9-yl)-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-D-alanine; Fmoc-Aal; **13**), N^{α}-Fmoc-N^{im}-trityl-L-histidine (= *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-1-(triphenylmethyl)-L-histidine; Fmoc-His), and N^{α}-Fmoc-N^{γ}-trityl-L-glutamine (= N^{2}-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-*N*-(triphenylmethyl)-L-glutamine; Fmoc-Gln).

3. *Amino Acid Building Blocks.* $N^{\alpha\text{-}Boc}$-*β*-*(lumazin-1-yl)*-D-*alanine* (= N-*[(*tert-*Butoxy)carbonyl]-3-(1,2,3,4-tetrahydro-2,4-dioxopteridin-1-yl)*-D-*alanine*; **1**). To a suspension of lumazine (= pteridine-2,4-(1*H*,3*H*)-dione; 3.57 g, 22 mmol, 1.1 equiv.) in anh. DMF (200 ml), NaH (0.48 g, 20 mmol, 1 equiv.) was added. The suspension was stirred at r.t. for 2 h and then cooled to −78°. A solution of *N*-Boc-serine *β*-lactone (3.74 g, 20 mmol) in DMF (50 ml) was added dropwise within 1 h. The mixture was stirred at r.t. for 18 h. After evaporation, the remaining solid was dissolved in H_2O, the soln. acidified to pH 2 with 2N HCl and extracted with AcOEt (3×), the org. layer dried ($MgSO_4$) and evaporated, and the residue submitted to CC (silica gel, CH_2Cl_2/MeOH/AcOH 97:3:0.1 → 90:10:0.1 (stepwise)): 3.33 g (47.5%) of **1**. ^{1}H-NMR: 1.46 (*s*, ^{t}Bu); 4.44 (*m*, CH(*α*) CH_2(*β*)); 6.93 (*d*, *J* = 8.2, OCONH); 8.59 (*d*, *J* = 2, H−C(7)); 8.74 (*d*, *J* = 2, H−C(6)); 11.99 (*s*, H−N(3)); long-range coupling of CH_2(*β*) (4.6 ppm) to C(9) and C(2) (149 and 150 ppm, resp.) ^{13}C-NMR: 172.49 (COOH); 160.27 (C(4)); 154.98 (OCONH(Boc)); 150.26 (C(2)); 149.84 (C(9)); 147.33 (C(7)); 139.52 (C(6)); 128.74 (C(10)); 77.74 (C−O(Boc)); 52.84 (CH(*α*)); 44.61 (CH_2(*β*)); 27.91 (Me(Boc)). HR-MS: 374.1067 ($C_{14}H_{17}N_5NaO_6^+$ [*M* + Na]$^+$; calc. 374.1076).

N^{α}-Boc-*β*-*(thymin-1-yl)*-D-*alanine* (= N-*[(*tert-*Butoxy)carbonyl]-3-(1,2,3,4-tetrahydro-5-methyl-2,4-dioxopyrimidin-1-yl)*-D-*alanine*; **2**). As described for **1**, with thymine (2.77 g, 22 mmol, 1.1 equiv.), DMF (200 ml), NaH (0.48 g, 20 mmol, 1 equiv.) and *N*-Boc-serine *β*-lactone (3.74 g, 20 mmol) in DMF (50 ml): 4.46 g (71%) of **2**. ^{1}H-NMR: 1.29 (*s*, ^{t}Bu); 1.70 (*s*, Me−C(5)); 3.48 (*d*, 1 H, CH_2(*β*)); 4.26 (*m*, 2 H, CH(α), CH(*β*)); 6.36 (*d*, *J* = 8.2, OCONH); 7.33 (*s*, H−C(6)); 11.15 (*s*, H−N(3)). ^{13}C-NMR: 172.08 (COOH); 164.68 (C(4)); 155.39 (OCONH); 151.17 (C(2)); 142.40 (C(6)); 107.65 (C(5)); 78.21 (C−O(Boc)); 52.71 (CH(*α*)); 50.04 (CH_2(*β*)); 28.13 (Me(Boc)); 12.07 (*Me*−C(5)). HR-MS: 336.1180 ($C_{13}H_{19}N_3NaO_6^+$, [*M* + Na]$^+$; calc. 336.1171).

