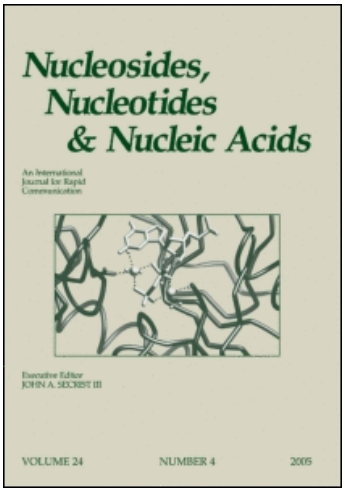

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A New Type of Fluorescence Labeling of Nucleosides, Nucleotides and Oligonucleotides

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A NEW TYPE OF FLUORESCENCE LABELING OF NUCLEOSIDES, NUCLEOTIDES AND OLIGONUCLEOTIDES

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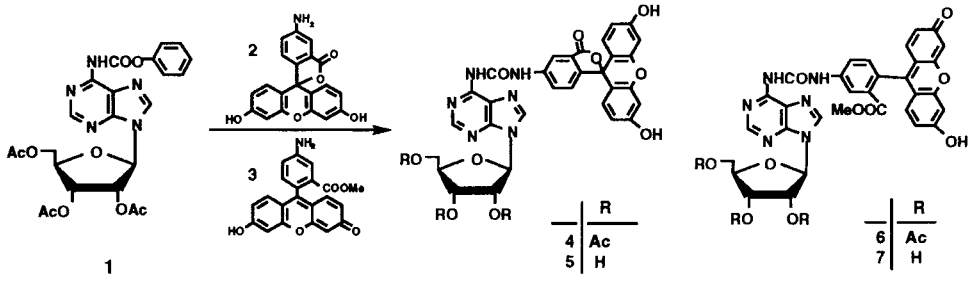
Abstract. Fluorescein has been coupled to the amino groups of the common nucleosides via a carbamoyl spacer to form a new type of conjugates. The corresponding phosphoramidites have been prepared with Npe and Npeoc protecting groups for application in oligonucleotide synthesis. Hybridizations have been studied in dependence of the fluorescing label as well as fluorescence quantum yields and fluorescence anisotropy effects.

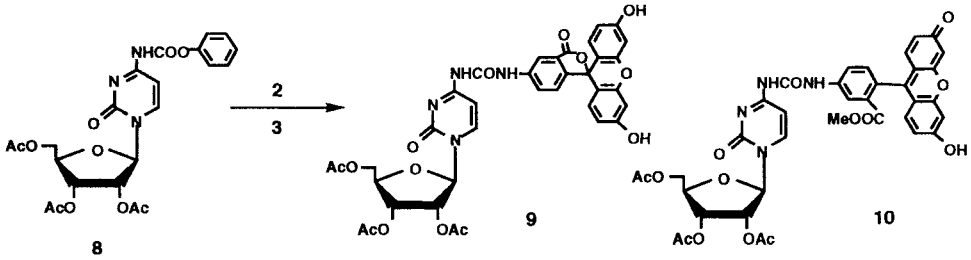
Introduction. DNA-sequencing and DNA-probing afford effective labeling which started by the use of radioactive isotopes[1,2] and has in recent years changed more and more towards fluorescent and biochemical markers [3-5], respectively, from obvious reasons. Also, indirect methods using the proteins avidin and streptavidin in combination with biotinylated alkaline phosphatase or oligonucleotide-digoxigenin-conjugates detectable by specific antibodies modified to induced color reactions have been applied successfully[6-9]. These and all the analogous methods have in common that the label is attached via a spacer either to the 3'- or 5'-end of an oligonucleotide,[10-12] the internucleotidic phosphate function,[13-15] the ribose moiety[16,17] or the nucleobase,[18-21] respectively. The most common fluorescent markers are the dansyl[20], pyrenyl[22], psoralenyl[23] and fluoresceinyl group[21]. Our intension was the direct coupling of the fluorescein molecule onto the amino functions of the adenine, guanine and cytosine moiety via a carbamoyl bridge in order to bring the fluorophor in close proximity to the nucleobases. We have shown recently[24] that urethane derivatives of adenosine can easily been converted into urea derivatives by nucleophilic displacement reactions applying aliphatic and aromatic amines, respectively. Analogously, we coupled 5-aminofluorescein with activated urethanes of the common nucleosides to obtain a new type of fluorescent conjugates which can be used in form of their phosphoramidites as monomeric building blocks in oligonucleotide synthesis.

