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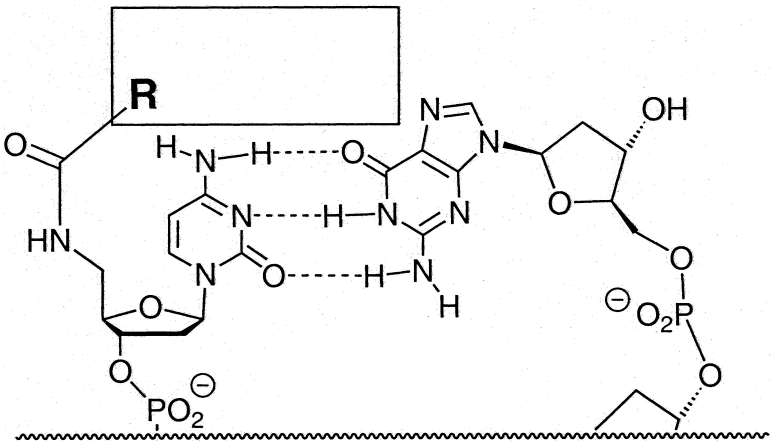

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Monitored Selection of DNA-Hybrids Forming Duplexes with Capped Terminal C:G Base Pairs

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Reported here are the results of a search for modified oligodeoxynucleotides with a 5′-terminal cytidine residue whose affinity for target strands is enhanced by 5′-acylamido groups. These acylamido groups were envisioned to act as molecular caps that bind to the exposed terminal base pair of the duplex with the target strand. A total of 52 capped oligonucleotides of the sequence R-C*GGTTGAC, where R denotes the 5′-appendage and C* a 5′-amino-2′,5′-dideoxycytidine residue, were tested. Among the building blocks employed to modify the 5′-amino group of the DNA strand were carboxylic acid residues, either appended directly or via an amino acid residue, and aromatic aldehydes, coupled via reductive amination. The carboxylic acids employed ranged from Fmoc-glycine to $(Fmoc)_2$-vancomycin and included a number of aromatic acids and bile acids. Small libraries were subjected to MALDI-monitored nuclease selection experiments, and selected compounds were tested in UV-melting assays with target strands. Cholic acid appendages stabilized terminal C:G base pairs to the greatest extent, with melting point increases of up to 10 °C. Further, the cholic acid residue enhanced base pairing fidelity at the terminus, as determined in melting analyses with target strands containing a mismatched nucleobase at the 3′-terminus.

Introduction

Oligonucleotides have a number of desirable properties, such as their ability to interact predictably with DNA and RNA target strands, but also possess a number of undesirable properties. Among the latter are their susceptibility to enzymatically catalyzed hydrolysis and the inability to cross cell membranes by diffusion. Therefore, modified oligodeoxyribonucleotides with high target affinity and enhanced bioavailability have been the target of many recent investigations.[1] Many oligonucleotide analogues reported to date bear nonnatural backbones.[2] Of those oligonucleotides whose termini have been modified, many bear moieties that facilitate detection, e.g. via autoradiography, fluorescence, or absorption of visible light without noticeably affecting the binding properties of the core portion of the strands.[3] However, the termini also offer an opportunity to append moieties that enhance target affinity and selectivity. The latter is particularly desirable, as base pairing fidelity at the termini of duplexes is poorer than in the interior.[4] For terminal substituents envisioned to enhance target affinity there are fewer constraints for binding without steric conflicts than for those in the interior of a strand. Further, the exposed terminal base pair of a duplex offers a large flat binding site, for which it is seemingly easy to design a ligand. Therefore, it is surprising that there are comparatively few reports on modifications for terminal residues of oligonucleotides that enhance duplex stability.[5]

In these laboratories, a research project was initiated that is aimed at finding non nucleic acid appendages for the termini of oligonucleotides that engage in binding with target strands.[6] Since it can be challenging to design modifications to biomacromolecules of up to several thousand Daltons molecular weight, it was considered desirable to search for the modified oligonucleotides with improved binding properties via combinatorial synthesis and selection assays. While very successful *in vitro* selection methodologies were known for natural oligo- and polynucleotides, few modifications are tolerated by the polymerases required for the critical amplification and sequencing steps in these selections.[7] Alternative technologies are therefore being required for selections with backbone modified oligonucleotides and DNA or RNA strands bearing substituents at their termini. With MALDI-monitored nuclease selection experiments, we have been able to report one such technology that is suitable for small libraries of short oligonucleotides with one unmodified terminus.[8]

An initial study, whose results were published recently in this journal,[9] was focused on establishing the methodology for monitored nuclease selections of affinity-enhanced oligonucleotides and gaining some insight into what appendages might act as molecular caps for terminal T:A base pairs. Here we report on a study aimed at identifying acylamido groups that can act as molecular caps for terminal C:G base pairs (Figure 1). The search for these caps was on a broader structural basis than that for T:A base pairs[9] and also included "double caps", whose branched structure features both an acyl group and an N-alkyl group introduced via reductive amination. Further, all selections were performed with a non-self-complementary sequence to make the optimization of the assay temperature less critical.[9]

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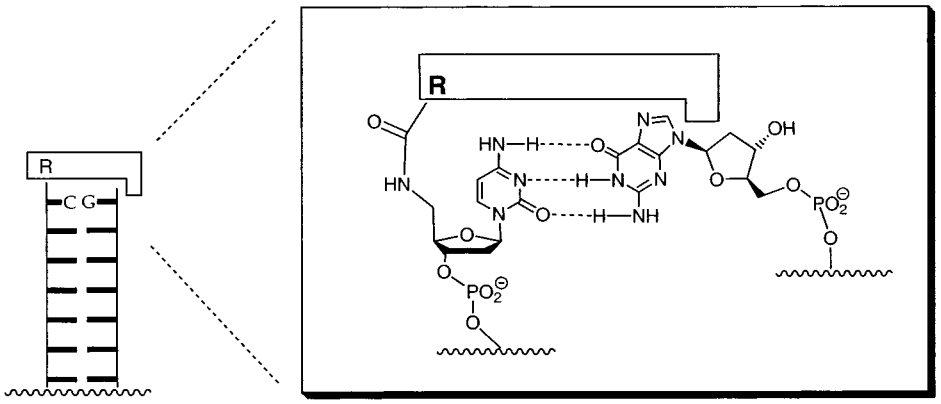
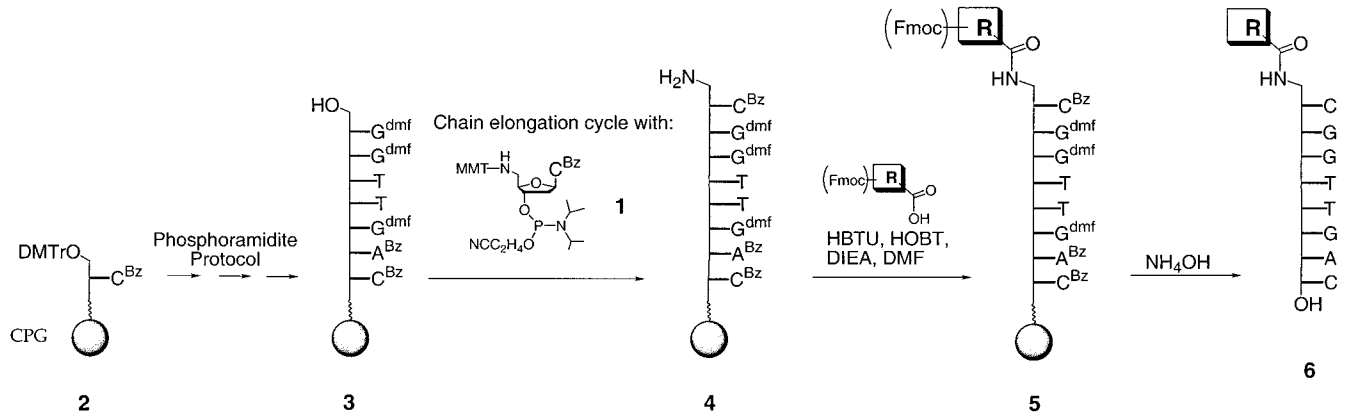


Figure 1. Proposed molecular cap for terminal C:G base pairs.

Scheme 1



Results

The synthesis of 5′-modified oligonucleotides started with the preparation of the cytidine building block with the terminal amino group required for amide bond formation with carboxylic acids. The phosphoramidite building block of 5′-amino-2′,5′-dideoxycytidine (**1**, Scheme 1) was prepared as previously described[10] with the following minor modifications: Sodium azide was used instead of lithium azide in the first step of the synthesis, DMAP was added to the reaction mixture employed for introducing the monomethoxytrityl group, and the phosphitylation was performed in CH_3CN, rather than CH_3CN/CH_2Cl_2, and with diisopropylammonium tetrazolide, rather than tetrazole, as the catalyst. Building block **1** was then used in standard DNA syntheses on controlled pore glass (CPG), starting from derivatized support **2**, producing **4** in uneventful syntheses (Scheme 1).

The carboxylic acid building blocks employed in the couplings with the amino-terminal DNA are shown in Figure 2. Besides amino acid building blocks, including a derivative of tryptophan,[6a] derivatives of nucleobases,[11] several aromatic acids, bile acids,[6b] quinolones, and vancomycin were chosen. Succinylated 5′-amino-5′-deoxynucleosides α and β were prepared under conventional acylation conditions (Scheme 2). Two of the quinolones and vancomycin were Fmoc-protected on a small scale, using the *N*-hydroxy succinimide "ester" of the protecting group (Fmoc-OSu) and basic, aqueous reaction conditions, to give building blocks π, ρ, and ψ. Protecting vancomycin required switching from the sodium carbonate used for the quinolones to bicarbonate and DMF as the only cosolvent, instead of DMF/dioxane/water.