N^{α}-*Boc-β-(1,2,3,4-tetrahydro-2,4-dioxo-3-yl)*-D-*alanine* (= N-*[(*tert-*Butoxy)carbonyl]-3-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)*-D-*alanine*; **5**). As described for **1**, with quinazoline-2,4 (1*H*,3*H*)-dione (3.61 g, 22 mmol, 1.1 equiv.), anh. DMF (200 ml), DBU (3 ml, 20 mmol, 1 equiv.) instead of NaH, and *N*-Boc-D-serine *β*-lactone (3.74 g, 20 mmol) in DMF (50 ml). Prior to CC, DMF (100 ml), Et_3N (2.2 ml, 15.6 mmol, 1 equiv.), and benzyl bromide (1.9 ml, 15.6 mmol, 1 equiv.) were added to the crude solid **5** (5.99 g, 15.6 mmol). After stirring for 18 h at r.t., the mixture was evaporated. Then 5% aq. $NaHCO_3$ soln. was added, and the product was extracted with AcOEt (3×). The org. layer was dried ($MgSO_4$) and evaporated, and the residue purified by CC (silica gel, hexane/Et_2O 3:7): 2.42 g (5.7 mmol) of N^{α}-*Boc-β-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)*-D-*alanine benzyl ester* (**6**). To a soln. of **6** in MeOH (50 ml), 10% Pd/C (*w*/*w*; 0.24 g, 10%) was added, and hydrogenation was performed for 5 h at 45 psi. After filtration of the mixture and washing the Pd extensively with MeOH, the filtrate was evaporated: 1.67 g (24%) of pure **5**. ^{1}H-NMR: 1.25 (*s*, ^{t}Bu); 4.26 (*m*, CH(*α*), CH_2(*β*)); 7.06 (*d*, *J* = 8.4, OCONH); 7.18 (*m*, H−C(6), H−C(8)); 7.64 (*t*, *J* = 7.8, H−C(7)); 7.91 (*d*, *J* = 7.6, H−C(5)); 11.45 (*s*, H−N(1)); long-range coupling of CH_2(*β*) (4.3 ppm) with C(2) and C(4) (150 and 162 ppm, resp.), and strong NOE from H−N(1) (11.5 ppm) to H−C(8) (7.2 ppm), both indicative for the N(3) position of the alanine moiety. ^{13}C-NMR: 172.08 (COOH); 162.34 (C(4)); 155.45 (Boc, OCONH); 150.44 (C(2)); 139.67

(C(8a)); 135.21 (C(7)); 127.65 (C(5)); 122.64 (C(6)); 115.23 (C(8)); 113.87 (C(4a)); 78.30 (C–O(Boc)); 50.98 (CH(α)); 40.69 (CH_2(β)); 28.03 (Me(Boc)). HR-MS: 394.1048 ($C_{16}H_{18}N_3Na_2O_6^+$, $[M-H+2\,Na]^+$; calc. 394.0991).

N$^{\alpha}$-Fmoc-*β-(lumazin-1-yl)*-D-*alanine* (**3**). A soln. of **1** (3.3 g, 9.4 mmol) in CF_3COOH/CH_2Cl_2/thioanisole 50:50:0.1 (150 ml) was stirred for 2 h. After evaporation and addition of 10% aq. Na_2CO_3 soln., dioxane, and FmocCl (1.1 equiv., 10.34 mmol, 2.66 g), the mixture was stirred for 18 h. The mixture was extracted with Et_2O (3×), acidified to pH 2 with 2N HCl and extracted with AcOEt. The combined org. layer was dried ($MgSO_4$) and evaporated, and the residue crystallized from EtOH: 2.54 g (57%) of **3**. M.p. 206°. ^{1}H-NMR: 4.33 (*m*, 6 H, CH(α), CH(β), CH_2O(Fmoc), H–C(9) (Fmoc)); 7.59 (*m*, 9 H, OCONH, arom. H (Fmoc)); 8.50 (br. *s*, H–C(7)); 8.69 (br. *s*, H–C(6)); 12.04 (*s*, H–N(3)). ^{13}C-NMR: 171.60 (COOH); 160.12 (C(4)); 156.03 (OCONH); 150.17 (C(2)); 149.47 (C(8a)); 147.56 (C(7)); 143.92 (C(8a), C(9a) (Fmoc)); 140.85 (C(4a), C(4b) (Fmoc)); 140.00 (C(6)); 128.83 (C(4a)); 127.83 (C(3), C(6) (Fmoc)); 127.31 (C(2), C(7) (Fmoc)); 125.37 (C(1), C(8) (Fmoc)); 120.27 (C(4), C(5) (Fmoc)); 65.88 (CH_2O) (Fmoc)); 51.59 (CH(α)); 46.58 (C(9) (Fmoc)); 41.54 (CH_2(β)). HR-MS: 472.1226 ($C_{24}H_{18}N_5O_6^-$, $[M-H]^-$; calc. 472.1257). Anal. calc. for $C_{24}H_{19}N_5O_6\cdot 1.5\,H_2O$: C 57.60, H 4.43, N 13.99; found: C 57.83, H 4.60, N 14.05.

N$^{\alpha}$-Fmoc-*β-(thymin-1-yl)*-D-*alanine* (**4**). As described for **3**, with **2** (4.4 g, 14.05 mmol): 3.38 g (55%) of **4**, after crystallization from acetone. M.p. 142°. ^{1}H-NMR: 1.60 (*s*, Me–C(5)); 3.62 (*d*, 1 H, CH_2(β)); 4.30 (*m*, 5 H, CH(α), CH_2(β), CH_2O (Fmoc), H–C(9) (Fmoc)); 7.35, 7.77 (*m*, 10 H, H–C(6), OCONH, arom. H (Fmoc)); 11.30 (*s*, H–N(3)). ^{13}C-NMR: 171.42 (COOH); 164.53 (C(4)); 156.27 (OCONH); 151.11 (C(2)), 143.83 (C(8a), C(9a) (Fmoc)); 142.27 (C(6)); 140.91 (C(4a), C(4b) (Fmoc)); 127.89 (C(3), C(6) (Fmoc)); 127.28 (C(2), C(7) (Fmoc)); 125.34 (C(1), C(8) (Fmoc)); 120.33 (C(4), C(5) (Fmoc)); 108.13 (C(5)), 60.04 (CH_2O (Fmoc)); 52.23 (CH(α)); 48.43 (CH_2(β)); 46.64 (C(9) (Fmoc)); 11.95 (*Me*–C(5)). HR-MS: 480.1108 ($C_{23}H_{20}N_3Na_2O_6^+$, $[M-H+2\,Na]^+$; calc. 480.1148). Anal. calc. for $C_{23}H_{21}N_3O_6\cdot H_2O$: C 60.92, H 5.11, N 9.27; found: C 61.03, H 5.41, N 9.03.