Synthesis. 5-Aminofluorescein (**2**) was synthesized according to literature[25-27] whereby the reduction of 5-nitro- to 5-aminofluorescein was best achieved by a sodium

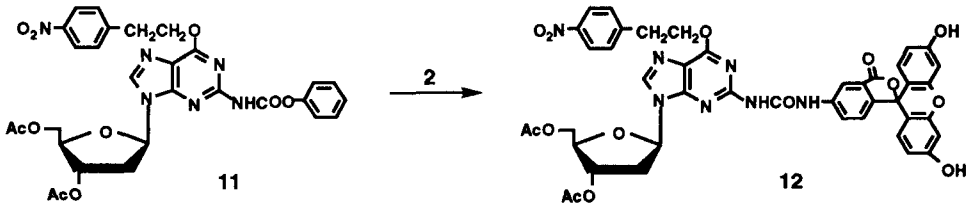
sulfide / sodium hydrosulfide mixture[28,29] and conversion of **2** into 5-aminofluorescein methylester (**3**) resulted from boiling in MeOH / H_2SO_4. Coupling of N^6-phenoxy-carbonyl-2',3',5-tri-O-acetyladenosine (**1**) with **2** and **3**, respectively, in pyridine at 70°C formed the dyestuff-conjugates **4** and **6** in excellent yields and mild deprotection at the sugar moiety by K_2CO_3 / MeOH led to **5** and **7**, respectively.



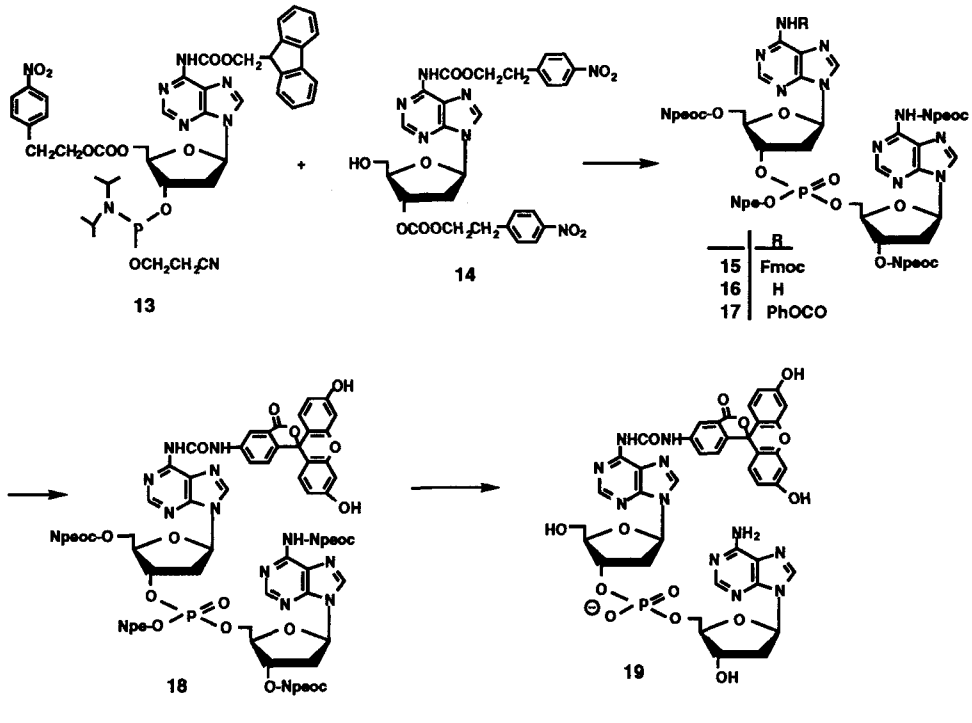
In a similar manner 2',3',5'-tri-O-acetylcytidine was first treated with phenoxy-carbonyltetrazolide to form N^4-phenoxycarbonyl-2',3',5',-tri-O-acetylcytidine (**8**) and then reacted with **2** and **3**, respectively to give the corresponding conjugates **9** and **10**.



