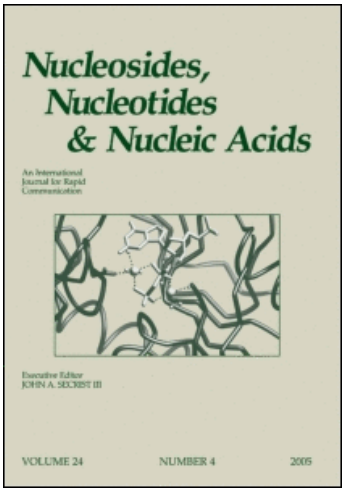

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Pteridine Nucleosides—New Versatile Building Blocks in Oligonucleotide Synthesis

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PTERIDINE NUCLEOSIDES - NEW VERSATILE BUILDING BLOCKS IN OLIGONUCLEOTIDE SYNTHESIS

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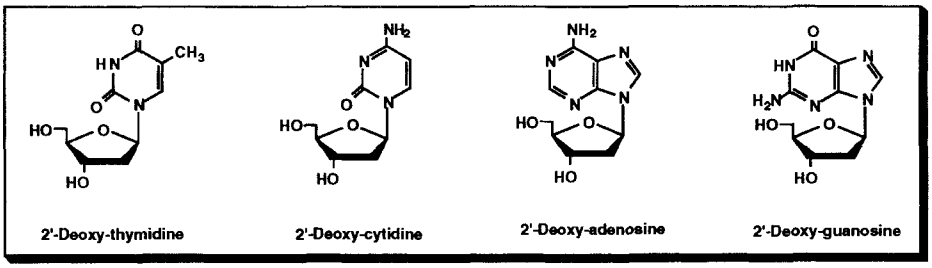
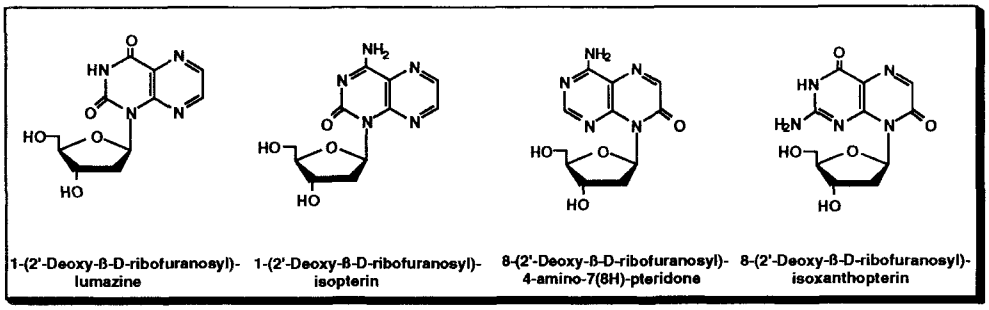
Abstract. Chemical syntheses of 1-(2-deoxy-β-D-ribofuranosyl)lumazines and isopterins as well as 8-(2-deoxy-β-D-ribofuranosyl)-4-amino-7(8H)pteridones and -isoxanthopterins have been developed to make the structural analogs of the naturally occurring 2'-deoxyribonucleosides in the pteridine series available. The corresponding phosphoramidites have been used in machine-aided solid-support syntheses leading to new types of fluorescence labeled oligonucleotides. The effects of the various fluorophors on duplex formation and as labels for enzyme reactions is demonstrated.

Introduction

Pteridine-N-1 and -N-8-nucleosides show a close structural relationship to the common naturally occurring pyrimidine and purine nucleosides and can therefore be regarded as potential substitutes in synthetic oligonucleotides. After synthesizing a large number of pteridine ribonucleosides in the past[1-13] we recently concentrated more on new synthetic approaches leading to the corresponding pteridine 2'-deoxyribonucleosides[14]. Due to substantial differences, however, in the chemistry between the pyrimidine/ purine and the pteridine ringsystems chemical reactivities and especially physical properties created problems and afforded new methodologies from a synthetic point of view. Much efforts were put into the regio- and stereoselective glycosylation step leading finally in most cases with 1-chloro-1,2-dideoxy-3,5-di-O-acyl-D-ribofuranoses in a highly stereospecific manner to pteridine-2'-deoxy-β-D-ribofuranosides in good yields. Tedious chromatographical separations of anomeric mixtures could be avoided and speeded up the accessibility of the

new building blocks. A striking feature of the pteridines is their strong fluorescence which can be applied for labelling experiments in biochemistry and molecular biology. Only





recently these fluorescence properties have been considered as an alternative possibility to label oligonucleotides at various sites of the chain by using pteridine phosphoramidites in a solid-support approach.