N$^{\alpha}$-*Fmoc-β-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)*-D-*alanine* (**7**). As described for **3**, with **5** (1.60 g, 4.6 mmol), CF_3COOH/CH_2Cl_2/thioanisole 50:50:0.1 (150 ml), 10% aq. Na_2CO_3 soln. (150 ml), dioxane (130 ml), and FmocCl (1.29 g, 5 mmol, 1.1 equiv.) in dioxane (20 ml). Crystallization from nitromethane yielded 1.6 g (74%) of **7**. M.p. 154°. ^{1}H-NMR: 4.29 (*m*, CH(α), CH_2(β), CH_2O (Fmoc), H–C(9) (Fmoc)); 7.29, 7.76 (*m*, OCONH, 12 arom. H); 11.48 (*s*, H–N(1)). ^{13}C-NMR: 171.93 (COOH); 162.40 (C(4)); 156.15 (OCONH); 150.47 (C(2)); 143.95 (C(8a), C(9a) (Fmoc)); 140.88 (C(4a), C(4b) (Fmoc)); 139.67 (C(8a)); 135.27 (C(7)); 127.82 (C(3), C(6) (Fmoc)); 127.73 (C(5)); 127.27 (C(2), C(7) (Fmoc)); 125.46 (C(1), C(8) (Fmoc)); 122.67 (C(6)); 120.27 (C(4), C(5) (Fmoc)); 115.27 (C(8)); 113.84 (C(4a)); 65.97 (CH_2O (Fmoc)); 51.41 (CH(α)); 46.64 (C(9) (Fmoc)); 40.42 (CH_2(β)). HR-MS: 516.1154 ($C_{26}H_{20}N_3Na_2O_6^+$, $[M-H+2\,Na]^+$; 516.1148). Anal. calc. for $C_{26}H_{21}N_3O_6\cdot 0.5\,H_2O$: C 65.00, H 4.62, N 8.75; found: C 64.75, H 4.72, N 8.95.

N^4-*(4-Methoxyphenyl)diphenylmethyl]cytosine* (**8**). In analogy to [22]. To a suspension of cytosine (2.22 g, 20 mmol) in anh. pyridine (100 ml), MeOTrCl (9.26 g, 30 mmol, 1.5 equiv.) and *N*-methylmorpholine (2.2 ml, 20 mmol, 1 equiv.) were added. After heating to 40° for 15 min, the mixture was stirred for 60 h at r.t. H_2O (35 ml) and CH_2Cl_2 (15 ml) were added to the suspension. The mixture was filtered and the precipitate washed extensively with Et_2O. Drying of the resulting product *in vacuo* for 24 h yielded 3.5 g (45%) of **8**.

N$^{\alpha}$-Z-*β-[*N^4*-(MeOTr)-cytosin-1-yl]*-D-*alanine* (= N-*[(Benzyloxy)carbonyl]-3-(1,2-dihydro-4-[[(4-methoxyphenyl)diphenylmethyl]amino}-2-oxopyrimidin-1-yl)*-D-*alanine*; **9**). NaH (0.24 g, 8 mmol, 1 equiv.) was added to a soln. of **8** (3.38 g, 8.8 mmol, 1.1 equiv.) in anh. DMF (150 ml). After stirring for 2 h, the mixture was cooled to −78°. A soln. of *N*-Z-serine β-lactone (1.76 g, 8 mmol) in DMF (50 ml) was added dropwise, and the mixture was stirred for 18 h. After evaporation, the remaining solid was dissolved in H_2O and the soln. carefully acidified to pH 3.5–4 by addition of 2N aq. $NaHSO_4$. The suspension was then extracted with AcOEt (3×), the org. layer dried ($MgSO_4$) and evaporated, and the residue purified by CC (silica gel, $CHCl_3$/MeOH/Et_3N 98:2:0.1 → 95:5:0.1). After evaporation of the collected fractions, the residue was dissolved in H_2O and the soln. carefully acidified to pH 3.5–4 by addition of aq. 2N $NaHSO_4$. The suspension was extracted with AcOEt (3×) and the org. layer dried ($MgSO_4$) and evaporated: 3.17 g (66%) of **9**. ^{1}H-NMR: 3.71 (*s*, *Me*OTr); 4.16 (*m*, CH(α), CH_2(β)); 5.02 (*s*, CH_2O(Z)); 6.12 (*d*, H–C(5)); 6.82 (br. *d*, H–C(6), OCONH); 7.22 (*m*, 19 arom. H); 8.28 (*s*, NH–C(4)). ^{13}C-NMR: 172.14 (COOH); 164.19 (C(4)); 157.79 (C(2)); 156.06 (C_p (MeOTr)); 155.18 (OCONH); 145.45 (C_{ipso} (MeOTr)); 145.13 (C(6)); 137.39 (C_{ipso} (Z)); 136.81 (C'_{ipso} (MeOTr)); 130.17 (C'_o(MeOTr)); 128.80–128.53 (C_m(MeOTr), C_m(Z), C_o(Z)); 127.86 (C_p(Z)); 127.62 (C_o(MeOTr)); 126.37 (C_p(MeOTr)); 112.93 (C'_m(MeOTr)); 95.32 (C(5)); 69.80 (C–N(MeOTr)); 65.27 (CH_2O(Z)); 55.08 (CH(α)); 53.99 (*Me*OTr); 50.49 (CH_2(β)). HR-MS: 649.2020 ($C_{35}H_{31}N_4Na_2O_6^+$, $[M-H+2\,Na]^+$; calc. 649.2039).