Couplings to **4** (Scheme 1) were performed with a mixture of "uronium salt" HBTU, hydroxybenzotriazole (HOBT), and diisopropyl ethylamine (DIEA) in DMF, i.e. under conditions previously established for coupling to protected oligonucleotides terminating in a 5′-amino-5′-deoxythymidine residue.[12] L-Amino acid building blocks were introduced in mixed couplings, using building block ratios similar to those recommended by Houghten and collaborators,[13] whereas the other carboxylic acids were coupled individually. Couplings with individual building blocks were performed with 1−5 mg of the solid support in conventional polypropylene cups of 1.6 mL capacity. Protected, coupled products (**5**, Scheme 1) were deprotected in concentrated ammonium hydroxide at room temperature, overnight, to yield acylamido DNA of general structure **6**.

Crude products were evaluated via MALDI-TOF mass spectrometry. While all building blocks shown in Figure 2 gave a discernible $[M - H]^-$ peak, no 5′-acylated full length product was observed under the coupling conditions chosen when Boc-D-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, fusidic acid, 9-fluorene carboxylic acid, glycolic acid, hippuric acid, (+)-*N*-(1-phenylethyl)phthalamic acid, 1-nitroanthraquinone-2-carboxylic acid, or *N*-phenylanthranilic acid were employed. Benzyl esters (building block **g**) and benzoyl amides (building block α) were hydrolyzed upon deprotection of the DNA from the solid support, yielding the free carboxylic acid and the free amine. Fmoc protecting groups were also removed during this base treatment, liberating the free amines of building blocks **a**−**h**, π, ρ, ψ, whereas the Boc protection groups of building blocks **i**, **j**,

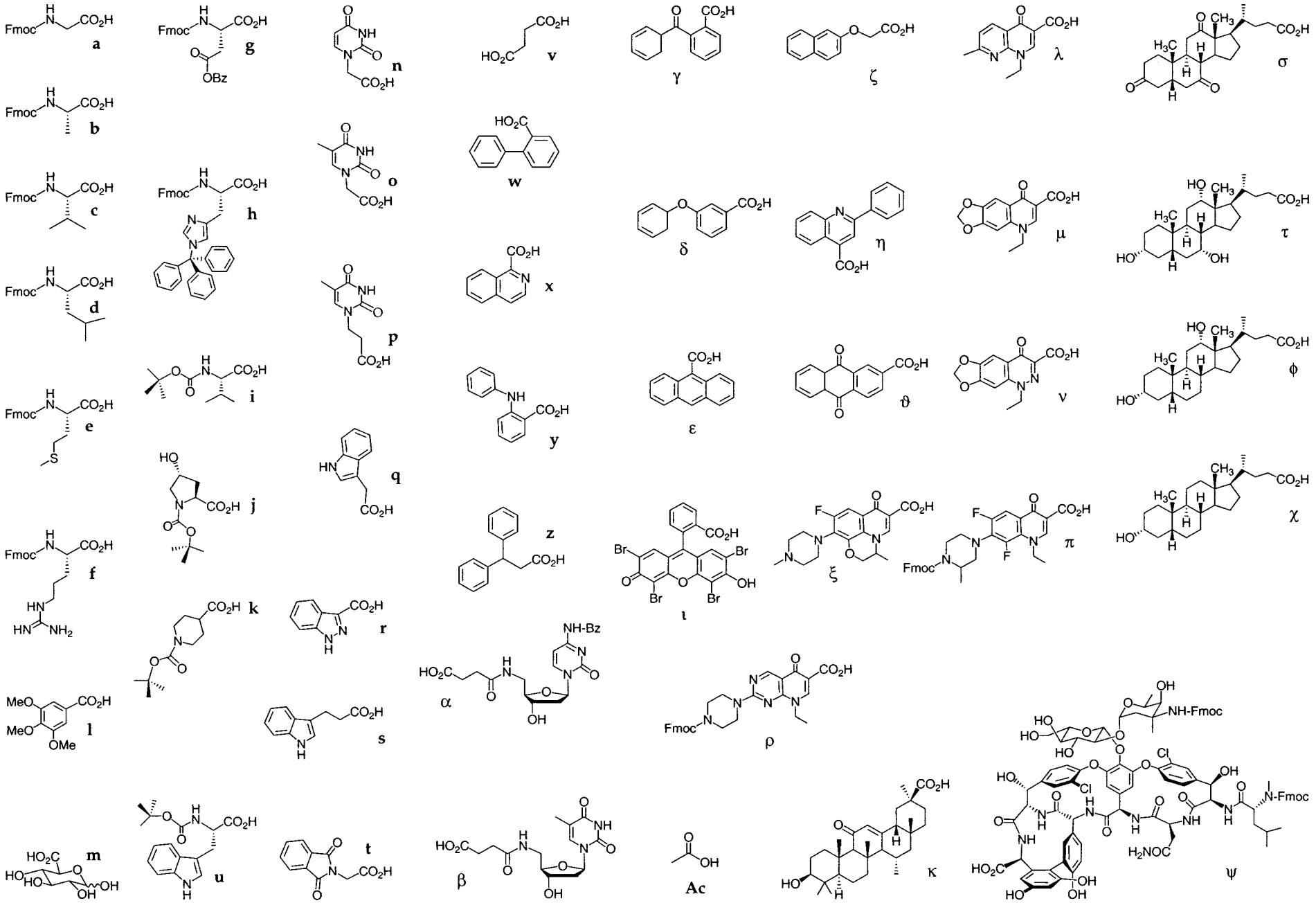
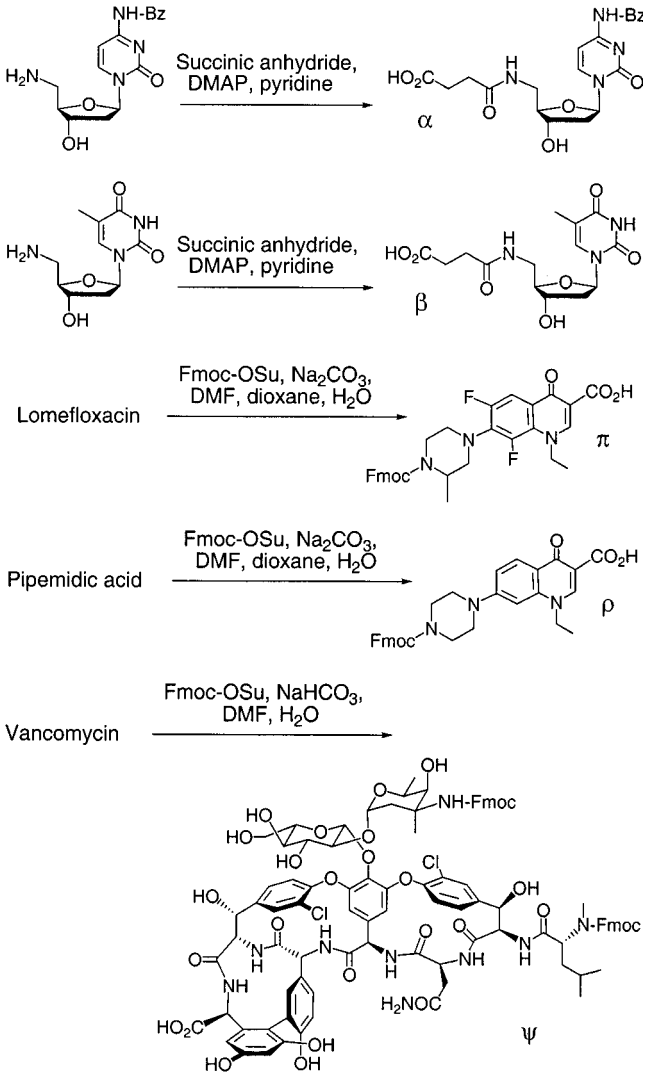


Figure 2. Carboxylic acid building blocks employed for the synthesis of 5′-acylamido oligonucleotides.

Scheme 2



k, **u**, and the trityl moiety of building block **h** were found intact in the products, as expected.