In the guanosine series a more complex blocking group strategy has to be employed in order to account for the low solubility of many of those derivatives in organic solvents. O^6-2-(4-nitrophenyl)ethyl-3',5'-di-O-acetyl-2'-deoxyguanosine was chosen as starting material which reacted with phenyl chloroformate at 0-25°C to the N^2-phenoxycarbonyl derivative **11** and gave subsequently on treatment with **2** the fully blocked dyestuff-conjugate **12** showing that the various amino functions in the common nucleosides can be modified in the same manner. The conjugates have been characterized by UV and NMR spectra, elemental analyses as well as pKa measurements offering a clear picture of the various equilibria between the different molecular forms including di- and monocations, neutral species, mono- and dianions in aequeous solution.



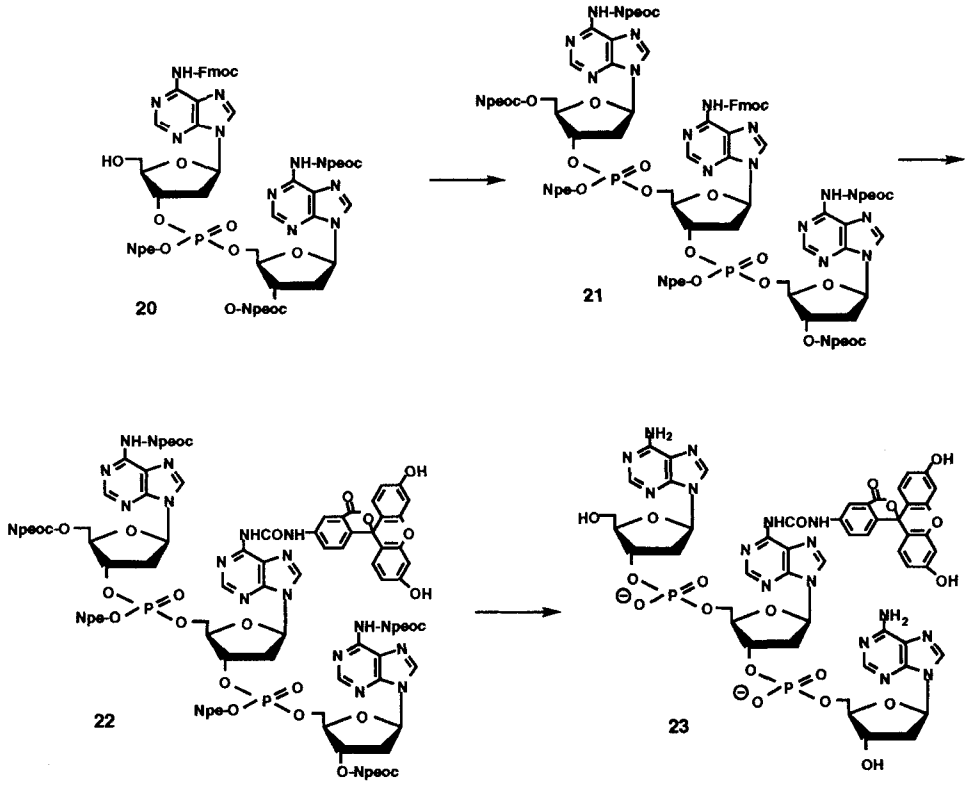
The easy coupling of 5-aminofluorescein with the phenylurethane function encouraged us to try this type of modification also on oligonucleotides by solution chemistry. In order to achieve this goal a special blocking chemistry based upon the relatively stable p-nitrophenylethyl (Npe) and p-nitrophenylethoxycarbonyl (Npeoc) group[30] has to be applied. Starting from 2'-deoxyadenosine 5'-O-p-nitrophenylethoxycarbonyl-N^6-fluorenylmethoxycarbonyl-2'-deoxyadenosine-3'-O-(p-nitrophenylethyl, N-diisopropyl)-phosphoramidite (**13**) and N^6,3'-O-bis-p-nitrophenylethoxycarbonyl-2'-deoxyadenosine (**14**) have been synthesized and condensed in the usual manner by phosphoramidite chemistry to the fully protected dimer (**15**). The Fmoc group can selectively be removed to form **16** under very mild conditions using triethylamine which does not harm the other blocking groups.