N$^{\alpha}$-*Fmoc-β-[*N^{4}-*(MeOTr)-cytosin-1-yl]*-D-*alanine* (**10**). To a soln. of **9** (3 g, 4.97 mmol) in MeOH (40 ml), 10% Pd/C (3 g) was added, and the mixture was hydrogenated for 10 h at 45 psi. The suspension was filtered and the catalyst washed extensively with MeOH. The filtrate and MeOH washings were evaporated, H_2O was added to the obtained foam (1.83 g, 3.89 mmol), and the mixture was acidified to pH 3.5 with 2N $NaHSO_4$. After extraction with AcOEt, Na_2CO_3 was added to the extracted aq. layer until a pH of 10 was obtained. This aq. soln. was concentrated to 100 ml, and an equal volume of dioxane was added. After adding a soln. of FmocCl (1.11 g, 4.28 mmol, 1.1 equiv.) in dioxane (30 ml), the mixture was stirred for 18 h, extracted with Et_2O (3×), acidified to pH 3.5 with 2N $NaHSO_4$, and extracted with AcOEt. The combined org. layer was dried ($MgSO_4$) and evaporated and the residue purified by CC (silica gel, MeOH/CH_2Cl_2 5:95 → 10:90) followed by crystallization from nitromethane: 1.13 g (33%) of **10**. M.p. 166°: ^{1}H-NMR: 3.68 (*s*, *Me*OTr); 4.28 (*m*, CH(α), CH_2(β), H–C(9)(Fmoc), CH_2O(Fmoc)); 6.13 (*d*, *J* = 8.0, H–C(5)); 6.74 (*d*, *J* = 8.0, H–C(6)); 6.83 (*d*, *J* = 8.8, OCONH); 7.24, 7.76 (2*m*, 22 arom. H); 8.30 (*s*, NH–C(4)). ^{13}C-NMR: 171.66 (COOH); 164.04 (C(4)); 157.57 (C(2)); 156.09 (C'_p(MeOTr)); 154.84 (OCONH); 145.07 (C_{ipso}(MeOTr)); 144.06 (C(6)); 143.80 (C(8a), C(9a) (Fmoc)); 140.88 (C(4a), C(4b) (Fmoc)); 136.85 (C'_{ipso}(MeOTr)); 130.11 (C_o'(MeOTr)); 128.74 (C_m(MeOTr)); 128.44 (C(3), C(6) (Fmoc)); 127.74 (C_o(MeOTr)); 127.50 (C(2), C(7) (Fmoc)); 127.19 (C_p(MeOTr)); 125.25 (C(1), C(8) (Fmoc)); 120.18 (C(4), C(5) (Fmoc)); 112.76 (C'_m(MeOTr)); 95.57 (C(5)); 69.77 (C–N(MeOTr)); 65.85 (CH_2O(Fmoc)); 54.96 (CH(α)); 52.32 (*Me*OTr); 49.74 (CH_2(β)); 46.70 (C(9) (Fmoc)). HR-MS: 691.2545 ($C_{42}H_{35}N_4O_6^-$, $[M-H]^-$; calc. 691.2556). Anal. calc. for $C_{42}H_{36}N_4O_6 \cdot 3\,H_2O$: C 67.55, H 5.67, N 7.50; found: C 67.47, H 5.11, N 7.30.

N^{6}-*[(*tert-*Butoxy)carbonyl]adenine* (**11**). To a suspension of adenine (10.8 g, 80 mmol) in anh. DMF (200 ml) and pyridine (100 ml), Boc_2O (35.95 g, 160 mmol, 2 equiv.) was added. The mixture was heated at 70–80° for 1 h and then stirred for 18 h at r.t. After evaporation, the remaining solid was dissolved in H_2O (100 ml) and MeOH (25 ml) by heating under reflux for 15 min. The mixture in H_2O/MeOH was evaporated, and to the remaining oil, MeOH (150 ml) and CH_2Cl_2 (25 ml) were added. After extensive stirring, the suspension was filtered. The filtrate evaporated, and the residue purified by CC (silica gel, CH_2Cl_2/MeOH 98:2), followed by crystallization from CH_2Cl_2: 7.9 g (42%) of **11**. ^{1}H-NMR: 1.51 (*s*, ^{t}Bu); 8.43 (*s*, H–C(2)); 8.57 (*s*, H–C(8)); 10.55 (*s*, NH–C(6)); 12.22 (*s*, H–N(9)). ^{13}C-NMR: 161.69 (C(6)); 152.64 (C(4)); 151.27 (C(2), OCONH); 145.62 (C(8)); 112.69 (C(5)); 81.15 (C–O(Boc)); 27.94 (Me(Boc)). HR-MS: 236.1133 ($C_{10}H_{14}N_5O_2^+$, $[M+H]^+$; calc. 236.1147).

