New dsDNA-Binding Hybrid Molecules Combining an Unnatural Peptide and an Intercalating Moiety

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In our search for new dsDNA-binding ligands, combinatorial chemistry was first applied to select unnatural oligopeptides with moderate affinity for dsDNA. To enhance the binding affinity of a heptapeptide lead structure, Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2 ($K_d = 4.9 \cdot 10^{-4}$ M), the compound was conjugated to different heteropolyaromatic moieties by means of a variety of linker arms. Glycine, β-alanine, glycyl-glycine, glycyl-β-alanine, γ-aminobutyric acid, and 6-aminocaproic acid were used as spacers, representing different lengths and/or flexibilities. The intercalators coupled to the oligopeptide were acridine, fluorenone, anthracene, anthraquinone, and 3,8-diamino-5-methyl-6-phenylphenantridinium (methidium). The binding capacities of these new hybrid molecules to dsDNA have been investigated by gel retardation and footprinting assays. The results show that, by conjugating the unnatural oligopeptide to intercalators, the affinity for dsDNA could be enhanced more than 100-fold. For methidium-β-alanyl-glycyl-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2 (K_d of $2.1 \cdot 10^{-6}$ M), the interaction with dsDNA was dominated by the intercalator in such a way that the sequence specificity of the heptapeptide was changed. The interaction with dsDNA of hybrid molecules of other intercalators was mainly governed by the oligopeptide, since the sequence selectivity of the heptapeptide was conserved. In general, the linker arm glycine (shortest spacer) and glycine-β-alanine were preferred over β-alanine, glycyl-glycine and the more-flexible spacers γ-aminobutyric acid and 6-aminocaproic acid. This way new hybrid molecules endowed with dsDNA affinities of *ca.* 10^{-6} M and displaying different sequence selectivities have been developed. Therefore, combinations of such unnatural peptides with intercalators can be used to broaden the knowledge about the sequence-selective recognition of dsDNA.

1. Introduction. – Inhibition of the expression of a specific gene by targeting dsDNA is a promising strategy in the combat against many diseases and can be a helpfull tool in molecular biology. Nature exploited this approach already in its defence mechanisms by using, for example, small minor-groove-binding ligands like netropsin or DNA-alkylating antibiotics like pyrrolo[2,1-*c*][1,4]benzodiazepines. Research in the field of synthetic sequence-specific dsDNA-recognition ligands has afforded many interesting molecules like the lexitropsins and the major-groove-binding peptide nucleic acids (PNA) [1][3]. An alternative and complementary approach to the lexitropsin, oligonucleotide, and PNA efforts is the development of hybrid ligands, combining two modes of binding to dsDNA. Such is the case for actinomycin D, a natural antitumor agent that bears two identical cyclic pentapeptides attached to a phenoxazone ring (*Fig. 1*). The chromophore intercalates between the base pairs of GpC sequences, leaving the two pentapeptides lying in the minor groove of the double helix [4][5]. Similar observations can be made for the bisintercalating quinoxaline antibiotics, of which echinomycin is the best-known member [6].

These naturally occuring hybrid molecules are models for the development of synthetic conjugates. Coupling of such a polyaromatic heterocycle to an addressing

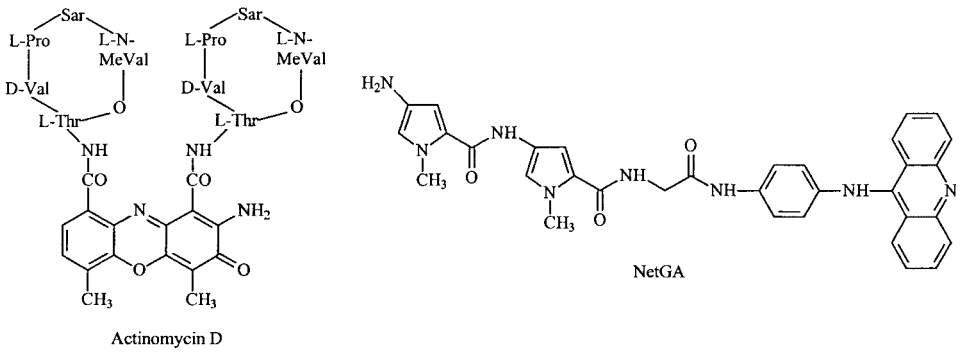

Fig. 1. *Structure of actinomycin D and NetGA* (a netropsin-acridine combilexin)

molecule does not only provide extra strength of binding, but can also influence the sequence selectivity of the conjugate, decrease degradation in biological fluids, and, furthermore, be helpful to address problems of poor cellular uptake.

In the combilexin approach, such a potentially intercalating heteropolyaromatic moiety is coupled to a peptidic or pseudopeptidic groove-binding entity. The first hybrid molecules of this class, distactins, consisted of an actinomycin like compound in which 1-methyl-1*H*-pyrrolecarboxamide units sandwiched a phenoxazone ring [7][8]. Many other combilexins like NetGA and derivatives are still the subject of considerable attention, and more-efficient analogues are being investigated [9] (*Fig. 1*). With such hybrid molecules, it has been demonstrated that DNA is able to accept two types of binding in close proximity, despite their unique distortions. Furthermore, the length and the flexibility of the spacer arms in conjugates seems to be critical for optimal positioning of both parts of the molecules. Intercalators have also been coupled to oligonucleotides, peptides, and other DNA-interacting agents [10][11].

In an effort to develop new dsDNA ligands, we screened libraries of unnatural heptapeptides for their interaction capacities with a 14-base-pair dsDNA target derived from the binding site of NF-IL6 [12][13]. This led to the selection of seven heptapeptides with affinities ranging from $7 \cdot 10^{-4}$ to $9 \cdot 10^{-5}$ M. Footprinting experiments with the oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2 ($K_d = 4 \cdot 10^{-4}$ M) indicated a preferential interaction with a six-base-pair-long mixed sequence 5′-CTGCAT-3′ [13]. To enhance the binding affinity of this lead compound, a strategy of conjugating intercalating molecules was explored. Therefore, conjugates with different intercalating agents connected by a variety of linker arms were prepared and investigated. Six linkers with different length and flexibility were used (gly, β-ala, gly-gly, gly-β-ala, γ-aminobutanoic acid, and ε-aminohexanoic acid). The intercalators coupled to the oligopeptide were acridine, fluorenone, anthraquinone, anthracene, and 3,8-diamino-5-methyl-6-phenylphenantridinium (methidium). Gel-retardation assays were used to determine the binding strength of the conjugates and sequence specificity was investigated with footprinting experiments (*Fig. 2* and *3*).