Treatment with phenoxycarbonyltetrazolide afforded modification of the free amino group to the corresponding urethane **17** which reacted with 5-aminofluorescein (**2**) in

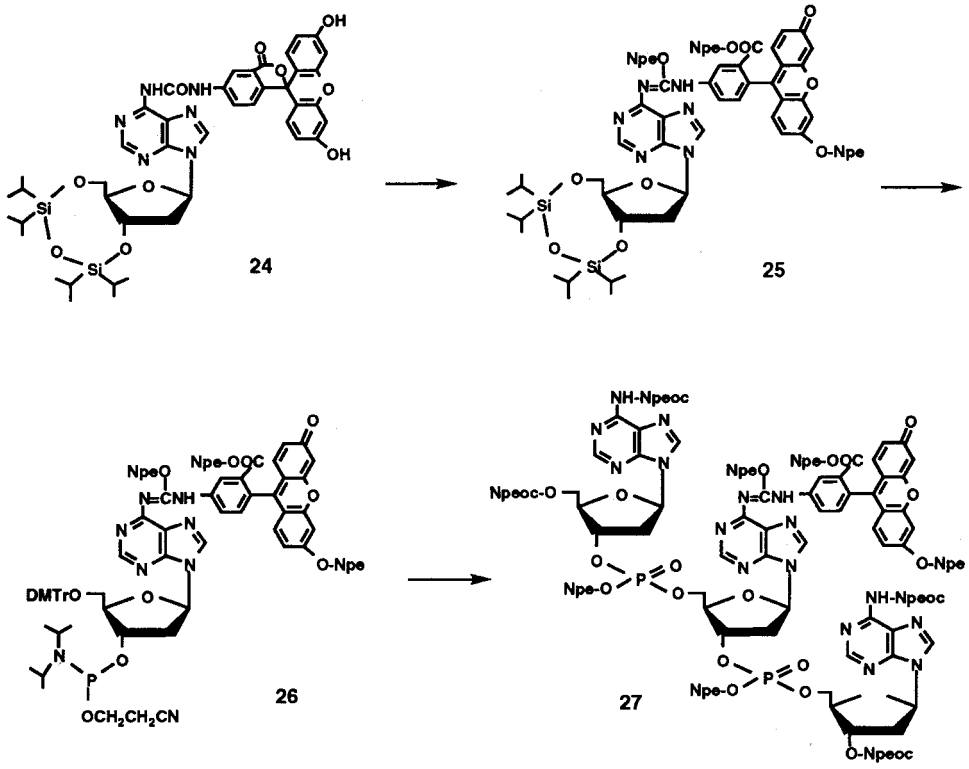
pyridine at 70°C in 68% isolated yield to the conjugate **18**. Final deprotection can then be performed by a β-elimination process using DBU in pyridine to remove quantitatively the Npe and Npeoc groups in one step yielding **19** after DEAE-sephadex chromatotography.

In a similar manner the partially deprotected dimer **20** was further condensed with N^6,5'-O-bis-p-nitrophenylethoxycarbonyl-2'-deoxyadenosine-3'-O-(p-nitrophenylethyl, N-diisopropyl)phosphoramidite to the trimer **21** which was then stepwise analogously modified at the middle 2'-deoxyadenosine moiety by the 5-aminofluorescein residue yielding **22**. Total deprotection by DBU treatment afforded the free fluorescein-labeled trimer **23**.