N$^{\alpha}$-*Z-β-(*N^{6}-*Boc-adenine-9-yl)*-D-*alanine* (= N-*[(Benzyloxy)carbonyl]-3-(6-[[(*tert-*butoxy)carbonyl]amino}-9*H-*purin-9-yl)*-D-*alanine*; **12**). A soln. of **11** (7.24 g, 30.8 mmol, 1.1 equiv.) in anh. DMF (300 ml) was stirred for 2 h after addition of DBU (4.2 ml, 28 mmol, 1 equiv.). The mixture was cooled to −78°, and a soln. of *Z*-serine β-lactone (6.16 g, 28 mmol) in DMF (50 ml) was added dropwise within 1 h. Stirring was continued at r.t. for 18 h. After evaporation, the obtained oil was dissolved in H_2O and carefully acidified to pH 2 with 2N HCl. The suspension was extracted with AcOEt (4×), the org. layer dried ($MgSO_4$) and evaporated, and the residue purified by CC (silica gel, CH_2Cl_2/MeOH 95:5 → 85:15): 3.7 g (29%) of **12**. ^{1}H-NMR: 1.47 (*s*, ^{t}Bu); 3.22 (*m*, CH(α), CH_2(β)); 5.03 (br. *s*, CH_2O(Z)); 6.82 (*d*, *J* = 8.4, OCONH); 7.20 (*m*, 5 arom. H (Z)); 8.29 (*s*, H–C(2)); 8.54 (*s*, H–C(8)); 10.01 (*s*, NH–C(6)). ^{13}C-NMR: 171.57 (COOH); 155.91 (C(6)); 152.48 (OCONH(Z)); 151.54 (C(2)); 151.39 (OCONH(Boc)); 149.84 (C(4)); 144.71 (C(8)); 137.18 (C_{ipso}(Z)); 128.50 (C_m(Z)); 127.86 (C_o(Z)); 127.68 (C_p(Z)); 123.28 (C(5)); 80.15 (C–O(Boc)); 65.37 (CH_2O(Z)); 55.05 (CH(α)); 44.82 (CH_2(β)); 27.19 (Me(Boc)). HR-MS: 455.1659 ($C_{21}H_{24}N_6O_6^-$, $[M-H]^-$; calc. 455.1679).

N$^{\alpha}$-Fmoc-*β-(*N^{6}-*Boc-adenin-9-yl)*-D-*alanine* (**13**). As described for **10**, with **12** (3.5 g. 7.69 mmol). Following hydrogenation, evaporation (2.27 g, 7.1 mmol), and addition of H_2O, the soln. was acidified to pH 2 with 2N HCl and extracted with AcOEt. The aq. soln. was concentrated to 100 ml and an equal volume of dioxane was added. The Fmoc group was introduced, and further workup was performed as described for **10**. The final compound was purified by CC (silica gel, CH_2Cl_2/MeOH 95:5 → 85:15), followed by crystallization from AcOEt: 1.75 g (42%) of **13**. M.p. 174°. ^{1}H-NMR: 1.46 (*s*, ^{t}Bu); 4.46 (*m*, CH(α), CH_2(β), H–C(9)(Fmoc), CH_2O(Fmoc)); 7.35, 7.56, 7.87 (3*m*, 9 arom. H (Fmoc), OCONH(Fmoc)); 8.31 (*s*, H–C(2)); 8.58 (*s*, H–C(8)); 10.10 (*s*, NH–C(6)). ^{13}C-NMR: 171.26 (COOH); 156.12 (C(6)); 152.23 (OCONH(Fmoc)); 151.72 (C(2)); 151.39 (OCONH(Boc)); 150.05 (C(4)); 144.49 (C(8)); 143.88 (C(8a), C(9a)(Fmoc)); 140.88 (C(4a), C(4b)(Fmoc)); 127.83 (C(3), C(6)(Fmoc)); 127.28 (C(2), C(7)(Fmoc)); 125.34 (C(1), C(8)(Fmoc)); 123.49 (C(5)); 120.27 (C(4), C(5)(Fmoc)); 80.18 (C–O(Boc)); 65.94 (CH_2O(Fmoc)); 53.68 (CH(α)); 46.61 (C(9)(Fmoc)); 43.94 (CH_2(β)); 27.91 (Me)(Boc)). HR-MS: 545.2148 ($C_{28}H_{29}N_6O_6^+$, $[M+H]^+$; calc. 545.2148). Anal. calc. for $C_{28}H_{28}N_6O_6 \cdot 2$ AcOEt: C 59.99, H 6.15, N 11.66; found: C 60.28, H 5.86, N 11.51.