2. Results. – 2.1. *Selection and Synthesis of the Oligopeptide–Intercalator Conjugates.* The heptapeptide Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2 was coupled *via* the

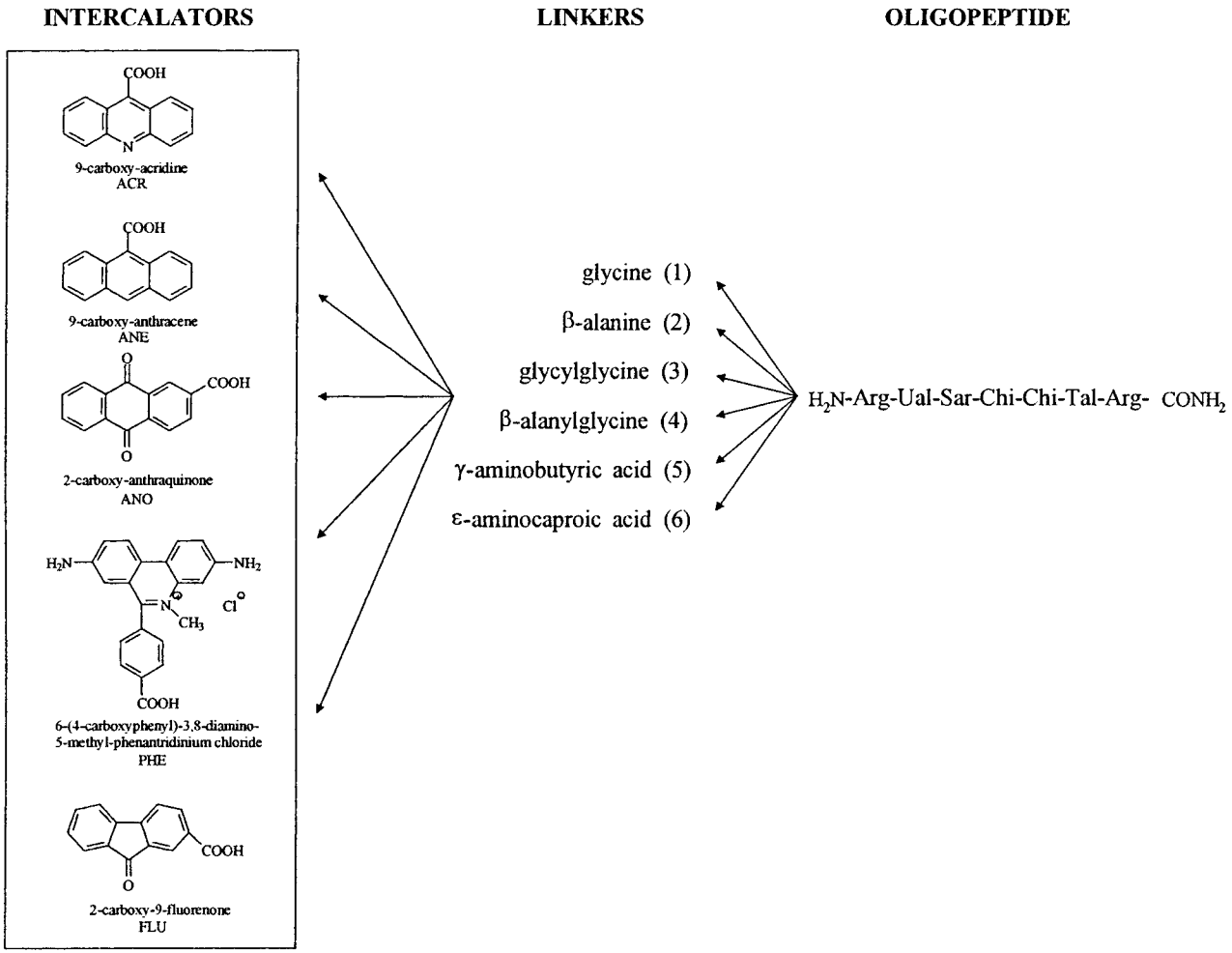

Fig. 2. *Overview of the different molecules selected for synthesis of the oligopeptide–intercalator conjugates*

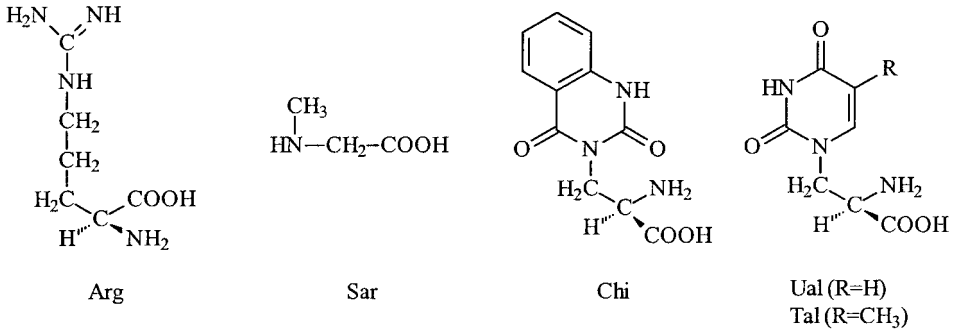

Fig. 3. *Structures of the amino acid building blocks of the peptide Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH$_2$.* The amino acids are L-arginine (Arg), β-(uracil-1-yl)-D-alanine (Ual), sarcosine (Sar), β-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-D-alanine (Chi), and β-(thymin-1-yl)-D-alanine (Tal).