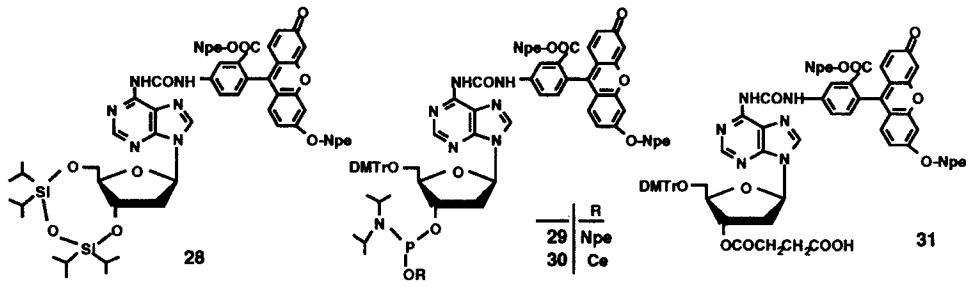


The next approach was dealing with the modification of the fluorescein moiety already attached to 2'-deoxyadenosine which was first protected by the Markiewicz reagent, then treated by phenoxycarbonyl tetrazolide and finally reacted with 5-amino-fluorescein (**2**) to form **24**. Treatment of compound **24** with excess of 2-(4-nitrophenyl)-ethanol in a Mitsunobu reaction afforded the introduction of three Npe groups which were localised by NMR spectroscopy at the xanthenyl, carboxyl- anf the urea functions forming the fully protected 2'-deoxyadenosine-conjugate **25**. The silyl group can be removed

selectively by tetrabutylammonium fluoride in presence of some AcOH and subsequent dimethoxytritylation and phosphitylation led to the new phosphoramidte **26** which was used in solution synthesis of oligonucleotides of type **27**. During deblocking studies with DBU, however, was noticed that all Npe and Npeoc group except the isourea protection were cleaved within the normal range of 2-4 hours whereas the latter one afforded at least 48 hours for complete removal. Obviously **26** is therefore not recommended as a monomeric building block in solid-support oligonucleotide synthesis due to its high chemical stability in the deprotection step.



Fortunately, compound **24** can also be protected selectively only at the fluorescein moiety using 2.1 - 2.3 equivalents of the components in the Mitsunobu reaction leading to the nucleoside **28** in 64% isolated yield. Conversion of **28** into the phosphoramidets **29** and **30**, respectively, was again achieved by desilylation, dimethoxytritylation at 5'-OH and phosphitylation at the 3'-OH group each step working in excellent yield. Furthermore, the 3'-OH function was also reacted with succinic anhydride to give the corresponding succinate **31** which was used for anchoring the first nucleoside onto the solid-support in the phosphoramidite approach towards synthetic oligonucleotides.



The Npe-protection of the fluorescein moiety turned out to be of excellent quality since solution and solid-support syntheses led to homogenous products which show in general high purity according to HPLC even in their isolated crude forms. Various oligodeoxyribonucleotides were assembled in a DNA-synthesizer with the adenosine-label at different positions in order to study the influence of the large fluorescein residue on the hybridization with the unmodified complementary oligomer strand. The following sequences (Table 1) have been synthesized and their melting temperatures on duplex formation compared in presence of 0.12 M NaCl. As expected hybridization between sequence 2 on one side and sequence 3, 5 and 6, respectively, on the other side is not much disturbed by the additional terminal adenosine-fluorescein-conjugate whereas introduction of the label in the chain (4 + 2) causes a strong decrease of T_m to 45.7°C in comparison to the unmodified duplex (1 +2) showing 59.3°C. Combinations of sequence 3 with the complementary strands (7,8,9) modified at the 3'- and 5'-end by different lengths of oligo-T sequences are relatively stable and differ from the model duplex by only 5 degrees.

An extension of our investigations was achieved by various modifications of the 5-aminofluorescein molecule to use it directly as a reagent of broader application. First 5-aminofluorescein (2) was treated with 2-(4-nitrophenyl)ethanol, triphenylphosphine and ethyl azodicarboxylate in a Mitsunobu reaction in dioxan to give 2-(4-nitrophenyl)ethyl 5-amino-2-{6-[-(4-nitrophenyl)ethoxy]-3-oxo-3H-xanthen-9-yl}-benzoate (**32**) in 88% yield and second, its reaction with phenyl chloroformate in pyridine and 4-dimethylaminopyridine as a catalyst yielded 93% of the corresponding activated urethane **33**.