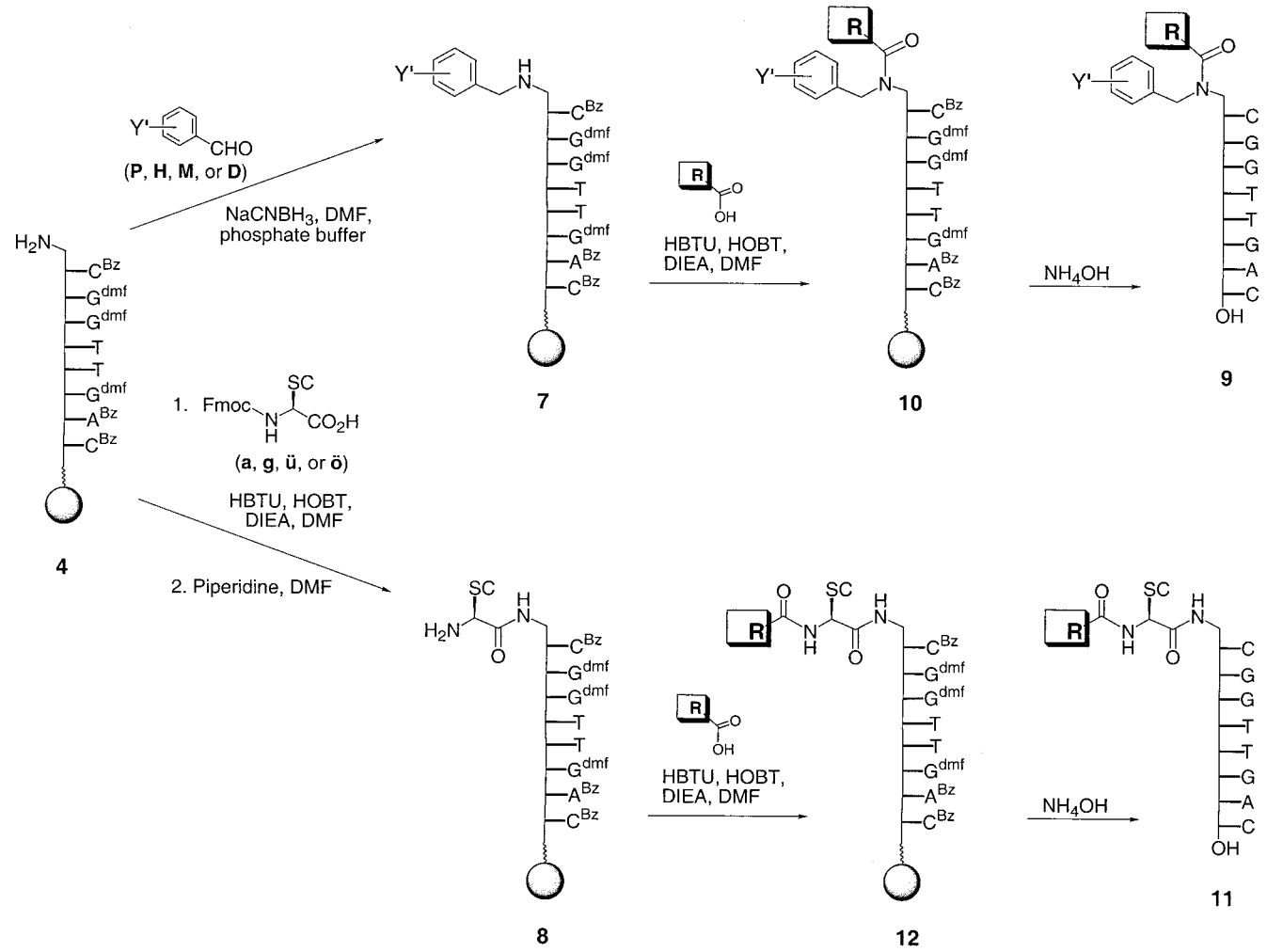
A number of oligonucleotide derivatives with a composite 5′-acylamido substituent were prepared (Scheme 3). These syntheses started from protected DNA octamer **4**, whose 5′-terminal amino group was either alkylated via reductive amination (**7**) or coupled to an Fmoc-protected L-amino acid, followed by removal of the Fmoc group with piperidine in DMF (**8**). The former route engaged aldehydes **P**, **H**, **M**, or **D** and the latter amino acid building blocks **a**, **g**, **ü**, or **ö** (Figure 3). Reductive amination with benzaldehyde (**P**) and its 4-hydroxy derivative (**H**) gave satisfactory conversion with cyano borohydride in a mixture of DMF, MeOH, and aqueous acetate buffer, pH 6. These conditions did not yield the desired alkylation products for mono- and dimethoxyphenyl aldehydes **M** and **D** in satisfactory yields, whose reaction with **4** required a mixture of DMF and phosphate buffer, pH 8. Alkylated amines of general structure **7** coupled well to give doubly capped oligonucleotides **9** via protected intermediates **10**. The same was true for aminoaclyated strands **8**, whose conversion to amino acid-bridged hybrids of general structure **11** (via **12**) proceeded in high yield, as determined via MALDI-TOF mass spectrometry.

Libraries of between two and eight compounds of general structure **6**, **9**, or **11** were obtained directly from the crude products of mixed coupling reactions or by pooling crude products from syntheses producing individual compounds. To these was added the acetylated version of general structure **6** (**Ac**-C*GGTTGAC) as a reference compound. In cases where pooling crude products containing the same low level side products led to MALDI spectra whose *m*/*z* range of interest (2000−3500 Da) was no longer dominated by the peaks of the compounds of interest, a prepurification via HPLC was performed. For this, the entire library was injected, and the fraction(s) enriched in the oligonucleotide hybrids were collected, lyophilized, and used for selection experiments. Some of the DNA hybrids, namely compounds of general structure **6** prepared with building blocks **m**, **u**, **t**, **y**, **z**, γ, σ, and ϕ, were lost during this process or desorbed so poorly in the MALDI mass spectrometer that monitoring them in the selection experiments was abandoned.

The libraries of 5′-capped oligonucleotides thus prepared were subjected to MALDI monitored nuclease survival selection experiments.[9] For a number of capped oligonucleotides of general structure **6**, calibration experiments were performed to ensure that the MALDI-TOF detection method established earlier[14,8,9] produced the linear relationship between relative signal intensity and concentration required for monitoring selection experiments. For this, and the actual selection experiments with compounds of general structure **6**, the DNA undecamer T_{11} was used as the internal standard. Experiments with compounds of general structure **9** and **11** were performed with internal standard 5′-GAAAAGAAAA-3′. All calibration experiments showed good linearity over the concentration range of interest (see Figure S29, Supporting Information, for examples). When control octamer Ac-C*GGTTGAC was added as an additional compound in the solutions at a constant concentration of 14.1 pmol/μL, its relative signal (analyte/internal standard) was constant, demonstrating that quantitation of individual compounds in libraries is feasible. It should be noted that high laser power and averaging over several spectra are required for quantitation. Further, the most popular matrixes for acquiring MALDI spectra of oligonucleotides (picolinic acid and hydroxypicolinic acid) have, in our hands, not been found suitable for quantitation. 2,4,6-Trihydroxy acetophenone and 6-aza-2-thiothymine, however, gave satisfactory results in all cases.

Nuclease survival selections provide a means of identifying modified oligonucleotides with increased target affinity by treating a library whose members compete for hybridization to a target strand with a single-strand specific nuclease and preferentially degrading those compounds that do not engage in duplex formation.[9] For the selections, 1 equiv of target DNA octamer 5′-GTCAACCG-3′ (**13**) was added to the libraries, the resulting mixtures were annealed at 65 °C, cooled, and then treated with snake venom phosphodiesterase (phosphodiesterase I, EC 3.1.4.1), an enzyme that attacks the 3′-terminus of oligonucleotides, i.e. at the terminus where all library components have identical structures. The rate of degradation of the individual compounds was then monitored via MALDI-TOF mass spectrometry. Representative spectra

Scheme 3



and the kinetics plot from a selection from an eight-member library are shown in Figures 4 and 5. The protection factors (PFs) obtained by integrating the areas under the individual degradation curves and dividing these areas by the integral under the corresponding curve of acetylated control compound Ac-C*GGTTGAC are compiled in Table 1.

The selections were performed by several operators and, in some cases, as part of a teaching laboratory for advanced students. As a result, there was some variability in the experimental conditions. Variability was tolerated, as, in the authors' opinion, this would test the ruggedness of the method. Some assays were performed at 0 °C, while others were performed at room temperature (21 °C). The number of strands competing for the target varied from two to nine. The concentration of the ammonium acetate buffer in the assay solution ranged between 143 and 250 mM. The strand concentration was not accurately determined, but rather adjusted iteratively to achieve roughly equal peak heights for the desired library members with estimated strand concentrations ranging from 2 to 75 pmol/μL. The amount of phosphodiesterase I employed ranged from 0.25 to 13 μU/μL. Nuclease selection experiments performed with unmodified oligonucleotides varying in sequence have shown that a high concentration of the strands and low temperature moderately increase the selectivity of the assay.[15] Despite the variability in the conditions in the present study, a validation of the results via correlation of protection factors with UV-melting points demonstrated the robustness of the method (vide infra). All protection factors calculated have statistical confidence values smaller than their absolute values. They range from 8% to 77%, with an average of 37.5%. Negative protection factors are, of course, not defined, since protection factors are ratios of integrated areas. See

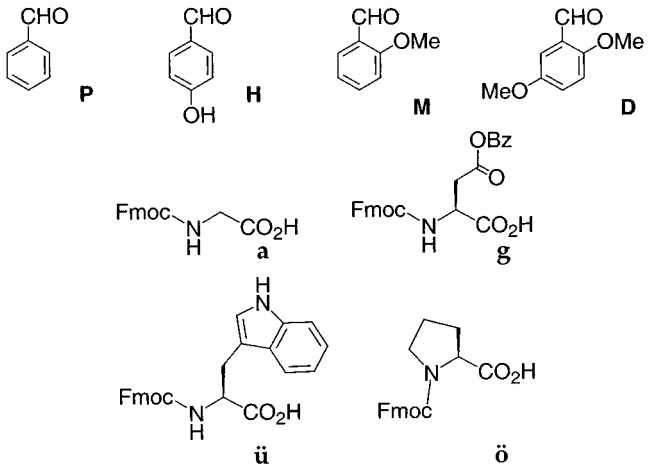


Figure 3. Aldehyde and carboxylic acid building blocks employed for the synthesis of oligonucleotides of general structure **R**-(**R**′)-C*GGTTGAC (**9**) and **R**-**R**″-C*GGTTGAC (**11**).