N-terminus to five intercalators with six different spacer arms (*Figs. 2* and *3*). The intercalators were selected on the basis of their commercial availability and relative sequence selectivity. Acridine (Acr), anthraquinone (Ano), anthracene (Ane), and 3,8-diamino-5-methyl-6-phenylphenantridinium (methidium) (Phe), an fluorenone (Flu) analogue, possess no sequence selectivity or a moderate-to-high preference for GC sequences. Fluorenone was chosen because it forms the core structure of tilorone, an AT-selective dsDNA intercalating ligand. It was suggested that a bridging H-bonded H_2O molecule between the intercalator C=O group and the thymine C=O group could account for the AT specificity [14]. All the intercalators were obtained as carboxyl-bearing derivatives. The distinct elements of the conjugates were coupled to each other with linker arms of differing length and flexibility. With glycine and β-alanine combinations, spacer lengths of three, four, six and seven atoms could be obtained. The more-flexible linker, γ-aminobutanoic acid (five atoms long), was added because it offers the optimum fit for several known hybrid molecules [15][16]. Finally, ε-aminohexanoic acid (seven atoms long) was selected to investigate the importance of the flexibility of the spacers, in comparison with glycine-β-alanine (seven atoms long).

Synthesis of the conjugates was performed on solid-support with standard coupling reagents for peptide formation and Fmoc-protected amino acids (N^{α}-Fmoc-β-(uracil-1-yl)-D-alanine, N^{α}-Fmoc-β-(thymin-1-yl)-D-alanine, N^{α}-Fmoc-N^{G}-(2,2,5,7,8-pentamethylchroman-6-sulfonyl)-L-arginine (G = guanidine moiety), N^{α}-Fmoc-β-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-D-alanine, *N*-Fmoc-sarcosine, *N*-Fmoc-glycine, *N*-Fmoc-β-alanine, *N*-Fmoc-γ-aminohexanoic acid, and *N*-Fmoc-ε-aminohexanoic acid). The quinazoline-2,4(1*H*,3*H*)-dione, uracil, thymine, and sarcosine derivatives were synthesized as described in [12][13], while the protected arginine, glycine, and β-alanine were commercially obtained. The γ-aminobutanoic acid and ε-aminohexanoic acid linker arms were Fmoc-protected under *Schotten–Bauman* conditions [12][13]. The intercalator building blocks anthracene-9-carboxylic acid, 8,10-dihydro-9,10-dioxoanthracene-2-carboxylic acid, 3,8-diamino-6-(4-carboxyphenyl)-5-methylphenantridinium chloride, acridine-9-carboxylic acid and 9-oxo-9*H*-fluoren-2-carboxylic acid were also commercially obtained (*Fig. 4*)

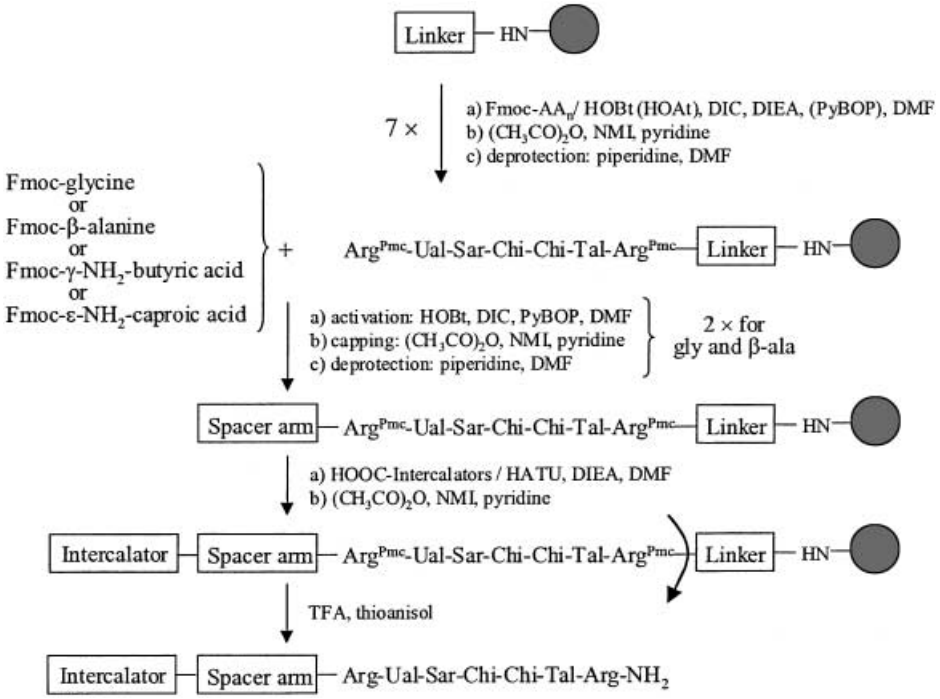

Fig. 4. *Schematic overview of the solid-phase synthesis of oligopeptide–intercalator conjugates*

The amino acid building blocks were coupled by means of activation mixtures consisting of 4 equiv. of DIC (diisopropylcarbodiimide), 4 equiv. of HOBt/HOAt (1-hydroxy-1*H*-benzotriazol/1-hydroxy-7-azabenzotriazol), 4 equiv. of $^{i}Pr_2EtN$ and/or 2 to 4 equiv. of *PyBOP*™ (*Fig. 4*). The carboxy intercalators were activated for amide formation with 4 equiv. of HATU (*O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and 4 equiv. of $^{i}Pr_2EtN$ (*Fig. 4*). The *Rink amide MBHA resin* was used so that the conjugates could be cleaved from the solid support after synthesis by TFA (CF_3COOH) treatment. The mixtures obtained after cleavage were purified by HPLC. The profiles of all conjugates showed one major peak (> 70%), which corresponded to the calculated mass of the desired compound. A more-polar side peak was always present, corresponding to the unconjugated oligopeptide-linker complex. *Table 1* gives an overview of all synthesized conjugates.

2.2 *Solution-Phase Screening.* The binding strength of the different hybrid molecules was investigated with gel-retardation assays as described before [13]. The experiments were 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′. After a 2-h incubation at 4° of the conjugates with the radioactively labelled dsDNA fragment in an appropriate buffer, the mixtures were applied on a 15% native polyacrylamide gel. After electrophoresis and imaging, the obtained signals were compared. A blank lane (no conjugate) and a reference lane (+ reference oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2) were

Table 1. *Overview of the Synthesized Oligopeptide–Intercalator Conjugates.* The abbreviations used for the hybrid molecules are given in parentheses.