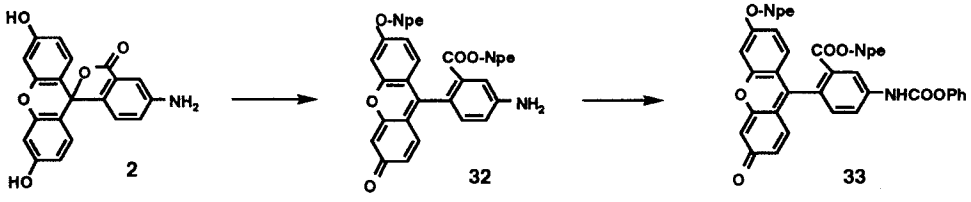
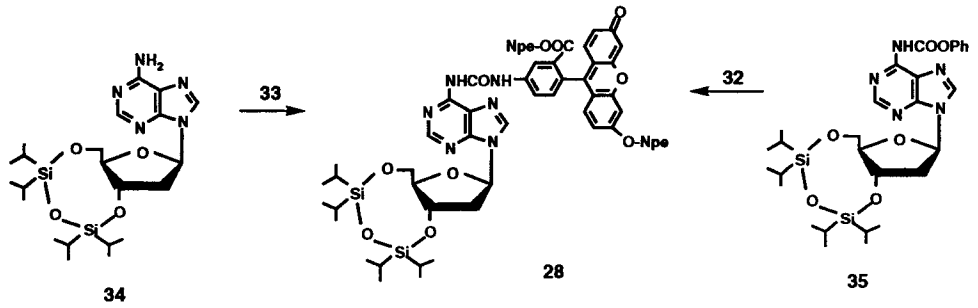


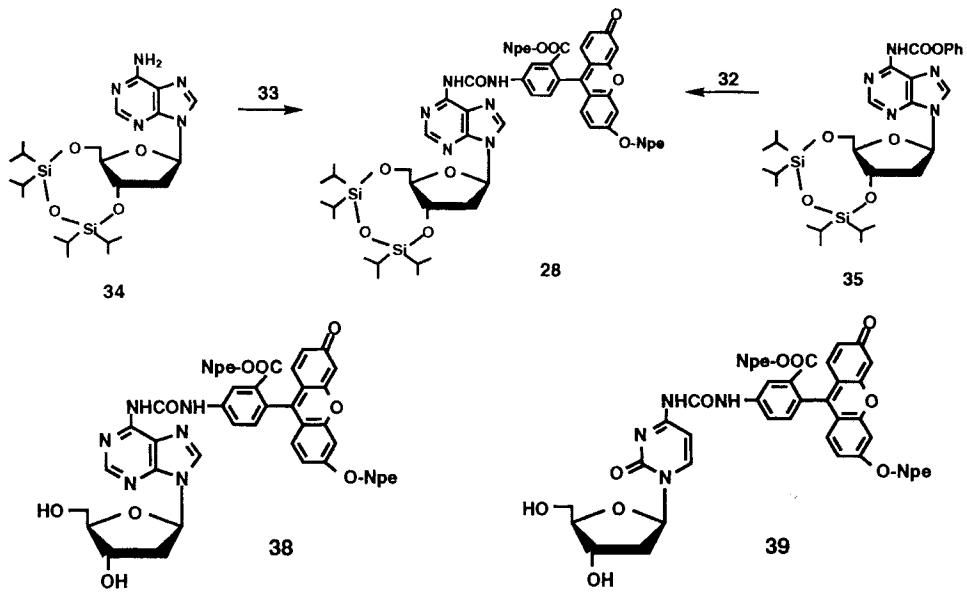
Table 1. Melting temperatures of modified oligonucleotide duplexes