4. *Evaluation of the Coupling Yield of Some Selected Amino Acid Building Blocks.* Two oligopeptides of random sequence were prepared for evaluation of the coupling conditions and to trace possible side reactions

during coupling (Ac-Arg-Chi-Tal-Cal-Cal-Hpr-Sar-Arg-NH_2 and Ac-Arg-Tal-Lum-Chi-Aal-Arg-NH_2). For peptide-bond formation, a mixture of DIC (4 equiv.), HOAt (4 equiv.), $^iPr_2EtN$ (4 equiv.), *PyBOP* (2 equiv., but 4 equiv. for Tal, Cal, and Aal) and the Fmoc-derivatized amino acids (4 equiv. with respect to the available amines) in DMF was used. A preactivation period of 15 min was applied. The peptides were synthesized on *Tentagel S-NH_2* with a trialkoxybenzhydrylamine derivative (*para*-{(*RS*)-α-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-2,4-dimethoxybenzyl}phenoxyacetic acid) as linker, which was loaded onto the *Tentagel S-NH_2* with the use of DIC (4 equiv.) and HOBt (4 equiv.) in DMF. The coupling yield was determined by UV spectrometry. If yields were not high enough, a second coupling was performed, and/or coupling conditions were changed and evaluated by synthesis of smaller peptides. Capping of the residual amines was performed with pyridine/Ac_2O/NMI 4 : 1 : 0.5, and for Fmoc deprotection, 20% piperidine/DMF was used. After synthesis of the peptides and final capping, the products were cleaved from the solid support with CF_3COOH/H_2O/thioanisole 10 : 0.5 : 0.5 and further treated as described for the individual oligopeptides. The purity was verified by reversed-phase HPLC (H_2O/MeCN/CF_3COOH 95 : 5 : 0.1 → 20 : 80 : 0.1), while the identity was determined by MS analysis (*Table 1*).

5. *Synthesis of the Libraries and Individual Oligopeptides.* For the synthesis of the libraries, the mix-and-split method was used. The amino acid building blocks retained from previously synthesized libraries were coupled as described before [17]. The newly added building blocks were coupled in the presence of a mixture of DIC (4 êquiv.), HOAt (4 equiv.), $^iPr_2EtN$ (4 equiv.), and *PyBOP* (2 equiv. or 4 equiv. for Tal, Cal, and Aal). For the sterically unhindered building blocks Thi and His, *PyBOP* was omitted. The benzhydrylamine linker was coupled to *Tentagel* in the presence of DIC (4 equiv.) and HOBt (4 equiv.) in DMF. The standard procedure used 4 equiv. of Fmoc-derivatized amino acids and linker. The libraries and individual peptides were assembled starting from 3.6 g of *Tentagel S-NH_2* (0.33 mmol $-NH_2$/g).

Before each coupling, deprotection was carried out with 20% piperidine/DMF for 15 min, and the support was washed 4 × with DMF and CH_2Cl_2. A mixture of the Fmoc-amino acids or linker and the coupling reagents in DMF was added to the amino-functionalized resin. The reaction vessels were shaken for 16 h, washed 3 × with DMF, 3 × with CH_2Cl_2, and twice with Et_2O. The beads were dried *in vacuo* and the Fmoc-substitution level was determined by dissolving an accurately measured quantity of resin (10 mg) in 20% piperidine/DMF (exactly 25.0 ml). After 10 min, the absorbance was measured at 300 nm *vs.* 20% piperidine/DMF as blank, allowing calculation of the substitution level (*Table 4*). If the coupling yields were satisfactory, all the beads were pooled in a 500-ml *Omnifit* bottle. In some cases, a double coupling was necessary to obtain an acceptable yield. Upon addition of DMF/CH_2Cl_2 1 : 1 (250 ml), the bottle was shaken in 3 dimensions for 30 min, after which the suspension was transferred back to a large sintered-glass filter. The solvent was removed by suction filtration, and the resins were washed twice with CH_2Cl_2 and twice with Et_2O, dried *in vacuo*, and redistributed. After each coupling, Fmoc determination, and, if necessary, recombining and mixing, the residual amines were acetylated by shaking the resins in pyridine/Ac_2O/NMI 4 : 1 : 0.5 (5 ml) to avoid formation of contaminant hybrid structures. After shaking for 10 min, the resins were washed 4 × with CH_2Cl_2 and DMF and once with 20% piperidine/DMF, before the Fmoc-protecting groups were removed by shaking the resins for 15 min in 20% piperidine/DMF to allow coupling of the next Fmoc-amino acid building block.

6. *Preparation of the Library and the Individual Oligopeptides for Solution-Phase Screening.* After the final assembly cycle, the resins were Fmoc-deprotected and acetylated. Since all the protecting groups were acid labile, the oligopeptides were deprotected and cleaved from the resins by stirring in CF_3COOH/H_2O 95 : 5 in the presence of thioanisole (5%) as carbocation scavenger. After 2 h of stirring, the resins were filtered, and the filtrate was captured in $^iPr_2O$ at −70°. The resins were washed with CF_3COOH and $^iPr_2O$. The obtained white cloudy suspension was centrifuged for 10 min at 9000 rpm. After removal of the supernatants, the remaining pellet was dried *in vacuo* and dissolved in H_2O/MeCN 9 : 1. The soln. was transferred in a tarred polypropylene tube and lyophilized. The final yield of synthesis was determined by mass, by means of the average molecular mass of the peptide mixture. As final step in the synthesis of the individual oligopeptides, purification was performed by reversed-phase semi-prep. HPLC (H_2O/MeCN/CF_3COOH 95 : 5 : 0.1 → 20 : 80 : 0.1). The identity of the individual peptides was determined by LSI-MS analysis (*Table 5*).