Intercalator	Oligopeptide (-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2)					
Acridine	glycine (Acr 1)	β-alanine (Acr 2)	glycyl-glycine (Acr 3)	β-alanyl-glycine (Acr 4)	γ-NH_2-butanoic acid (Acr 5)	6-NH_2-hexanoic acid (Acr 6)
Anthracene	glycine (Ane 1)	β-alanine (Ane 2)	glycyl-glycine (Ane 3)	β-alanyl-glycine (Ane 4)	γ-NH_2-butanoic acid (Ane 5)	6-NH_2-hexanoic acid (Ane 6)
Anthraquinone	glycine (Ano 1)	β-alanine (Ano 2)	glycyl-glycine (Ano 3)	β-alanyl-glycine (Ano 4)	γ-NH_2-butanoic acid (Ano 5)	6-NH_2-hexanoic acid (Ano 6)
Methidium	glycine (Phe 1)	β-alanine (Phe 2)	glycyl-glycine (Phe 3)	β-alanyl glycine (Phe 4)	γ-NH_2-butanoic acid (Phe 5)	6-NH_2-hexanoic acid (Phe 6)
Fluorenone	Glycine (Flu 1)	β-alanine (Flu 2)	glycyl-glycine (Flu 3)	β-alanyl-glycine (Flu 4)	γ-NH_2-butanoic acid (Flu 5)	6-NH_2-hexanoic acid (Flu 6)

included. In most cases, however, the band of DNA·conjugate complex was not observed. Since 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.

Investigation of the binding revealed that the formation of a shifted band depended mainly on the applied concentrations of conjugates. At a concentration of 100 μM a clear shift was obtained (*Fig. 5*). Furthermore, the gel-retardation method proved to be reliable for the selection of dsDNA binding ligands, as an ethidium bromide displacement assay yielded comparable results (*Fig. 5*).

Experiments with a 20-μM concentration of conjugates showed that incorporation of any intercalator with any linker arm leads to higher dsDNA affinities in comparison with the reference oligopeptide. However, experiments with reduced concentrations of conjugates show that there is a large difference in binding strength between the different derivatives.

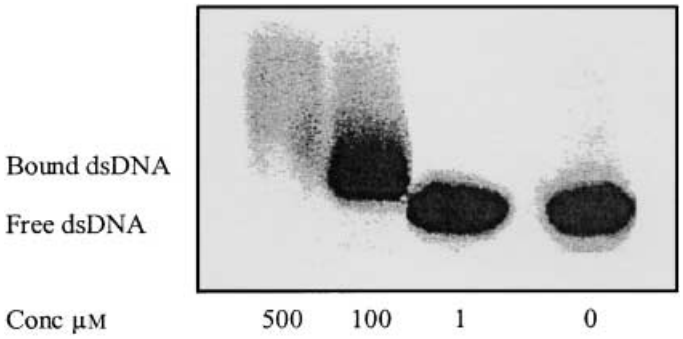

Fig. 5. *Gel-shift experiments with the oligopeptide–intercalator conjugate Acr 1.* The gel-shifted band obtained with 100 μM Acr 1, is clearly visible. At higher concentrations, a smear or precipitation is obtained.

The concentration of hybrid molecules leading to retardation of the dsDNA target could be reduced to 5 μM for the methidium conjugates. For other conjugates, higher concentrations had to be used to obtain binding signals in the screening assays. This suggests that compounds with the highest affinity are among the methidium conjugates. The additional positive charge of 3,8-diamino-5-methyl-6-phenylphenantridinium could be responsible for this stronger interaction. In the group of the methidium derivatives, Phe 1 and Phe 4 possessed a pronounced stronger interaction (*Fig. 6*).

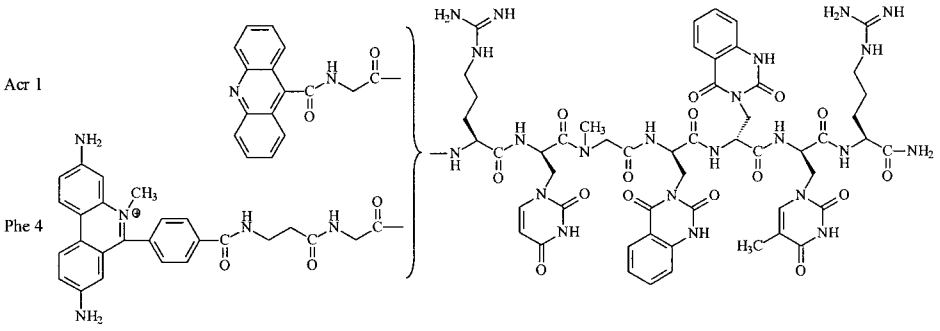

Fig. 6. *Structures of the oligopeptide-glycine-carboxyacridine conjugate Acr 1* (top) *and of oligopeptide-glycine-β-alanine-methidium* (Phe 4, bottom)

For the acridine conjugates, Acr 1, the hybrid with the shortest linker glycine (*Fig. 6*), was the most-active compound, followed by Acr 2 and Acr 4. Flu 3 and Flu 4 possessed the highest interaction capacity in the group of fluorenone conjugates, while Ano 1 displayed the strongest binding of all the anthraquinone derivatives. For the anthracene conjugates, Ace 3 and Ace 4 displayed the strongest interaction capacities, Ace 1 and Ace 2 were of medium strength, and Ace 5 and Ace 6 showed low activity. These results demonstrate that the spacer arm as well as the intercalator itself influence the binding strength of the peptide, and that, in every case, an optimal combination can be selected. However, as a general rule, the γ-aminobutanoic acid spacer and the ε-aminohexanoic acid linker are not suited for this purpose (*Fig. 6*).

2.3. *Determination of Apparent Dissociation Constants* (K_d). To evaluate the affinities of the hybrid molecules in relation to one another and in comparison with the oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2, apparent dissociation constants of the strongest interacting conjugates were determined. The gel-retardation assays used for this purpose were performed as described in [13]. The concentration of the conjugates was increased from 1 to 20 μM. Results of the K_d determinations are shown in *Table 2*.