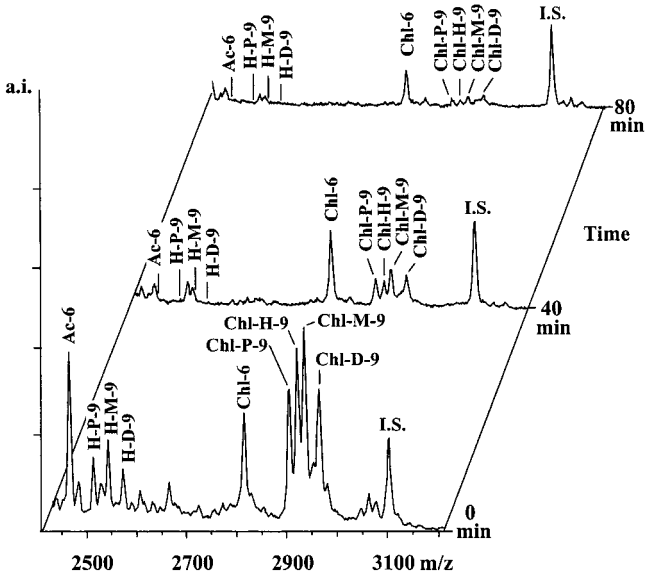


Figure 4. Representative MALDI-TOF mass spectra acquired from samples of the assay solution during nuclease selection from a library composed of 5′-modified oligonucleotides **Ac**-C*GGTTGAC (**Ac-6**), H-(**P**)-C*GGTTGAC (**P-9**), H-(**M**)-C*GGTTGAC (**M-9**), H-(**D**)-C*GGTTGAC (**D-9**), **Chl**-C*GGTTGAC (**Chl-6**), **Chl**-(**P**)-C*GGTTGAC (**Chl-P-9**), **Chl**-(**H**)-C*GGTTGAC (**Chl-H-9**), **Chl**-(**M**)-C*GGTTGAC (**Chl-M-9**), and **Chl**-(**D**)-C*GGTTGAC (**Chl-D-9**). The solution was approximately 75 pmol/μL in every oligonucleotide, 250 mM NH_4OAc, 0 °C, and contained 0.7 μU of snake venom phosphodiesterase.

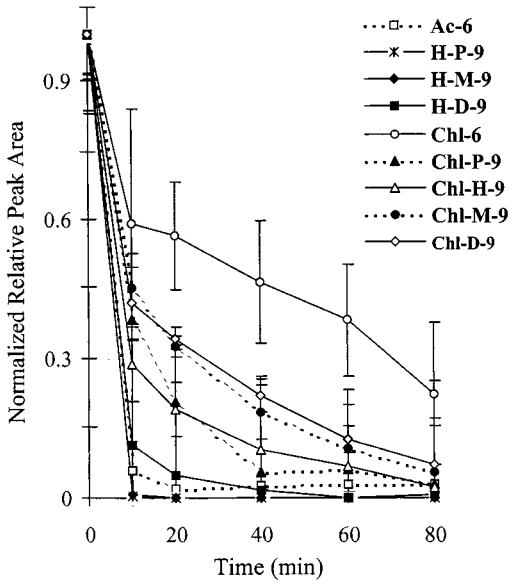


Figure 5. Representative kinetics of the degradation of the full length oligonucleotides during nuclease selection. Shown are the results of the very selection whose spectra are presented in Figure 4. Data points are averages of relative signal intensities (analyte/internal standard) from four spectra normalized to the signal of the respective full length oligomer at time 0 of the assay. Lines are point-to-point fits between data points, error bars are ± one standard deviation.

ref 9 for a more detailed description of statistical analyses with AUTOMATON.

Some compounds of general structure **6** were members of more than one library. For these, an averaged protection factor is being reported in Table 1. Results from assays in which the most nuclease resistant strand was less than 60%

Table 1. Oligonucleotide Hybrids Prepared and Results from Spectrometrically Monitored Nuclease Selection Experiments

sequence[a] / compound	building blocks employed R	R′	R″	PF[b]	$PF_{co+trunc}$[c]
R-C*GGTTGAC (**6**)					
Chl	τ			3.7	4.7
OA	μ			2.2	2.4
AQ	ϑ			2.0	2.5
OF	ξ			2.0	2.3
LO	π			2.0	1.8
CI	ν			1.8	2.3
T#	β			1.8	2.1
PM	ρ			1.5	1.2
NO	ζ			1.4	1.5
NA	λ			1.3	1.5
C#	α			1.3	1.3
IA	**s**			1.2	1.0
Van	ψ			1.2	1.0
Ala	**b**			1.2	1.3
Leu	**d**			1.1	1.2
Met	**e**			1.1	1.2
u*	**n**			1.1	1.1
t	**o**			1.1	1.1
Ac	**Ac**			1.0	1.0
BE	**w**			1.0	0.9
t*	**p**			1.0	0.9
IZ	**r**			1.0	0.9
Arg	**f**			0.9	1.3
BP	**k**			0.9	0.9
PB	δ			0.9	0.9
IQ	**x**			0.8	0.7
TM	**l**			0.8	0.7
PQ	η			0.6	0.3
AT	ϵ			0.6	0.6
IA	**q**			0.6	0.5
EO	ι			0.6	0.7
SU	**v**			0.6	0.4
Lch	χ			0.3	0.2
5′-TGGTTGAC-3′				0.4	0.3
R-(**R′**)-C*GGTTGAC (**9**)					
Chl-(25Di)	τ	D		3.0	3.3
Chl-(2MeO)	τ	M		2.8	3.1
Chl-(Phe)	τ	P		2.0	2.0
Chl-(4HOP)	τ	H		2.0	1.8
T#-(Phe)	β	P		1.4	1.5
T#-(25Di)	β	D		1.4	1.5
H-(25Di)		D		1.0	1.1
C#-(25Di)	α	D		1.0	0.8
C#-(Phe)	α	P		0.9	0.8
SU-(Phe)	v	P		0.8	0.7
SU-(25Di)	v	D		0.9	0.8
H-(P)		P		0.7	0.9
H-(2MeO)		M		0.7	0.9
R-R″-C*GGTTGAC (**11**)					
Chl-Trp	τ		ü	1.9	1.8
Chl-Asp	τ		g	1.9	2.2
NO-Asp	ζ		g	1.9	2.2
OA-Pro	μ		ö	1.5	1.5
NO-Gly	ζ		a	1.3	1.0
NO-Trp	ζ		ü	0.9	0.7
OA-Asp	μ		g	0.9	0.9

[a] Sequences are given 5′ to 3′ terminus and are deoxyribonucleotides, except for C*, denoting the 5′-amino-2′,5′-dideoxycytidine residue. [b] Protection factor. [c] Protection factor after correction with a cutoff reflecting the percentage of residual full length oligomer and truncation correction. See ref 9 for a detailed discussion of protection factors and correction functions. See Figures 2 and 3 for structures of building blocks and Schemes 1 and 3 for design of the modified oligonucleotides.

Table 2. UV-Melting Points and Hyperchromicities of Duplexes

duplex[a]	building blocks employed R	R′	R″	T_m at [salt] (°C)[b] 10 mM	160 mM	1 M	hyperch. (%)[c]
GTCAACCG:							
CGGTTGAC				23.2 ± 0.2	36.3 ± 0.7	41.1 ± 0.4	13.7 ± 1.3
GTCAACCG:							
R-C*GGTTGAC (**6**)							
Ac	**Ac**			22.3 ± 0.7	36.9 ± 1.1	40.8 ± 0.9	14.7 ± 0.3
Chl	τ			33.1 ± 0.5	45.5 ± 0.7	50.7 ± 0.5	15.9 ± 1.7
OA	μ			26.7 ± 0.1	41.0 ± 1.0	46.6 ± 0.8	19.2 ± 0.9
AQ	ϑ			25.8 ± 0.2	39.9 ± 0.4	45.3 ± 0.6	24.2 ± 3.5
OF	ξ			26.4 ± 0.1	38.8 ± 0.7	43.1 ± 0.2	15.1 ± 2.1
CI	ν			27.1 ± 1.0	41.9 ± 1.2	46.7 ± 1.0	15.6 ± 0.7
NO	ζ			25.3 ± 0.6	38.4 ± 0.8	43.9 ± 0.6	13.3 ± 0.6
LO	π			26.7 ± 0.9	39.9 ± 0.8	44.0 ± 0.6	18.3 ± 4.6
PM	ρ			26.2 ± 1.2	38.8 ± 1.0	42.7 ± 0.6	15.0 ± 0.6
T#	β			23.6 ± 1.3	38.1 ± 1.0	42.2 ± 0.9	16.4 ± 0.3
THis	**h**			22.6 ± 0.7	37.1 ± 1.0	41.1 ± 0.6	15.6 ± 1.0
R-(**R**′)-C*GGTTGAC (**9**)							
Chl-(2-**2MeO**)	τ	**M**		29.1 ± 0.5	43.0 ± 0.8	47.8 ± 1.2	13.8 ± 1.2
R-**R**″-C*GGTTGAC (**11**)							
GY-Gly	κ		**a**	28.9 ± 1.0	39.3 ± 0.5	47.3 ± 0.3	14.0 ± 1.0
OA-Asp	μ		**g**	23.8 ± 0.8	38.0 ± 1.2	43.1 ± 1.0	15.2 ± 0.4
(**R**-C*GTACG)$_2$							
Ac	**Ac**			13.5 ± 0.6	20.7 ± 1.0	21.2 ± 0.6	9.7 ± 0.1
Chl	τ			nd[d]	46.4 ± 0.8	49.0 ± 0.7	14.4 ± 0.7
(**R**-C*TTTTAAAAG)$_2$							
Ac	**Ac**			11.7 ± 0.5	27.0 ± 1.1	34.5 ± 0.9	30.8 ± 0.2
Chl	τ			nd[d]	38.8 ± 1.2	48.1 ± 0.9	35.4 ± 2.0