7. *Solution Screening of the Libraries and Individual Oligopeptides.* The solution-screening process was performed by gel shift experiments. Both strands of the target oligonucleotide were radiolabeled (^{32}P) at the 5′-end by standard procedures [30], purified on a *NAP-5*® column, and dissolved in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) at a 2 μM concentration. Equal volumes of both solns. were mixed and subsequently heated for 3 min at 80°, placed for 5 min at r.t. and for 20 min at 4° to allow hybridization of the DNA strands. The peptides were dissolved in an appropriate volume of PBS. If mixtures had

Table 4. *Coupling Yields* [%] *for Positions X¹, X², and X³* (determination of the Fmoc-substitution level)[a])

	First library			Second library			Individual peptides		
	X^1	X^2	X^3	X^1	X^2	X^3	X^1	X^2	X^3
Ual	101	98	101	–	96	106	–	–	100
Tal	101	100	103	100[c])	100	104	100[c])	–	103
Chi	99	100[b])	104	99[c])	103	104	101[c])	100[c])	99
Lum	101	99	104	–	98	102	–	–	103
Aal	93[b])	92[b])	93[b])	–	98	102	–	–	99
Cal	99	102[b])	101[b])	–	104	105	–	–	106
Inp	95	95	102	–	95	98	–	–	101
Hpr	97	98	100	–	95	97	–	–	100
Gln	99	100	98	–	96	102	–	–	95
Thi	99	95	100	–	97	101	–	–	98
Sar	96	97	97	–	94	97	–	–	96
His	98	95	101	–	102	101	–	–	101

[a]) The coupling of the linker to the beads (100% yield) and of Arg to the linker (99% yield) was nearly quantitative. The average coupling yield in all the libraries of Sar to X^3 was 96%, but several double couplings were necessary, of Ual to Sar 102% and of Arg to Ual 98%. [b]) Yield after two couplings. [c]) Average yield of 12–24 couplings.

Table 5. *Yield of Synthesis and Calculated and Observed Molecular Mass of the Individual Peptides as Determined by LSI-MS.* Masses were obtained as $[M+H]^+$ or $[M+2H]^{2+}$.

Individual peptides with the sequence -Tal-Chi-X^3-			
X^3	Yield [%]	M_r, calc.	M_r, found
Ual	55	1230	1232
Tal	29	1244	1246
Chi	58	1280	1282
Lum	41	1282	1284
Aal	29	1253	1254
Cal	26	1229	1231
Inp	45	1160	1162
Hpr	62	1162	1163
Gln	41	1177	1179
Thi	42	1202	1204
Sar	41	1120	1121
His	50	1186	1188
Individual peptides with the sequence -Chi-Chi-X^3-			
X^3	Yield [%]	M_r, calc.	M_r, found
Ual	52	1266	1267
Tal	47	1280	1281
Chi	47	1316	1317
Lum	35	1318	1319
Aal	43	1289	1291
Cal	47	1265	1266
Inp	57	1196	1197
Hpr	63	1198	1199
Gln	54	1213	1215
Thi	51	1238	1239
Sar	36	1156	1157
His	57	1222	1223

to be screened, the amount of precipitated peptide and the average molecular mass of the peptides were used to calculate the required amount of peptide.

In a total reaction volume of 5 µl (PBS), 1 µl of the oligonucleotide soln. (containing 1 pmol of dsDNA) was mixed with 1 µl of oligopeptide solns. resulting in peptide concentrations ranging from 0.01 to 5 mM. The mixture was stored at 4° for 2 h. To the control tube, no peptide was added. The mixtures were resolved at 10° on a 15% native polyacrylamide gel with TBE running buffer (pH 7.4; 90 mM H_3BO_3, 30 mM *Tris*; 2 mM Na_2EDTA) at 2 W/gel within 3 h. The gels were quantitatively imaged by means of a *Cyclone Phosphorimager* (*Packard*). The degree of complex formation was quantified by measuring the residual amount of free DNA with *Optiquant*™, an image analysis software program (*Packard*).

Also an ethidium bromide displacement test was used. These experiments were performed as described in [27] with a 10 mM *Tris*/10 mM NaCl (pH 7.4) buffer. The *FL600 Microplate Fluorescence* reader was used with 530/25 nm as excitation wavelength and 590/35 nm as emission wavelength.

8. *Apparent Dissociation Constant Determination*. To determine the apparent dissociation constants K_d of oligopeptide · dsDNA complexes, gel mobility-shift assays were used. These gel shift experiments were performed as described earlier and according to [31]. To determine the apparent dissociation constants, a series of concentrations of the investigated peptides were used, from 0.002 to 2 mM, together with a fixed concentration of the dsDNA target (0.2 µM). The apparent K_d was then determined as the peptide concentration at which 50% of the target is mobility-shifted. At least 3 independent gel shift assays were performed for each peptide to determine the apparent K_d. Concentration-response curves, obtained by analysis of the gel shifts, were fitted by the equation $Y = E_{max}/[1 + 10^{(\log K_d - X)nH}]$, where Y stands for the response (% shift), E_{max} for the maximal response, and nH for the *Hill* coefficient, by using *Graphpad Prism* (*Graphpad Software Inc.*).