Experiments with the strongest-interacting hybrid compound, Phe 4, yielded a K_d of $2.4 \cdot 10^{-6}$ M. This is a more than 100-fold improvement in comparison with the reference oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2, which possessed a K_d of $4 \cdot 10^{-4}$ M. Also conjugates with other intercalators displayed a high increase of the affinity for the dsDNA target and possessed K_d values ranging from $5.6 \cdot 10^{-6}$ to $7.1 \cdot 10^{-6}$ M (*Table 2* and *Fig. 6*).

Table 2. *Comparison of the* K_d *Values of the Reference Oligopeptide with Oligopeptide–Intercalator Conjugates.* The K_d values are averaged over three experiments.

	Apparent K_d [M]
Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2	$4.0 \times 10^{-4} \pm 0.12$
Antracene-β-Ala-Gly-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2 (Ane 4)	$7.1 \times 10^{-6} \pm 0.20$
Fluorenone-β-Ala-Gly-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2 (Flu 4)	$6.5 \times 10^{-6} \pm 0.15$
Antraquinone-Gly-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2 (Ano 1)	$6.3 \times 10^{-6} \pm 0.08$
Acridine-Gly-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2 (Acr 1)	$5.6 \times 10^{-6} \pm 0.10$
Methidium-β-Ala-Gly-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2 (phe 4)	$2.4 \times 10^{-6} \pm 0.10$

2.4. *Footprinting Experiments.* To investigate the influence on the sequence specificity of binding an intercalating agent to the oligopeptide, DNase-I-footprinting experiments were performed. The results were obtained as described previously with a 271-base-pair fragment, derived from the pUC 19 plasmid, which contained the 14-mer target site used for screening (nucleoside positions 139–152). Patterns of DNase-I digestion in the absence and presence of Phe 4 are shown in *Fig. 7.* The DNase-I cleavage patterns of all conjugates differ clearly from that seen in the control lanes. Moreover, there is a pronounced difference between the cleavage patterns of the methidium derivative Phe 4 and conjugates of other intercalators. The distinction in the cleavage pattern at nucleotide positions 137 to 142 is clearly visible (*Fig. 8,a*). While there is an enhanced cleavage at those positions with Phe 4, there is inhibition of cleavage with the reference oligopeptide and conjugates of other intercalators (*Fig. 7*).

Differential cleavage plots revealed more-detailed information (*Fig. 8*). The hybrid molecules Acr 1, Flu 4, and Ano 1 show, in comparison with the control lane, enhanced inhibition of cleavage at three sites: nucleotide positions 106–113 (5′-TCATAGCT-3′), 134–140 (5′-CTGCATG-3′), and 153–159 (5′-CCTCTAG-3′). Two other sequences display a less-pronounced decrease of cleavage, namely positions 95–99 (5′-TGTGT-3′) and 146–149 (5′-TTGC-3′). This cleavage pattern is comparable to that of the unconjugated oligopeptide, obtained at higher concentrations, which exhibit considerable inhibition at nucleotide positions 134–140 and less-pronounced inhibition at the four other locations (*Fig. 8,a*). Therefore, it seems that the sequence selectivity of the intercalator is not playing an important role, and that the intercalating agent mainly further stabilizes the ligand–DNA complex. Furthermore, the cleavage patterns show that, at positions 106–113 and 153–159, the recognition site of Acr 1 is extended by several base pairs compared to the reference peptide, although this is not visible at other locations. Closer investigation reveals, however, that the increased binding size is present only at those locations where the differential cleavage intensity of the conjugate is much lower compared to the original peptide. The interpretation might be that, at these locations, the intercalator itself plays a more important role in the binding process.

The cleavage pattern of the methidium derivative Phe 4 on the other hand, differs clearly from the patterns obtained with the oligopeptide and with conjugates of other intercalators. Noteworthy is the enhanced cleavage at positions 139–141, in contrast to the decrease of cleavage at those locations with the oligopeptide itself and hybrid molecules with other intercalators. Sequences that are protected against cleavage are

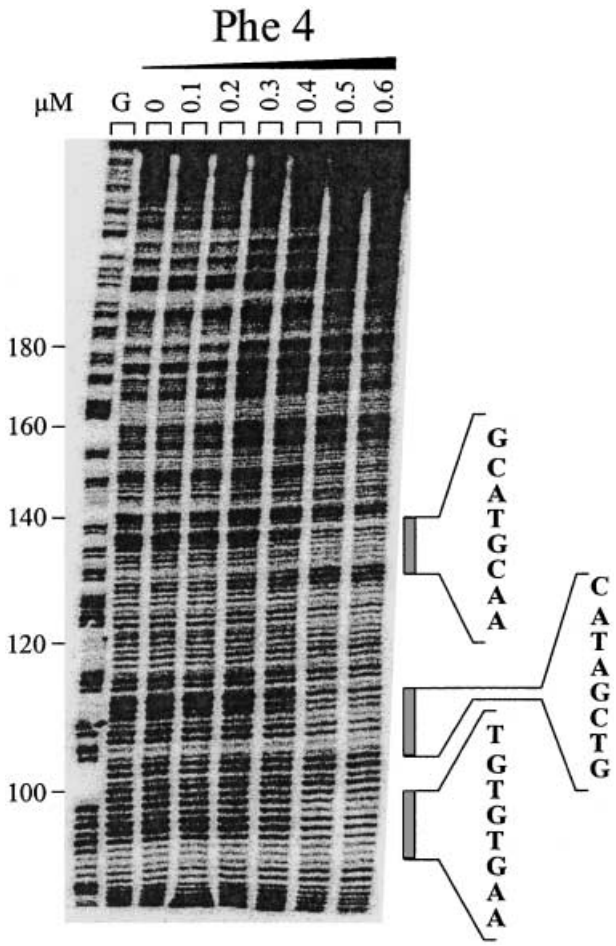

Fig. 7. *DNase-I Footprinting on the 271-base-pair Pvu II-Ase I restriction fragment, cut out from the cloned plasmid pUC 19, in the presence of the conjugate Phe 4* (oligopeptide-gly-β-ala-methidium). The duplex DNA was 3′-end labeled at the Asn I site with [α-^{32}P]dTTP in the presence of AMV-reverse 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 (0) contained no conjugates. Numbers at the side of the gel refer to the numbering scheme of the fragment. The sequences and positions of the most important oligopeptide–intercalator conjugate binding sites is indicated

located around nucleotide positions 94–99 (5′-TGTGTG-3′), 106–112 (5′-CATAGCT-3′), and 131–138 (5′-GCATGCAA-3′). Positions of less-pronounced protection against cleavage are 120–123 (5′-GTAA-3′) and 146–148 (5′-TGC-3′). This cleavage pattern shows, however, a remarkable resemblance to the digestion patterns of EtBr itself (*Fig. 8,b*), although the binding strength of EtBr is lower than of the conjugate Phe 4.