Results and Discussion

Glycosylation reactions of lumazine and its 6,7-diphenyl, benzo[g], naphtho[g] and anthra[g] derivatives worked best in a Hilbert-Johnson-Birkofer approach reacting the silylated heterocycle with 3,5-di-O-p-toluoyl-α-D-ribofuranosyl chloride in $CHCl_3$ to give in a quaternization process always the β-anomer in excess. The various 1-(2-deoxy-β-D-ribofuranosyl)lumazines have then been converted by known procedures in the corresponding 5'-O-dimethoxytrityl-3'-O-(β-cyanoethyl, N-diisopropyl)phosphoramidites which have then been applied for the synthesis of oligonuceotides. The sequence of a self-complementary 18-mer was labeled at the 3' and 5'-end as well as in various position of the chain and then the melting curves and T_m values messured (Table 1.) It is interesting to note that the T_ms increase considerably with the introduction of the condensed lumazine base moieties due to a stabilizing stacking effect. The use of 6- and 7-phenyl as well as 6- and 7-diphenyllumazine-2'-deoxyribofuranosides show somewhat similar but smaller effects depending on the site in the oligonucleotide chain and the nature of the substituent at the lumazine moiety (Table 2).

TABLE 1. Self-complementary oligonucleotie sequences and T_m values

5'-d(GG-TT-CC-AT-GC-AT-GG-AA-CC)-3	**60.4**			
5'-d(GG-TT-CC-AT-GC-AT-GG-AA-CC-**Lu**)-3'	61.2	62.5	62.3	61.2
5'-d(**Lu**-GG-TT-CC-AT-GC-AT-GG-AA-CC)-3'	61.2	62.3	61.9	61.5
5'-d(GG-TT-CC-A**Lu**-GC-AT-GG-AA-CC)-3'	61.5	66.4	66.4	65.5
5'-d(GG-TT-CC-AT-GC-A**Lu**-GG-AA-CC)-3'	61.4	65.5	65.4	66.4
5'-d(GG-TT-CC-A**Lu**-GC-A**Lu**-GG-AA-CC)-3'	60.3	> 70	74	>70

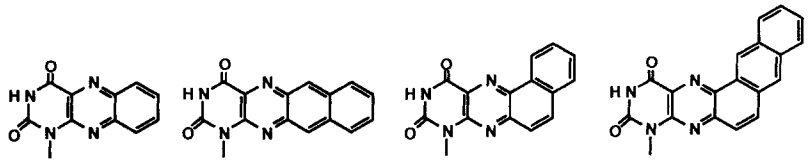
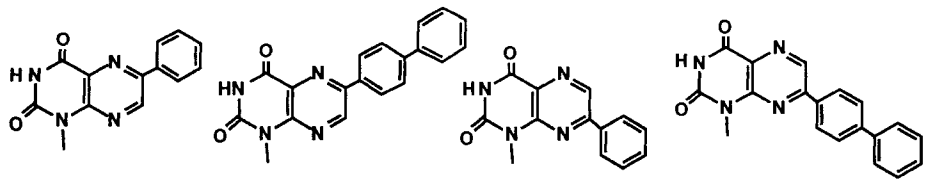
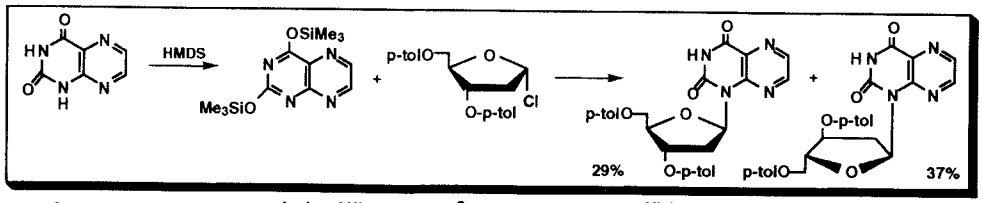


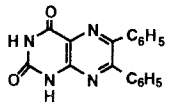
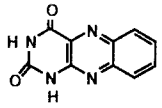
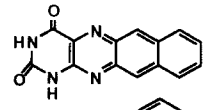
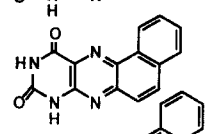
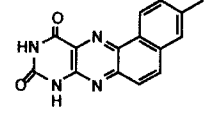
TABLE 2. Self-complementary oligonucleotides sequences and T_m values

Sequence	Lu	Tm ^{o}C
5'-d(GG-TT-CC-AT-GC-AT-GG-AA-CC)-3'	-	**60.4**
5'-d(GG-TT-CC-AT-GC-A**Lu**-GG-AA-CC)-3'	6-Ph	60.0
5'-d(GG-TT-CC-A**Lu**-GC-A**Lu**-GG-AA-CC)-3'	6-Ph	65.5
5'-d(**Lu**-GG-TT-CC-AT-GC-AT-GG-AA-CC)-3'	6-Ph	60.8
5'-d(GG-TT-CC-AT-GC-A**Lu**-GG-AA-CC)-3'	7-Ph	61.5
5'-d(GG-TT-CC-A**Lu**-GC-A**Lu**-GG-AA-CC)-3'	7-Ph	63.4
5'-d(**Lu**-GG-TT-CC-AT-GC-AT-GG-AA-CC)-3'	7-Ph	60.9
5'-d(GG-TT-CC-AT-GC-A**Lu**-GG-AA-CC)-3'	6-Ph-Ph	57.3
5'-d(GG-TT-CC-A**Lu**-GC-A**Lu**-GG-AA-CC)-3'	6-Ph-Ph	-
5'-d(**Lu**-GG-TT-CC-AT-GC-AT-GG-AA-CC)-3'	6-Ph-Ph	59.4
5'-d(GG-TT-CC-AT-GC-A**Lu**-GG-AA-CC)-3'	7-Ph-Ph	64.3
5'-d(GG-TT-CC-A**Lu**-GC-A**Lu**-GG-AA-CC)-3'	7-Ph-Ph	70.2
5'-d(**Lu**-GG-TT-CC-AT-GC-AT-GG-AA-CC)-3'	7-Ph-Ph	60.4