[a] Sequences are given 5′- to 3′-terminus; C* denotes a 5′-amino-2′,5′-dideoxycytidine residue. [b] Average of four melting points ± one standard deviation (SD) at 3.1 ± 0.3 μM strand concentration. Salt concentrations in the solutions were as follows: 10 mM phosphate buffer ("10 mM"), 150 mM NaCl, 10 mM phosphate buffer ("160 mM"), and 1 M NaCl, 10 mM phosphate buffer ("1 M"), pH 7.1. [c] Hyperchromicity at 260 nm upon duplex dissociation (average of four melting curves ± SD). [d] Not determined.

degraded at the end of the monitoring period were discarded. Inclusion of octamer 5′-TGGTTGAC-3′ (**12**), whose 5′-terminal residue is not Watson−Crick complementary to the 3′-terminal guanosine residue of the target (though the formation of a wobble base pair is a possibility), led to the expected low protection factor of 0.4 (uncorrected) and 0.3 (corrected for incomplete degradation). Only the derivative bearing the residue of lithocholic acid (χ) gave a lower protection factor, presumably because this strongly amphiphilic compound was slowly adsorbed on surfaces[16] or because it formed aggregates that failed to desorb in source of the mass spectrometer.

Of the 52 hybrids of general structures **6**, **9**, and **11** tested in nuclease selections, 14 were HPLC purified from samples set aside prior to pooling to give the libraries. This group of compounds included representatives from each type of general structure. The purified strands were mixed with 1 equiv of target strand 5′-GTCAACCG-3′, and the resulting duplex was subjected to UV melting analyses at three different salt concentrations. The results from these melting experiments are compiled in Table 2. Also included in this study was the duplex of unmodified DNA strand 5′-CGGTTGAC-3′ with target strand 5′-GTCAACCG-3′ (**13**). As expected, its melting points did not differ significantly from those of the duplex formed by the acetylated acetamido derivative **Ac**-C*GGTTGAC. Capped strands that survived preferentially in the nuclease selection experiments, however, did give the increased duplex stability sought.

The highest melting point increase was observed for **6** with a cholic acid residue (**Chl-6,** Figure 6). Compared to the acetylated control, its duplexes with **13** melted between 8.6 and 10.8 °C higher. To validate this result, two other DNA sequences were synthesized with and without this bile acid residue. The first was the self-complementary hexamer CGTACG. Compared to the acetylated control duplex, **Chl**-C*GTACG (**14**), where **Chl** stands for the cholic acid residue, gave a melting point that was 27.8 °C higher, i.e. 13.9 °C per modification (Table 2). The other, longer, and A/T-rich test sequence, decamer CTTTTAAAAG, gave a melting point increase of up to 13.6 °C for the cholic acid derivative (**15**), or 6.8 °C per modification. In all cases, this

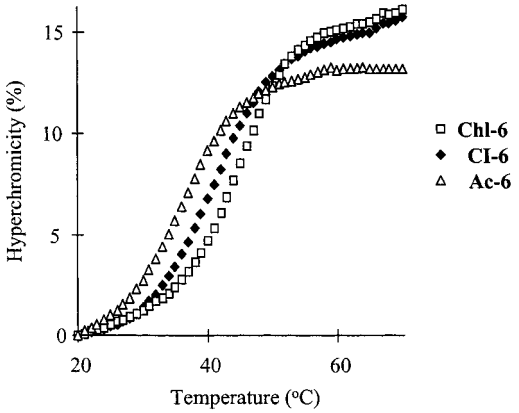


Figure 6. Overlay of UV-melting curves of duplexes of GTCAACCG with **Ac**-C*GGTTGAC (triangles, **Ac-6**), **CI**-C*GGTTGAC (filled diamonds, ν-**6**), and **Chl**-C*GGTTGAC (squares, **Chl-6**). Melting curves were measured at 260 nm at 1.5 ± 0.3 μM strand concentration and 1.0 M NaCl, 10 mM phosphate buffer, pH 7.1.

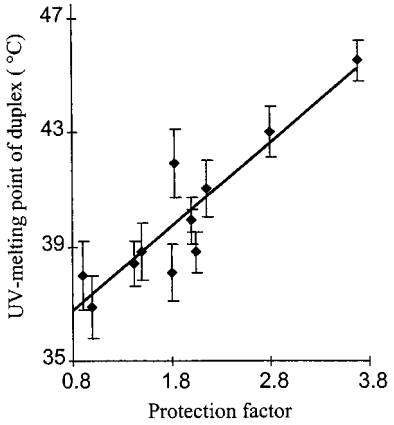


Figure 7. Plot showing the correlation between UV-melting points and nuclease protection factors for those compounds for which both types of data were obtained. Melting points were determined at 1.5 ± 0.3 μM strand concentration and 1.0 M NaCl, 10 mM phosphate buffer, pH 7.1. Protection factors are without cutoff and truncation correction, from selection experiments performed at 2−75 pmol/μL strand concentration and 143 or 250 mM NH_4OAc buffer, pH 7.0.

increase in the melting point was accompanied by an increase in the hyperchromicity during duplex dissociation (see the last column in Table 2 for high salt data). The hexamer did, again, give the greatest relative increase over the value of the control duplex.

Since the cholic acid residue does not absorb at 260 nm, where the melting was monitored, the increase in the hyperchromicity points toward an indirect structural effect, most likely improved stacking in the DNA portion of the duplex. Exploratory melting curve experiments with **Chl-6** and undecamer 5′-GTCAACCGTCA-3′, which contains an overhang of three residues at its 3′-terminus, indicate that the stabilizing effect of the cholic acid residue is not significantly impaired when it is faced with a longer target strand. At 1 M salt and 3.1 μM strand concentration (same conditions as those for the third column of melting points given in Table 2), the melting point of **Chl-6**:GTCAAC-CGTCA was 48.3 ± 0.8 °C, whereas the control duplex CGGTTGAC:GTCAACCGTCA gave a melting point of 40.0 ± 1.0 °C. The hyperchromicities were 14% for the steroid-capped duplex and 12% for the control duplex.

A number of other capped duplexes gave increased melting points and hyperchromicities, with several quinolones and the residue of anthraqinone carboxylic acid (ϑ) the most noteworthy in terms of increased hyperchromicity. Interactions between anthraquinones and nucleic acids have previously been noted, and PNA-anthraquinone conjugates can be used for photoinduced cleavage.[17]

Acquisition of the UV-melting points allowed a comparison of the results from nuclease selections with those of this conventional hybridization experiment. Figure 7 shows the correlation between the melting points and the protection factors. The value for **6** with a vancomycin residue (**Van**-C*GGTTGAC) was excluded from this analysis since this compound gave a significant melting curve in the single-stranded state, indicating that it was folding into an intramolecular structure. For the remaining compounds tested, a satisfactory correlation between the values obtained from the kinetic readout (nuclease selection) and equilibrium experiment (UV melting) was obtained.

Table 3. UV-Melting Points of Duplexes Containing a Mismatched Nucleobase, as Obtained at 1.5 ± 0.3 μM Strand Concentration and 1.0 M NaCl, 10 mM Phosphate Buffer, pH 7.1

oligonucleotide[a]	R	target strand	T_m (°C)[b]	discrimination (ΔT_m to fully matched duplex)
R-C*GGTTGAC				
Ac	**Ac**	GTCAACC**G**	38.7 ± 0.8	
Chl	τ	GTCAACC**G**	48.8 ± 0.3	
R-C*GGTTGAC				
Ac	**Ac**	GTCAACC**A**	33.8 ± 0.6	4.9
Chl	τ	GTCAACC**A**	35.8 ± 0.7	13.8
R-C*GGTTGAC				
Ac	**Ac**	GTCAACC**C**	32.1 ± 0.6	6.6
Chl	τ	GTCAACC**C**	35.2 ± 0.7	13.6
R-C*GGTTGAC				
Ac	**Ac**	GTCAACC**T**	32.2 ± 0.3	6.5
Chl	τ	GTCAACC**T**	35.5 ± 0.8	13.3

[a] Sequences are given 5′- to 3′-terminus; C* denotes a 5′-amino-2′,5′-dideoxycytidine residue. [b] Average of four melting points ± SD.