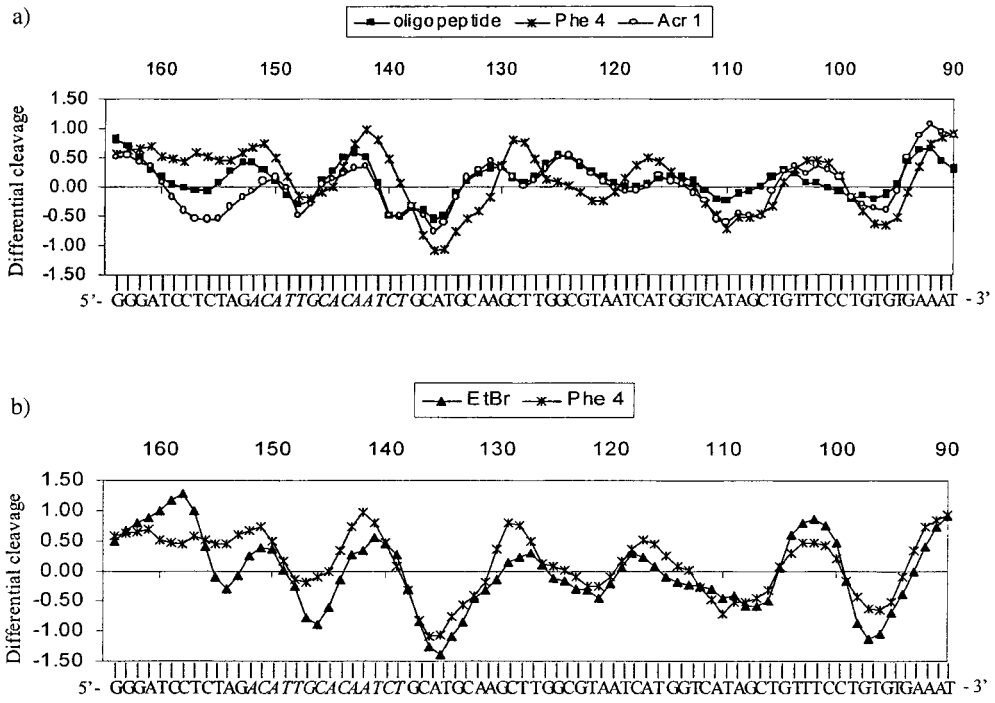

Fig. 8. *Differential cleavage plot comparing the susceptibility of the 3'-labeled 271-base-pair fragment to Dnase-I cutting in the absence and in the presence of* a) *oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2, methidium-β-ala-gly-oligopeptide* (Phe 4) *and acridine-gly-oligopeptide* (Acr 1); b) *methidium-β-ala-gly-oligopeptide* (Phe 4; 0.5 μM) *and ethidium bromide* (5 μM). 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. Data are compiled from quantitative analysis of several sequencing gels like the one shown in *Fig. 7* and must be considered a set of average values.

3. Discussion. – In our search for sequence-specific dsDNA-binding ligands, we synthesized new hybrid molecules consisting of different intercalators and an unnatural oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2, obtained from screening of an unnatural peptide library. The distinct parts of the conjugates were coupled with six linker arms differing in length and flexibility. After synthesis and purification of the hybrid compounds, their dsDNA binding capacity was investigated by means of gel-retardation assays and with a 14-base-pair-long dsDNA target, a partially palindromic recognition sequence of NF-IL6. The results show that incorporation of an intercalator leads to enhanced affinity for the dsDNA target, in comparison with the oligopeptide. There are, however, large differences between the distinct hybrid molecules. This means that both parts of the conjugates, the intercalator as well as the oligopeptide, are of importance for the interaction with dsDNA. It was found that the less-flexible linker arms were preferred. In general, there even seemed to be a preference for the shortest spacer glycine and the glycine-β-alanine linker (and not for the 6-aminohexanoic acid

linker). This in contrast with many other conjugates where relatively long and more flexible linker arms (*e.g.*, butanoyl chain) are preferred [15][16].

The compounds with the strongest dsDNA interaction were found in the group of the methidium derivatives, namely Phe 1 (oligopeptide-glycine-methidium) and Phe 4 (oligopeptide-glycine-β-alanine-methidium). Phe 4 possessed an apparent dissociation constant of $2.4 \cdot 10^{-6}$ M, which represents a more than 100-fold improvement in dsDNA affinity in comparison with the unnatural oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2 ($K_d = 4 \cdot 10^{-4}$ M). Furthermore, the strongest-binding conjugate of each intercalator was selected. The hybrid molecules Acr 1, Ano 1, Flu 4, and Ane 4 were selected, and they possessed a strongly enhanced affinity for the dsDNA target, with K_d values ranging from $5.6 \cdot 10^{-6}$ M to $7.1 \cdot 10^{-6}$ M. The strength of binding of these conjugates is close to the affinity of known dsDNA-interacting molecules like netropsin, distamycin ($K_a \approx 10^5$ to 10^8 M^{-1}), and the combilexin NetGA [6][17].