No.	Sequence	Combination	T_m °C
1	5'-d(TCC CAG TCA CGA CGT)-3'	1 + 2	59.3
2	5'-d(ACG TCG TGA CTG GGA)-3'		
3	5'-d(**A** TCC CAG TCA CGA CGT)-3'	3 + 2	58.5
4	5'-d(TCC CAG TCA **C**GA CGT)-3'	4 + 2	45.7
5	5'-d(TCC CAG TCA CGA CGT **A**)-3'	5 + 2	57.6
6	5'-d(**A** TCC CAG TCA CGA CGT **A**)-3'	6 + 2	59.8
		6 + 7	55.0
7	5'-d(T_9 ACG TCG TGA CTG GGA T_9)-3'	3 + 7	54.6
8	5'-d(T_{20} ACG TCG TGA CTG GGA T_{20})-3'	3 + 8	54.3
9	5'-d(T_{30} ACG TCG TGA CTG GGA T_{30})-3'	3 + 8	54.0

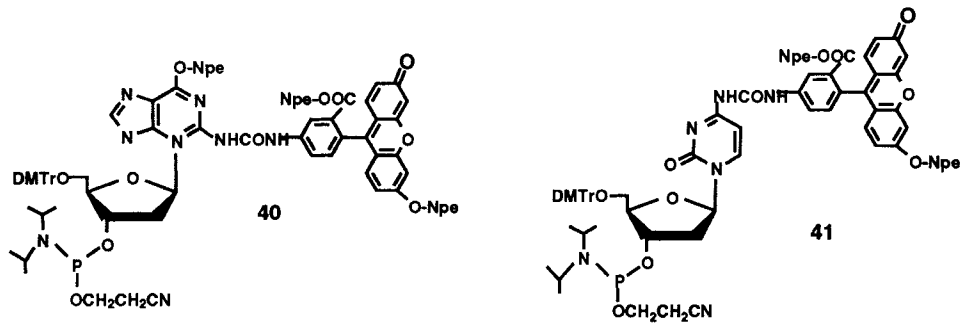
Both reagents **32** and **33**, respectively, can be used to modify nucleosides at their amino functions. Compound **28** can now be synthesized directly either from 2'-deoxy-3',5'-O-(tetraisopropyldisiloxan-1,3-diyl)adeosine (**34**) with **33** or from the carbamate **35** by reaction with **32** giving excellent yields over 90%.



In a similar fashion reacted O^6-[2-(4-nitrophenyl)ethyl-3',5'-O-(tetraisopropyldisila-oxan-1,3-diyl)-2'-deoxyguanosine (**36**) to give **37** in 91% yield and 2'-deoxyadenosine and 2'-deoxycytidine, respectively, could also been converted into their fluorescein-conjugates using the transient protection method of Jones[31] and followed by acylation with **33** leading on normal work-up to the urea derivatives **38** and **39**.



The base modified 2'-deoxyribonucleosides **38** and **39** as well as the corresponding 2'-deoxyguanosine resulting from desilylation of **37** have then been transformed into the fully protected phosphoramidites **30**, **40** and **41**, respectively, by dimethoxytritylation and phosphitylation in the usual manner to give valuable monomeric building blocks for general oligonucleotide synthesis.



The availability of the three phosphoramidites **30**, **40** and **41** enabled us to synthesize a series of fluorescein-label oligonucleotides of which the hybridization properties with the unmodified complementary strand offered some first indications about the magnitude of disturbance on duplex formation.

The few hybridization experiments indicate already clearly that there is a striking influence on the melting temperature in dependence of the nature of the labeled nucleoside

Table 2. Melting temperatures of oligonucleotide duplexes

No	Sequence	T_m °C
1	3'-d(TTT CCC TTG TTT TCG ACC CAT)-5'	
2	5'-d(AAA GGG AAC AAA AGC TGG GTA)-3'	60.5
3	5'-d(**A**AA GGG AAC AAA AGC TGG GTA)-3'	57.0
4	5'-d(AAA GGG AAC AAA AGC TGG GT**A**(-3'	58.7
5	5'-d(AAA GGG AAC **A**AA AGC TGG GTA)-3'	33.0
6	5'-d(AAA GGG AA**C** AAA AGC TGG GTA)-3'	52.0
7	5'-d(AAA GGG AAC AAA AG**C** TGG GTA)-3'	55.7
8	5'-d(**AAA** GGG AAC AAA AGC TGG GTA)-3'	56.7
9	5'-d(AAA **GGG** AAC AAA AGC TGG GTA)-3'	54.9
10	5'-d(AAA **GGG** AAC AAA A**G**C TGG GTA)-3'	50.9
11	5'-d(AAA GGG AA**C** AAA AG**C** TGG GTA)-3'	47.8
12	5'-d(AAA GGG **A**AC AAA **A**GC TGG GT**A**)-3'	29.9