The most strongly duplex-stabilizing acylamido substituent identified in this study, the cholic acid residue, was then tested for its ability to increase base pairing fidelity at the terminus of a duplex. The results from this series of UV melting experiments are compiled in Table 3. When faced with a terminal base pair that cannot form a canonical Watson−Crick base pair, the cholic acid cap provides little of the stabilization that it confers upon the C:G pair. As a result, the cholic acid capped strand **Chl**-C*GGTTGAC shows a substantially increased mismatch discrimination, a property that may make it useful for applications that require high fidelity hybridization probes. Work with a steroid-capped oligonucleotide duplex terminating in a T:A base pair has shown that base pairing fidelity increases both at the terminal and at the penultimate position of the duplex.[6b]

Discussion

The selections performed in this study were aimed at finding modified oligonucleotides with favorable binding properties. For this, building blocks were chosen that were believed to have the potential to bind to the exposed terminal base pair without creating oligonucleotides with undesirable physicochemical properties. Oligocationic appendages were avoided, since these were shown earlier to lose their stabilizing effect at salt concentrations relevant, e.g. for hybridization experiments involving DNA chips.[5g] Further, it is known that lengthening cationic peptides does not necessarily increase their affinity for nucleic acids, as enthalpic gains can be matched by entropic cost.[18] Very large lipophilic appendages, such as porphyrins,[19] can lead to capped oligonucleotides that aggregate or become very difficult to handle because of their propensity to adsorb on surfaces. The majority of the hybrids prepared with the building blocks shown in Figure 2 did not induce such unfavorable effects. The pentacyclic building blocks, such as 18β-glycyrrhetinic acid (κ), and even the macrocycle

vancomycin (ψ) gave tractable compounds (though the latter apparently induced "folding"). The more lipophilic of the bile acid building blocks, however, were more problematic, possibly defining one of the upper limits of what is tolerated without obtaining compounds whose binding properties are no longer dominated by the DNA portion. The quinolones, compounds known to bind to DNA[20] and previously found to enhance duplex stability when appended to a terminal 5′-amino-5′-deoxythymidine residue,[9] did indeed stabilize duplexes. As expected for successful drugs, they possess the required balance of polar, hydrophilic groups, lipophilicity, and rigidity that is required for binding to biomacromolecules without high entropic penalty and without being trapped in nonproductive states, such as aggregation or the solid state (due to insolubility).

The exposed terminal base pair offers a lipophilic patch on the surface of the double helix that, in principle, should be readily bound via π-stacking.[21] The observation that neither electron-rich aromatic building blocks, such as ζ or δ, nor their electron-depleted counterparts, such as ϑ, gave a strong stabilizing effect indicates that π-stacking alone may not be a very strong force, unless one employs entire nucleotide analogues with a pyrene moiety rigidly fixed to the ribose ring.[5d] Instead, the cholic acid residue gave the strongest duplex stabilizing effect, an effect that is indeed stronger than that of an additional A:T base pair,[5g] even though the appended residue is smaller than two additional nucleotides and is not commonly thought of as a DNA-binding molecule. Apparently, rigidity, the correct linker length, and a tight fit to the terminal base pair are important for molecular caps, since all composite 5′-acylamido substituents tested fared more poorly than directly appended cholic acid. The NMR evidence gathered from a complex involving a terminal T:A base pair and the cholic acid cap points toward a stacking interaction between the methyl groups on the β-face of the steroid and the surface of the terminal base pairs.[6b] Since the cholic acid residue seems to have the ability to enhance target affinity and base pairing fidelity at the terminus for both T:A and C:G base pairs, the results presented here move it one step closer to what may eventually become a "universal cap" for high fidelity hybridization probes. Since there is little doubt that the bile acid residue also prevents nuclease attack at the 5′-terminus, such probes would combine several desirable properties, without being difficult to synthesize.

Finally, it may be worthwhile discussing the advantages and disadvantages of the approach chosen to find oligonucleotide derivatives with desirable binding properties. Despite the variability in the assay conditions (vide supra), the selection assay picked up stabilizing substituents that induce a melting point increase of ≥ 5 °C reliably (Figure 7). This value may be improved by employing the strategies developed during optimization of the related nuclease assay for rapid genotyping.[15] Two main concerns considered when first developing this assay, namely using a kinetic readout when trying to measure binding affinity (i.e. a thermodynamic parameter) and searching just for affinity when trying to find a moiety that binds with good selectivity, seem to be allayed by the results from the present study. The latter concern may not have been a concern at all, if high affinity does indeed lead to high specificity automatically.[22] This argument that, in our opinion, does have to take the unspecific electrostatic interactions into account that can be quite important in binding to polymers with repetitive charges such as nucleic acids, as evidenced e.g. by the binding of polyamines to DNA. On the other hand, the facilitated diffusion of cationic proteins along genomic DNA[23] shows how compounds capable of adopting a defined three-dimensional structure can engage in low affinity, electrostatically driven interactions and in high specificity/high affinity complex formation. In any event, the nuclease selection technology for selecting oligonucleotides with increased target affinity from small libraries employed here for a search in an admittedly confined structure space does seem to provide new derivatives efficiently.

Experimental Section

General. Chemicals employed as building blocks or for building block synthesis were the best commercially available grade from Acros (Geel, Belgium), Aldrich/Fluka/Sigma (Deisenhofen, Germany), Merck (Darmstadt, Germany), or Advanced ChemTech (Louisville, KY) and were used without purification. DNA synthesis reagents were from Proligo (Hamburg, Germany). Reversed phase HPLC chromatography was performed with a gradient of acetonitrile in 0.1 M triethylammonium acetate, pH 7, on a 250 × 4.6 mm EcoChrom, Macherey Nagel (Düren, Germany) 5 μm column (C4). Yields of hybrids were determined from the integration of the product peaks in the HPLC trace of the crude products. The integration was not corrected for the absorbance caused by the solvent front. MALDI-TOF mass spectra were acquired on a Bruker BIFLEX III mass spectrometer with a drift tube extension, a dual microchannel plate detector, a 1 GHz digitizer, and delayed extraction capabilities. Spectra were acquired at pressures $<1 \times 10^{-6}$ mbar in the drift tube. Calculated masses are average masses, *m*/*z* found are those for the pseudomolecular ions ($[M - H]^-$), detected as the maximum of the unresolved isotope pattern. The accuracy of mass determination with the external calibration used is ca. ±0.1%, i.e. ±2 Da at *m*/*z* 2000.

Fmoc-lomefloxacin, Building Block π. Lomefloxacin hydrochloride (50 mg, 0.13 mmol) was suspended in Na_2CO_3 solution in water (9%, 0.5 mL) and cooled to 0 °C. Fmoc-OSu (53 mg, 0.16 mmol) in DMF/dioxane (3:1, 0.8 mL) was added. The mixture was vigorously stirred for 20 min and allowed to warm to room temperature. A precipitate formed that was removed by filtration. The filtrate was diluted with water (2 mL) and extracted with EtOAc (2 × 10 mL). The water solution was acidified with aqueous HCl (10%) to pH 2 and extracted with EtOAc (2 × 10 mL). The EtOAc solutions were combined, dried over Na_2SO_4, and dried *in vacuo*. The residue was taken up in $CHCl_3$/EtOH/AcOH (95:4.5:0.5) and purified by flash chromatography using the same eluant. Yield: 35 mg (61 μmol, 47%) of a white amorphous solid. TLC (silica, $CHCl_3$/EtOH/AcOH, 95:4.5:0.5): R_f 0.45. ^{1}H NMR ($CDCl_3$, 300 MHz, two rotamers) δ 8.68/8.60 (2 s, 1H); 8.10/7.98 (d, J = 13.4 Hz and dd J = 1.5, 11.4 Hz, 1H); 7.77 (d, 2H, J = 7.3 Hz); 7.58 (d, 2H, J

= 7.7 Hz); 7.40 (t, 2H, J = 7.3 Hz); 7.32 (t, 2H, J = 8.0 Hz); 4.50/4.32/4.24 (3 m, 5H); 3.93/3.67/3.45−3.14 (3 m, 7H); 1.55 (m, 3H); 1.26 (m, 3H). MALDI-TOF MS (positive mode, THAP/diammonium citrate matrix) for $C_{32}H_{30}F_2N_3O_5$ $[M + H]^+$: calcd 574.2, found 572.5.

Fmoc-pipemidic Acid, Building Block *ρ*. Pipemidic acid (50 mg, 0.17 mmol) was dissolved in an aqueous Na_2CO_3 solution (9%, 0.5 mL) and cooled to 0 °C. Fmoc-OSu (67 mg, 0.20 mmol) in DMF/dioxane (3:1, 0.8 mL) was added. The mixture was vigorously stirred for 20 min and allowed to warm to room temperature, upon which a precipitate formed. Filtration yielded a solution that was diluted with water (2 mL) and extracted with EtOAc (2 × 10 mL). The aqueous solution was acidified to pH 2 with HCl (10%) and extracted with EtOAc (2 × 10 mL). The organic phases were combined, dried over Na_2SO_4, and dried *in vacuo*. The residue was redissolved in a $CHCl_3$/EtOH/AcOH (95:4.5:0.5) mixture, and purified by flash chromatography with the same eluant. Yield: 30 mg (57 μmol, 34%) of a white solid. TLC (silica, $CHCl_3$/EtOH/AcOH (95:4.5:0.5): R_f 0.4. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.32 (s, 1H); 8.67 (s, 1H); 7.78 (m, 2H); 7.57 (m, 2H); 7.42 (m, 2H); 7.33 (m, 2H); 4.53 (d, 2H, J = 6.0 Hz); 4.33 (q, 2H, J = 7.7 Hz); 4.26 (m, 1H); 3.97/3.88 (2m, 4H); 3.55 (m, 4H); 1.49 (t, 3H, J = 7.7 Hz). MALDI-TOF MS (positive mode, THAP/diammonium citrate matrix) for $C_{29}H_{28}N_5O_5$ $[M + H]^+$: calcd 526.2, found 525.1.