DNase-I-Footprinting experiments showed that the hybrid molecules recognize the same sequences as the oligopeptide, but, at lower concentrations, except the conjugates of 3,8-diamino-5-methyl-6-phenylphenantridinium. The cleavage patterns of the methidium hybrid molecules differ clearly from that of the unconjugated oligopeptide and, in consequence, also from the other conjugates. There is, however, a remarkable similarity to the cleavage pattern of ethidium bromide, although differences are clearly present. This means that for the conjugates of 3,8-diamino-5-methyl-6-phenylphenantridinium, the binding specificity is governed by the intercalator, while the peptide only plays a role by enhancing the affinity of the intercalator. This enhancement of affinity in comparison with EtBr is clearly visible in the footprinting experiments. The inhibition of cleavage with Phe 4 appears already at a concentration of 0.5 μM, while, with EtBr, this decreased cleavage is only apparent at 5 μM. Conjugates of other intercalators, on the other hand, are governed by the oligopeptide, while the intercalator enhances the binding strength of the latter one. These results confirm that, in hybrid molecules, the sequence-selectivity can be determined as well by the intercalator agent as by the non-intercalating moiety, while both parts can contribute to the strength of binding. It is furthermore demonstrated that hybrid molecules with the same spacer can, however, possess different sequence selectivities, depending on the conjugated intercalator. Apparently, the part with the higher affinity will dominate the sequence-selective interaction with the target, provided that it is combined with an appropriate linker arm. Therefore, selection of the right combination of peptide, spacer, and intercalator is necessary in order to optimally improve binding and retain sequence selectivity.

4. Conclusions. – In previous work, we selected dsDNA ligands from a library of unnatural heptapeptides. Their dissociation constants ranged from $7 \cdot 10^{-4}$ M to $9 \cdot 10^{-5}$ M, and they showed sequence-selective interaction with a six-base-pair-long mixed sequence. To enhance the binding strength of the oligopeptide Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-NH_2 ($K_d = 4.9 \cdot 10^{-4}$ M), conjugates with five intercalators coupled by six different linker arms were synthesized. Their interaction capacity with a 14-base-pair dsDNA fragment was investigated by gel-shift assays.

All hybrids showed enhanced interaction in comparison with the original oligopeptide. The glycine-β-alanine-methidium conjugate showed the highest affinity

with a K_d of $2.4 \cdot 10^{-6}$ M, which reflects an enhancement of two orders of magnitude over the reference oligopeptide. The binding selectivity for the dsDNA fragment was, however, dominated by the intercalator in such a way that the compound became selective for the 5′-GCATGC-3′ sequence. Other hybrid molecules selected for their strong dsDNA binding (OP-glycyl-acridine, OP-glycyl-anthraquinone, OP-glycyl-β-alanyl-fluorenone, and OP-glycyl-β-alanyl-anthracene) possessed apparent K_d values ranging from $5.6 \cdot 10^{-6}$ to $7.1 \cdot 10^{-6}$ M. The interaction with the dsDNA target of these conjugates was governed by the oligopeptide, since the sequence selectivity of the heptapeptide was conserved. In general, the shorter and less-flexible linker arms glycine and glycine-β-alanine were preferred over β-alanine, glycyl-glycine, and the more flexible spacers γ-aminobutanoic acid and ε-aminohexanoic acid. Selection of the right combination of peptide, spacer, and intercalator is necessary to optimize binding and retain sequence selectivity.

By combining different intercalating moieties with an unnatural oligopeptide entity, new dsDNA-interacting hybrid molecules have been developed that possess dissociation constants in the μM range. This approach opens the possibility to develop, by combinatorial selection, a new class of dsDNA-binding agents with a wide range of sequence selectivity. The sequence specificity is, at this moment, not yet understood, and further research is needed to investigate the binding mode of these molecules. However, the sequence recognition seems to be more diverse (including mixed purine/pyrimidine sequences) than that of antigene oligonucleotides (poly-pu/poly-py tracts) and more broad than that of pure intercalating agents, the latter recognizing mainly dimers or trimers.

Exprimental Part

1. *General.* Anh. solvents were obtained as follows: CH_2Cl_2 was stored over CaH_2, refluxed and distilled; pyridine and ethyl(diisopropyl)amine ($^iPr_2EtN$) were refluxed overnight over KOH and distilled. 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. *Rink amide MBHA resin* was obtained from *Novabiochem* (CH-Läufelfingen). CH_2Cl_2, DMF, Ac_2O, and pyridine were obtained from *BDH* (Poole, England). Piperidine, CF_3COOH, diisopropylcarbodiimide (DIC), 1-hydroxy-1*H*-benzotriazole (HOBt), and 1-methyl-1*H*-imidazole (NMI) were supplied by *Acros* (Geel, Belgium). 1-hydroxy-7-azabenzotriazole (=1-hydroxy-1*H*-1,2,3-triazolo[4,5-*b*]-pyridine; HOAt) and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were purchased from *Perspective Biosystems.* (1*H*-Benzotriazol-1-yloxy)tri(pyrrolidin-1-yl)phosphonium hexafluorophosphate (*PyBOP*®) was obtained from *Advanced Chemtech.* Oligonucleotides were purchased from *Eurogentec.* T4 Polynucleotide kinase and DNase I were purchased from *Gibco BRL* 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*® column (250 × 9 mm, 100 Å, 15–20 μm). Liquid secondary-ion (LSI) MS: Cs^+ as primary ion beam, *Kratos Concept-IH* spectrometer (*Kratos*, Manchester, UK).

2. *Synthesis of the Oligopeptide–Intercalator Conjugates.* Amino acid building blocks *N*-Fmoc-sarcosine (= *N*-{[(9*H*-fluoren-9-yl)methoxy]carbonyl}-*N*-methylglycine; Fmoc-Sar), N^{α}-Fmoc-β-(uracil-1-yl)-D-alanine (= *N*-{[(9*H*-fluoren-9-yl)methoxy]carbonyl}-3-(1,2,3,4-tetrahydro-5-methyl-2,4-dioxopyrimidin-1-yl)-D-alanine; Fmoc-Ual), N^{α}-Fmoc-β-(thymin-1-yl)-D-alanine (= *N*-{[(9*H*-fluoren-9-yl)methoxy]carbonyl}-3-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-alanine; Fmoc-Tal) and N^{α}-Fmoc-β-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-D-alanine (= *N*-{[(9*H*-fluoren-9-yl)methoxy]carbonyl}-4-(1,2,3,4-tetrahydro-2,4-dioxoquinazolin-3-yl)-D-alanine; Fmoc-Chi) were synthesized as described in [13]. N^{α}-Fmoc-N^6-(2,2,5,7,8-pentamethylchroman-6-sulfonyl)-*L*-arginine (= N^2-{[(9*H*-fluoren-9-yl)methoxy]carbonyl}-N^5-{[(3,4-dihydro-2,2,5,7,8-pentamethyl-