Measured in phosphate buffer pH = 8.0 ; conc = 0.12 M NaCl. Bold letters = fluorescein labeled.

and especially by its location in the oligonucleotide chain. The introduction of the fluorescein-label at the 3'- or 5'-end does not lower much the T_m but putting the labeled nucleoside in the middle like in sequence 5 causes a tremendous decrease of almost 30 degrees. However, it is interesting to note that labeling of the adjacent **C**-(sequence 6) or the more distant **G**-position (sequence 7) show only a minor effect as does also multiple labeling of neigboring **A**- (sequence 8) or **G**-units (sequence 9). More studies have to be performed to unravel the individual effects regarding site and sequence dependence.

We, furthermore, determined the fluorescence quantum yields Θ and the fluorescence anisotropy $\mathbf{r}$[32] to get more detailed information about fluorescence properties of labeled oligonucleotides single- and double-stranded (Table 3). The fluorescence quantum yields of the labeled nucleosides (1-3) and short oligonucleotide trimers (4-6) and pentamers (7-10) as well as monolabeled oligomers (11-15) differ not much from fluorescein itself but adjacent labeling causes a strong quenching effect (16,17) which is partially compensated by an isolated labeled unit (18-20).

Table 3. Fluorescence quantum yields and fluorescence anisotropies of nucleosides and oligonucleotides

No	Sequence	Fluorescence quantum yield Θ single	duplex	Fluoresecence anisotropy r single	duplex
	Fluorescein	1.00		0.008	
1	dC^{Flu}	0.99		0.012	
2	dA^{Flu}	1.00		0.011	
3	dG^{Flu}	0.92		0.025	
4	$d(CC^{Flu}C)$	0.98		0.019	
5	$d(AA^{Flu}A)$	1.00		0.020	
6	$d(GG^{Flu}G)$	0.80		0.038	
7	$TTT\,TdC^{Flu}$	0.81		0.037	
8	$TTT\,TdA^{Flu}$	0.97		0.025	
9	$TTT\,TdG^{Flu}$	0.87		0.044	
10	$dA^{Flu}T\,TTT$	0.96		0.026	
11	d(AAA GGG AAC AAA AGC TGG GTA)	0.80	0.82	0.095	0.105
12	d(AAA GGG AAC AAA AGC TGG GTA)	0.78	0.87	0.057	0.096
13	d(AAA GGG AAC AAA AGC TGG GTA)	0.95	0.84	0.062	0.089
14	d(AAA GGG AAC AAA AGC TGG GTA)	0.92	0.80	0.060	0.086
15	d(AAA GGG AAC AAA AGC TGG GTA)	0.89	0.87	0.115	0.122
16	d(AAA GGG AAC AAA AGC TGG GTA)	0.05	0.05	0.05	0.06
17	d(AAA GGG AAC AAA AGC TGG GTA)	0.05	0.06	0.09	0.10
18	d(AAA GGG AAC AAA AGC TGG GTA)	0.35	0.52	0.096	0.108
19	d(AAA GGG AAC AAA AGC TGG GTA)	0.53	0.67	0.068	0.088
20	d(AAA GGG AAC AAA AGC TGG GTA)	0.32	0.43	0.054	0.081

Phosphate buffer pH 8; conc. (Na^+) = 0.12 M; excitation at 493 nm; determintion of the fluorescence anisotropy **r** at 515 nm.

The fluorescence anisotropy is also depending on the site and number of the fluorescence label and indicates that the substantial increase of the signal on duplex formation can be used for detection and probing of oligonucleotides and DNA sequences.

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