Fmoc$_2$-vancomycin, Building Block *ψ*. Vancomycin hydrochloride hydrate (13 mg, 8.8 μmol) was dissolved in a solution of $NaHCO_3$ (3.8 mg, 45.2 μmol in water and DMF, 9:1, v/v, 0.5 mL at 50 °C) and cooled to 0 °C. Fmoc-OSu (9 mg, 26.4 μmol) in DMF (0.2 mL) was added in one portion. The solution was vigorously stirred for 20 min, allowed to warm to room temperature, and allowed to stand for 2 h. Then, two additional portions of Fmoc-OSu (9 mg, 26.4 μmol each) in DMF (0.2 mL) were added after 2 h time increments. After another 2 h, TLC and MALDI analyses indicated full conversion of the starting material. The solution was diluted with water (5 mL), acidified to pH 2 with 1% HCl, and extracted with EtOAc (3 × 10 mL). The organic extracts were combined, washed with 1% aqueous HCl (10 mL) and brine (2 × 10 mL), and dried over Na_2SO_4. After evaporation, the residue was purified by preparative TLC (silica, MeOH/CH_2Cl_2 15/85). Yield: 10 mg (5.3 μmol, 60.0%). TLC (silica, MeOH/CH_2Cl_2 15/85): R_f 0.5; MALDI-TOF MS (positive mode, THAP/diammonium citrate matrix) for $C_{96}H_{95}Cl_2N_9O_{28}Na$ $[M + Na]^+$: calcd 1912.4, found 1912.4.

T#, Building Block *β*. A sample of 5′-amino-5′-deoxythymidine[12a] (20 mg 60 μmol) was dissolved in pyridine (2 mL). The solution was cooled to 0 °C, and DMAP (0.3 mg, 0.3 μmol) was added, followed by addition of a solution of succinic anhydride (6.5 mg, 65 μmol) in pyridine (1 mL). The reaction was followed by TLC (silica, CH_2Cl_2/MeOH/H_2O 69:30:1; R_f product 0.49, R_f educt 0.04). After full conversion of the starting material (1.5 h), the solvent was removed under reduced pressure. The residue was freed of pyridine by coevaporation with toluene and purified via column chromatography (silica, CH_2Cl_2/MeOH/H_2O 69:30:1), yielding 8.7 mg (25 μmol, 44%) of the title compound. NMR analysis indicated that the product contained 1.5 equiv of succinate after column purification. Rather than performing additional rounds of chromatography, succinate was added to the list of building blocks (**v**, Figure 2). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.03 (t, J = 5.6 Hz, 1H); 7.48 (s, 1H); 6.61 (s, 1H); 6.13 (t, J = 7.6 Hz, 1H); 4.15 (m, 1H); 3.73 (m, 1H); 3.31 (m, 2H); 2.43 (m, 2H); 2.35 (m, 2H); 2.08 (m, 2H); 1.81 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.98, 164.27, 151.02, 149.57, 136.73, 110.24, 85.62, 84.34, 71.67, 41.37, 39.04, 30.70, 29.87, 12.58. FAB MS: 342.0 $[M + H]^+$. UV−vis: λ_{max} 263 nm.

C#, Building block α. A solution of 5′-amino-*N*4-benzoyl-2′,5′-dideoxycytidine[10] (50 mg, 116 μmol) in pyridine (3 mL) at 0 °C was treated with DMAP (0.3 mg, 0.3 μmol) and a solution of succinic anhydride (13.9 mg, 139 μmol) in pyridine (0.5 mL). The reaction was monitored by TLC (silica, CH_2Cl_2/MeOH/acetic acid/H_2O 64:35:1:0.5; R_f product 0.44, R_f educt 0.05) and ended after 2 h by evaporating the solvent. After coevaporation with toluene, the residue was purified by column chromatography (silica, CH_2Cl_2/MeOH/H_2O 64:35:1). Yield: 29.1 mg (70 μmol, 60%). ^{1}H NMR (DMSO-d_6): δ 8.22 (d, J = 7.6 Hz, 1H); 8.08 (t, J = 5.9 Hz, 1H); 8.01 (m, 2H); 7.63 (m, 1H); 7.52 (m, 2H); 7.36 (d, J = 7.6 Hz, 1H); 6.12 (t, J = 6.8 Hz, 1H); 5.29 (s, 1H); 4.16 (m, 1H); 3.87 (m, 1H); 3.34 (m, 2H); 2.44 (m, 2H); 2.37 (m, 2H); 2.31 (m, 1H); 2.05 (m, 1H). ^{13}C NMR (DMSO-d_6): δ 174.35, 171.98, 168.01, 163.51, 154.81, 149.94, 145.77, 133.80, 133.25, 129.00, 96.88, 87.01, 86.53, 71.71, 41.46, 30.61, 29.71. FAB MS: 431 $[M + H]^+$. UV−vis: λ_{max} 260 nm (100%), 302 nm (sh, 39%).

Reductive Amination, General Procedure. A sample of 2-methoxybenzaldehyde (**M**, 0.3 mg, 2 μmol) was dissolved in DMF (0.3 mL), and phosphate buffer (pH 8, 0.1 M, 0.1 mL) was added. The mixture was thoroughly vortexed and then added to DNA-bearing CPG (3 mg, 0.1 μmol based on the loading of the first nucleoside), followed by vortexing for another 5 min. $NaB(CN)H_3$ (0.2 mg, 3 μmol) in DMF (0.1 mL) was added. The reaction mixture was left vortexing for 25 min (polypropylene reaction vessel taped down on vortexer). The supernatant was aspired, the CPG was washed with DMF (2 × 1 mL) and methanol (2 × 1 mL), and dried under high vacuum. A small portion (10−20 beads) of CPG was treated with concentrated ammonia solution for 2 h at 55 °C to analyze for complete conversion. This solution was analyzed by MALDI-TOF MS after evaporation and rehydration. The conversion of the starting material was found to be greater than 90%. MALDI-TOF MS for $C_{86}H_{107}N_{31}O_{47}P_7$ $[M - H]^-$: calcd 2543.4, found 2546.1. The bulk of the sample was used for acylation with a carboxylic acid building block without modification. 2,5-Dimethoxybenzaldehyde (**D**) derivatives were obtained using an identical procedure. Derivatives of aldehydes **P** and **H** were obtained under similar conditions, except that the reductive aminations were performed in a mixture of DMF/MeOH (1:3, 0.3 mL) and NaOAc/HOAc buffer (pH 6, 0.05 M, 0.1 mL). MALDI-TOF MS for an analytical sample of C*GGTTGAC alkylated with **P** $C_{85}H_{105}N_{31}O_{46}P_7$ $[M - H]^-$: calcd 2514.4, found 2513.7.

Synthesis of Acylamidooligonucleotides, General Procedure. The coupling protocol is similar to one reported

earlier.[12a,12b,9] A mixture of the carboxylic acid (100 μmol), HBTU (34.1 mg, 90 μmol), and HOBT (15.3 mg, 100 μmol) was prepared in a polypropylene reaction vessel (1.6 mL capacity) and dried at 0.1 Torr for 30 min. The mixture was dissolved in DMF (600 μL), treated with DIEA (40 μL, 234 μmol), vortexed, and immediately introduced to the reaction vessel containing the DNA bearing-CPG (**4**, 2−5 mg). The resulting slurry was vortexed once after addition of the coupling mixture and then every 5 min during the coupling time. The reaction was usually allowed to proceed for 40 min, unless Fmoc-protected chiral amino acids were used, in which case the reaction time was 20 min. At the end of the reaction time, the supernatant was aspired with a syringe, using a small gauge needle, and the CPG was washed with DMF (2 × 1.5 mL) and MeOH (2 × 1.5 mL), followed by drying at 0.1 Torr. For Fmoc-protected building blocks synthesized in-house, the following coupling mixtures were employed: $Fmoc_2$-vancomycin (ψ, 2.6 μmol), HBTU (2.4 μmol), HOBT (2.6 μmol), DIEA (5.8 μmol), CPG (3 mg, 0.1 μmol loading for the first 3′-terminal nucleotide); Fmoc-lomefloxacin (61 μmol), HBTU (55 μmol), HOBT (61 μmol), DIEA (134 μmol), CPG (3 mg, 0.1 μmol loading for the first 3′-terminal nucleotide); Fmoc-pipemidic acid (57 μmol), HBTU (51 μmol), HOBT (57 μmol), DIEA (125 μmol), CPG (3 mg, 0.1 μmol loading for the first 3′-terminal nucleotide).

Analytical data for oligonucleotides that were HPLC purified:

Ac-C*GGTTGAC. Yield: 42%. HPLC: 0% B for 5 min, in 40 min to 20% B, in 7 min to 90% B, 90% B for 10 min, R_t = 27.5 min. MALDI-TOF MS for $C_{80}H_{102}N_{31}O_{47}P_7$ [M − H]$^-$: calcd 2465.5, found 2466.6.

AQ-6 (**AQ**-C*GGTTGAC). Yield: 35%. HPLC: 0% B for 5 min, in 35 min to 25% B, in 10 min to 100% B, 100% B for 10 min, R_t = 34.6 min. MALDI-TOF MS for $C_{93}H_{105}N_{31}O_{49}P_7$ [M − H]$^-$: calcd 2656.5, found 2658.7.

OF-6 (**OF**-C*GGTTGAC). Yield: 52%. HPLC: 0% B for 5 min, in 35 min to 25% B, in 10 min to 100% B, 100% B for 10 min, R_t = 38 min. MALDI-TOF MS for $C_{96}H_{117}FN_{34}O_{49}P_7$ [M − H]$^-$: calcd 2765.6, found 2766.6.

NO-6 (**NO**-C*GGTTGAC). Yield: 16%. HPLC: 0% B for 5 min, in 25 min to 20% B, in 10 min to 100% B, 100% B for 10 min, R_t = 29 min. MALDI-TOF MS for $C_{90}H_{107}N_{31}O_{48}P_7$ [M − H]$^-$: calcd 2606.5, found 2609.9.