2*H*-1-benzopyran-6-sulfonyl)amino]iminomethyl}-L-ornithine) was obtained from *Novabiochem*. *N*-Fmoc-glycine and *N*-Fmoc-*β*-alanine were purchased from *Advanced Chemtech*. Fmoc Protection of *γ*-aminobutanoic acid and *ε*-aminohexanoic acid was performed under *Schotten–Bauman* conditions according to literature [13]. Anthracene-9-carboxylic acid and 9,10-dihydro-9,10-dioxoanthracene-2-carboxylic acid were purchased from *Sigma-Aldrich*. The 3,8-dimino-6-(4-carboxyphenyl)-5-methylphenantridinium chloride was supplied by *Fluka*. Acridine-9-carboxylic acid and 9-oxo-9*H*-fluoren-2-carboxylic acid were obtained from *Acros*. The conjugates were synthesised on *Rink amide MBHA resin*. For peptide bond formation, a mixture of DIC (4 equiv.), HOAT (4 equiv.), $^{i}Pr_2EtN$ (4 equiv.), *PyBoP* (2 or 4 equiv. for Tal), and the Fmoc-derivatized amino acids (4 equiv. with respect to the available amines) in DMF was used. The intercalators were coupled with the use of a mixture of HATU (4 equiv.), $^{i}Pr_2EtN$ (4 equiv.), and 4 equiv. of the heteropolyaromatic moieties in DMF. The hybrid molecules were assembled starting from 1.2 g of *Rink amide MBHA resin* (0.54 mmol/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 carboxy intercalators, and the coupling reagents in DMF was added (without preactivation) 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. After each coupling, 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 or intercalator.

3. *Isolation of the Hybrid Compounds for Solution-Phase Screening.* After the final assembly cycle, the resins were acetylated. Since all the protecting groups were acid-labile, the oligopeptides were deprotected and cleaved off 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 $^{i}Pr_2O$ at −70°. The resins were washed with CF_3COOH and $^{i}Pr_2O$. The white cloudy suspension obtained was centrifuged for 10 min at 9000 rpm. After removal of the supernatants, the remaining pellet as dried *in vacuo* and dissolved in H_2O/MeCN 9:1. The soln. was transferred to a tared polypropylene tube and lyophilized. The final yield of synthesis was determined by weight. 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 to 20:80:0.1). The identity of the individual oligopeptide-intercalator conjugates was determined by LSI-MS analysis (*Table 3*).

4. *Solution Screening of the Hybrid Molecules.* The soln.-screening process was performed by gel-shift experiments. Both strands of the target oligonucleotide were radiolabelled (^{32}P) at the 5′-end by standard procedures, 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 minutes at 4° to allow hybridization of the DNA strands. The conjugates and the reference oligopeptide were dissolved in an appropriate volume of PBS. In a total reaction volume of 5 μl (PBS), 1 μl of the oligonucleotide soln. (containing 1 pmol of dsDNA) was mixed with the appropriate volume of conjugate or oligopeptide soln., to acquire the desired concentrations. PBS Buffer was added to obtain a total volume of 5 μl. 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 with a *Cyclone Phosphorimager* (*Packard*). The degree of complex formation was quantified by measuring the residual amount of free DNA by means of *Optiquant*™, an image analysis software program (*Packard*).

5. *Apparent-Dissociation-Constant Determination.* To determine the apparent dissociation constants of conjugate–dsDNA complexes, gel-mobility-shift assays were used. These gel-shift experiments were performed as described in [13]. To obtain the apparent K_d, a series of concentrations of the investigated conjugates were used, going from 1 to 50 μM, together with a fixed concentration of dsDNA target (0.2 μM). The apparent K_d was then determined as the conjugate concentration at which 50% of the target is mobility-shifted. At least three independent gel-shift assays were performed for each conjugate to determine the apparent K_d. Concentration-response curves, obtained by analysis of the gel shifts, were fitted to 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 means of *Graphpad Prism* (*Graphpad Software Inc.*).

6. *Footprinting Experiments.* Cleavage reactions by DNase I were performed essentially according to the protocol described *Bailly*, *Waring*, and co-workers [11]. The 271 dsDNA fragment used in these experiments was derived from the vector pUC 19, which contained the 14-mer target sequence for the screening assays. The

Table 3. *Calculated and Observed Molecular Weights of the Peptide–Intercalator Conjugates as Determined by LSI-MS.* Masses were obtained as $[M+H]^+$ or $[M+2H]^{2+}$.

Conjugates	M_r, calc.	M_r, found	Conjugates	M_r, calc.	M_r, found
Acr 1	1500	1501	Ane 1	1500	1500
Acr 2	1514	1515	Ane 2	1513	1513
Acr 3	1557	1558	Ane 3	1556	1556
Acr 4	1571	1572	Ane 4	1570	1570
Acr 5	1528	1528	Ane 5	1527	1527
Acr 6	1556	1556	Ane 6	1555	1555
Conjugates	M_r, calc.	M_r, found	Conjugates	M_r, calc.	M_r, found
Ano 1	1529	1529	Flu 1	1501	1502
Ano 2	1543	1543	Flu 2	1515	1516
Ano 3	1586	1586	Flu 3	1558	1560
Ano 4	1600	1600	Flu 4	1572	1573
Ano 5	1557	1557	Flu 5	1529	1529
Ano 6	1585	1585	Flu 6	1557	1557
Conjugates	M_r, calc.	M_r, found			
Phe 1	1621	1621			
Phe 2	1635	1635			
Phe 3	1678	1678			
Phe 4	1692	1692			
Phe 5	1649	1649			
Phe 6	1677	1677			

preparation of the vector and the 271-base-pair fragment was performed as described in [13]. 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 investigated molecules 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., the concentration of which was adjusted to obtain an enzyme attack of *ca.* 30% of the starting material (final enzyme concentrations of 0.0005–0.1 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.

7. *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 phosphorus screen. Each resolved band was assigned to a particular bond within the DNA fragment by comparison of its position 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).

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