9. *Circular Dichroism Experiments*. CD Spectra were measured between 180 and 330 nm in a temp. range of 10 to 70°. All oligopeptides were measured in H_2O as solvent at a 10 µM concentration.

10. *Construction of Plasmid DNA*. The vector pUC 19 was constructed by ligating the hybridized insert 5′-AGATTGTGCAATGT-3′ and 5′-CTAGACATTGCACAATCTCATG-3′ into linear pUC19 Sph I/Xba I plasmid by using T_4 DNA ligase. The resulting construct was transformed into competent DH5-α *E. coli* cells. Ampicillin-resistant white colonies were selected from 25 ml *Luria-Bertani* (LB) medium agar plates (containing 50 µg/ml ampicillin and treated with XGAL and IPTG). Large-scale plasmid purification was performed with *Qiagen Midi* purification kits. Dideoxy sequencing was used to verify the presence of the insert. The concentration of the resulting vector was determined at 260 nm with the relationship 1 *OD* unit = 50 µg/ml.

11. *Purification and Radiolabeling of the 271-Base-Pair DNA Fragment*. The 271-base-pair fragment derived from the vector pUC 19 was rendered radioactive by 3′-^{32}P-end labeling of the Asn I-Pvu II double digest of the plasmid with [α-^{32}P]dTTP and AMV reverse transcriptase. The 271-mer was separated from the remainder of the vector by running on a nondenaturing 6% polyacrylamide gel in TBE buffer. After autoradiography, the requisite band of DNA was excised, crushed, and soaked in elution buffer (500 mM NH_4OAc, 10 mM $Mg(OAc)_2$) overnight at 4°. The suspension was filtered through a *Millipore* 0.4 µm filter, and the DNA was precipitated with EtOH. Following washing with 70% EtOH/H_2O and vacuum drying of the precipitate, the labeled DNA was dissolved in H_2O.

12. *Footprinting Experiments*. Cleavage reactions by DNase I were performed essentially according to the protocol described previously by *Bailly* and *Waring* [32]. Reactions were conducted in a total volume of 10 µl. Samples (2 µl) of the labeled DNA fragment were incubated with 5 µl of the buffered soln. containing the oligopeptide at the appropriate concentration. The solns. were allowed to equilibrate for 30 min at r.t. Cleavage was initiated by the addition of 3 µl of a DNase-I soln. of which the concentration was adjusted to obtain an enzyme attack of *ca*. 30% of the starting material (final enzyme concentrations 0.0005 – 0.05 units/10 µl reaction mixture). After 3 min, the reaction was stopped by freeze-drying. Samples were lyophilized and resuspended in 5 µl of an 80% formamide soln. containing tracking dyes. The DNA samples were then heated at 90° for 4 min and chilled on ice for 5 min prior to electrophoresis.

13. *Electrophoresis and Quantitation by Storage Phosphor Imaging for Footprinting Experiments*. DNA Cleavage products were resolved by polyacrylamide-gel electrophoresis under denaturing conditions (0.4-mm thick, 8% acrylamide containing 8M urea). After electrophoresis (*ca*. 2.5 h at 65 W in TBE (pH 8.0)-buffered soln.), gels were soaked in 10% AcOH/H_2O for 5 min, transferred to *Whatman 3-MM* paper, dried under vacuum at 80°, and then exposed to a storage phosphor screen. Each resolved band was assigned to a particular bond within the DNA fragment by comparison of its positon relative to sequencing standards generated by treatment of the DNA with dimethyl sulfate followed by piperidine-induced cleavage at the modified guanine bases in DNA (G track).

14. *NMR Experiments.* Quadrature detection was achieved by *States-Haberkorn* hypercomplex model [33]. Spectra were processed by the FELIX 97.00 software package (*Biosym Technologies*, San Diego, SA) running on a *Silicon-Graphics Indigo2-R 10000* workstation (Irix, version 6.2). The sample was prepared by dissolving 10 mg of peptide in 750 µl of D_2O and adjusting the pD to 7.0. Chemical-shift assignment of the individual residues in both conformers of Ac-Arg[1]-Ual[2]-Sar[3]-Chi[4]-Chi[5]-Chi[6]-Arg[7]-NH_2 was obtained by means of a gradient multiple-quantum-filtered phase-sensitive $^1H,^1H$ correlation experiment [34] (GMQFCOPS), a $^1H,^1H$ total correlation experiment [35] (TOCSY) with a MLEV17 [36] transfer time of 65 ms and a gradient heteronuclear single-quantum correlation spectrum [37] (GHSQC). A two-dimensional rotating-frame nuclear-*Overhauser* experiment [38] (ROESY) was used to determine which residues were interconverting with each other and to identify the exchange cross-peaks in the two-dimensional $^1H,^1H$ nuclear-*Overhauser* experiment [39] (NOESY) with mixing time 150 ms. The latter was used to complete the assignment in Chi residues through their weak H–C(α) to H–C(4) NOE and to obtain the sequential assignment through intra-residue NOE interactions.

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