Chl-6 (**Chl**-C*GGTTGAC). Yield: 14%. HPLC: 0% B for 5 min, in 25 min to 20% B, in 10 min to 100% B, 100% B for 10 min, R_t = 38 min. MALDI-TOF MS for $C_{102}H_{137}N_{31}O_{50}P_7$ [M − H]$^-$: calcd 2812.7, found 2814.9.

OA-6 (**OA**-C*GGTTGAC). Yield: 22%. HPLC: 0% B for 5 min, in 25 min to 25% B, in 10 min to 90% B, 90% B for 20 min, R_t = 59 min. MALDI-TOF MS for $C_{91}H_{108}N_{32}O_{50}P_7$ [M − H]$^-$: calcd 2665.5, found 2667.1.

LO-6 (**LO**-C*GGTTGAC). Yield: 23%. HPLC: 0% B for 5 min, in 35 min to 25% B, in 10 min to 100% B, 100% B for 10 min, R_t = 31.9 min. MALDI-TOF MS for $C_{95}H_{116}F_2N_{34}O_{48}P_7$ [M − H]$^-$: calcd 2755.6, found 2755.8.

PM-6 (**PM**-C*GGTTGAC). Yield: 28%. HPLC: 0% B for 5 min, in 35 min to 25% B, in 10 min to 100% B, 100% B for 10 min, R_t = 29.8 min. MALDI-TOF MS for $C_{92}H_{114}N_{36}O_{48}P_7$ [M − H]$^-$: calcd 2707.6, found 2707.8.

Van-6 (**Van**-C*GGTTGAC). Yield: 5%. HPLC: 0% B for 5 min, in 35 min to 25% B, in 10 min to 100% B, 100% B for 10 min, R_t = 35.2 min. MALDI-TOF MS for $C_{144}H_{172}Cl_2N_{40}O_{69}P_7$ [M − H]$^-$: calcd 3851.9, found 3853.4.

CI-6 (**CI**-C*GGTTGAC). Yield: 64%. HPLC: 0% B for 5 min, in 25 min to 25% B, in 10 min to 100% B, 100% B for 10 min, R_t = 32.5 min. MALDI-TOF MS for $C_{90}H_{107}N_{33}O_{50}P_7$ [M − H]$^-$: calcd 2666.5, found 2665.6.

SU-6 (**SU**-C*GGTTGAC). Yield: 72%. HPLC: 0% B for 5 min, in 30 min to 25% B, in 10 min to 100% B, 100% B for 10 min, R_t = 34.6 min. MALDI-TOF MS for $C_{92}H_{116}N_{34}O_{52}P_7$ [M − H]$^-$: calcd 2745.6, found 2743.4.

THis-6 (**THis**-C*GGTTGAC). Yield: 72%. HPLC: 0% B for 5 min, in 25 min to 25% B, in 10 min to 100% B, 100% B for 10 min, R_t = 42.0 min. MALDI-TOF MS for $C_{103}H_{123}N_{34}O_{47}P_7$ [M − H]$^-$: calcd 2804.6, found 2804.7.

Chl-(**P**)-**9** (**Chl**-(**P**)-C*GGTTGAC). Yield: 4%. HPLC: 0% B for 5 min, in 35 min to 25% B, in 10 min to 90% B, 90% B for 10 min, R_t = 48.5 min. MALDI-TOF MS for $C_{109}H_{143}N_{31}O_{50}P_7$ [M − H]$^-$: calcd 2902.8, found 2906.1.

Chl-(**D**)-**9** (**Chl**-(**D**)-C*GGTTGAC). Yield: 25%. HPLC: 0% B for 5 min, in 25 min to 25% B, in 10 min to 95% B, 95% B for 10 min, R_t = 42 min. MALDI-TOF MS for $C_{111}H_{147}N_{31}O52P_7$ [M − H]$^-$: calcd 2962.8, found 2962.9.

H-(**P**)$_2$-**9** (H-(**P**)$_2$-C*GGTTGAC). Yield: 6%. HPLC: 0% B for 5 min, in 35 min to 25% B, in 10 min to 90% B, 90% B for 10 min, R_t = 41 min. MALDI-TOF MS for $C_{92}H_{111}N_{31}O_{46}P_7$ [M − H]$^-$: calcd 2602.5, found 2604.8.

OA-Asp-11 (**OA-Asp**-C*GGTTGAC). Yield: 75%. HPLC: 0% B for 5 min, in 25 min to 25% B, in 10 min to 100% B, 100% B for 10 min, R_t = 27.6 min. MALDI-TOF MS for $C_{95}H_{113}N_{33}O_{52}P_7$ [M − H]$^-$: calcd 2764.5, found 2763.0.

Chl-Asp-11 (**Chl-Asp**-C*GGTTGAC). Yield: 15%. HPLC: 0% B for 5 min, in 35 min to 25% B, in 10 min to 90% B, 90% B for 10 min, R_t = 38 min. MALDI-TOF MS for $C_{106}H_{142}N_{32}O_{53}P_7$ [M − H]$^-$: calcd 2927.8, found 2928.4.

Chl-Trp-11 (**Chl-Trp**-C*GGTTGAC). Yield: 12%. HPLC: 0% B for 5 min, in 30 min to 20% B, in 10 min 50%, in 5 min to 90% B, 90% B for 10 min, R_t = 45 min. MALDI-TOF MS for $C_{113}H_{147}N_{33}O_{51}P_7$ [M − H]$^-$: calcd 2998.8, found 2999.9.

Ac-C*GTACG. Yield: 42%. HPLC: 0% B for 5 min, in 35 min to 20% B, in 10 min to 100% B, 100% B for 10 min, R_t = 33.2 min. MALDI-TOF MS for $C_{60}H_{77}N_{24}O_{34}P_5$ [M − H]$^-$: calcd 1832.4, found 1829.1.

Compound 14 (**Chl**-C*GTACG). Yield: 42%. HPLC: 0% B for 5 min, in 25 min to 25% B, in 10 min to 100% B, 100% B for 10 min, R_t = 37.9 min. MALDI-TOF MS for $C_{82}H_{112}N_{24}O_{37}P_5$ [M − H]$^-$: calcd 2179.6, found 2180.6.

Ac-C*TTTTAAAAG. Yield: 31%. HPLC: 0% B for 5 min, in 35 min to 20% B, in 10 min to 100% B, 100% B for 10 min, R_t = 35.0 min. MALDI-TOF MS for $C_{101}H_{128}N_{37}O_{58}P_9$ [M − H]$^-$: calcd 3065.6, found 3065.4.

Compound 15 (**Chl**-C*TTTTAAAAG). Yield: 57%. HPLC: 0% B for 5 min, in 25 min to 25% B, in 10 min to

100% B, 100% B for 10 min, $R_t = 34.6$ min. MALDI-TOF MS for $C_{123}H_{163}N_{37}O_{61}P_9$ $[M - H]^-$: calcd 3412.8, found 3411.6.

Nuclease Selection. An aliquot (2 μL) of a solution containing each of the compounds to be assayed (2 pmol/μL each, prepared by titrating compounds under MALDI control) was transferred to a polypropylene reaction vessel. To this were added aqueous solutions of ammonium acetate (1 μL, 0.5 M, pH 7) and target strand GTCAACCG (0.5 μL, 10 pmol/μL, 5 pmol). The solution was heated to 65 °C for 5 min, then cooled to 0 °C, at which temperature it was kept for 5 min and finally centrifuged for 1 min at 6000 rpm to collect residual fluid lost due to condensation. An aliquot of the mixture (0.5 μL) was removed for a control spectrum, followed by addition of a solution of snake venom phosphodiesterase (phosphodiesterase I, EC 3.1.4.1, 0.5 μL, 1 μU/μL). The reaction solution was vortexed briefly and placed in an ice bath. Aliquots (0.5 μL) were removed at 5, 30, 45, 75, 180, and 680 min and mixed with the matrix solution on the MALDI target. The matrix solution (0.5 μL) contained the DNA sequence T_{11} (1.3 pmol/μL) or GAAAA-GAAAA (6.6 pmol/μL) in a mixture of 0.1 M aqueous diammonium citrate/saturated 6-aza-2-thiothymine in acetonitrile (1:2). The spotted liquid of analyte sample/matrix solution was mixed via pipetting and then dried under reduced pressure (approximately 0.5 Torr), resulting in a semicrystalline residue. The target was kept under reduced pressure during the time when samples were not being spotted. After the last time point of an assay, the MALDI target was introduced to the spectrometer, and four spectra were acquired per time point in linear, negative mode at high laser power. Spectra with less than 1000 ion counts for the peak of the internal standard were discarded.

UV-Melting Experiments. UV-melting experiments were performed with DNA solutions in 10 mM phosphate buffer, pH 7, and different NaCl concentrations (0, 150, and 1000 mM). Melting and cooling profiles were recorded on a Perkin-Elmer Lambda 10 UV−visible spectrophotometer by measuring changes in absorbance at 260 nm in 1 cm path length microcuvetts at a heating or cooling rate of 1 °C per min. Prior to aquisition of the melting curves, samples were annealed by heating to 90 °C and cooling to 5 °C at a rate of 2 °C per min. Melting temperatures were determined with UV Winlab 2.0 as the extrema of the first derivative of the 91 point smoothed melting curves. Melting points reported are the average of two heating and two cooling curves.

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Supporting Information Available. MALDI-TOF mass spectra of 23 oligonucleotide derivatives that were HPLC purified, MALDI-TOF mass spectra of building blocks ψ, ρ, and π, NMR spectra of building blocks α and β, calibration plots for the detection of three oligonucleotide hybrids via MALDI-TOF mass spectrometry, and a table with thermodynamic parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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