Buffer pH 7 ; Na+ conc. 0.03 M ; Wavelength 260 nm

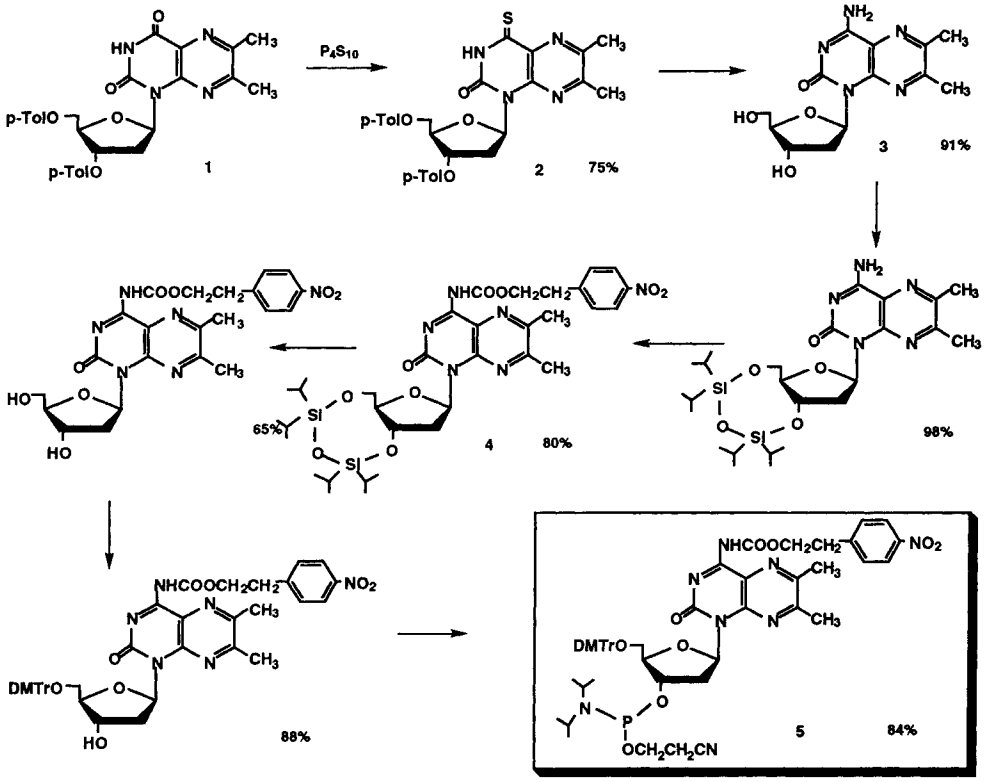




	Isolated Yield	α : β	Yield after Chromatographical Separation	
	80%	1 : 3	49%	16%
	75%		75%	-
	78%	1 : 6	58%	10%
	75%	1 : 3	57%	18%
	84%	1 : 2	48%	18%

The synthesis of an isopterin phosphoramidite (**5**) was achieved in a sequence of reactions starting from 6,7-dimethyl-1-(2-deoxy-3,5-di-p-toluoyl-β-D-ribofuranosyl)-lumazine (**1**) which was first thiated in 4-position to **2** and treated with ammonia to give the 6,7-dimethylisopterin-2'-deoxyriboside **3**. Markiewicz protection was then necessary to introduce the 2-(4-nitrophenyl)ethoxycarbonyl (NPEOC) residue (**4**) followed by common procedures to give the fully blocked phosphoramidite **5**. Oligonucleotide syntheses with this building block proceeded very well in a DNA-synthesizer applying the common protocoll of the NPE / NPEOC strategy[15] developed several years ago in our laboratory. Duplex formation was achieved with the complementary sequence and the resulting T_m values indicate that one mismatch does not harm the stability very much by lowering the T_m by about 2°, three mismatches adjacent, however, has a dramatic destabilizing effect as seen in the last example of table 3.

The 2'-deoxyadenosine analog 4-amino-8-(2-deoxy-β-Dribofuranosyl)7(8H)-pteridone (**9**) can be synthesized from 4-amino-7(8H)pteridone (**6**) which has to be protected, however, at the amino group from solubilitity reasons. Reaction with

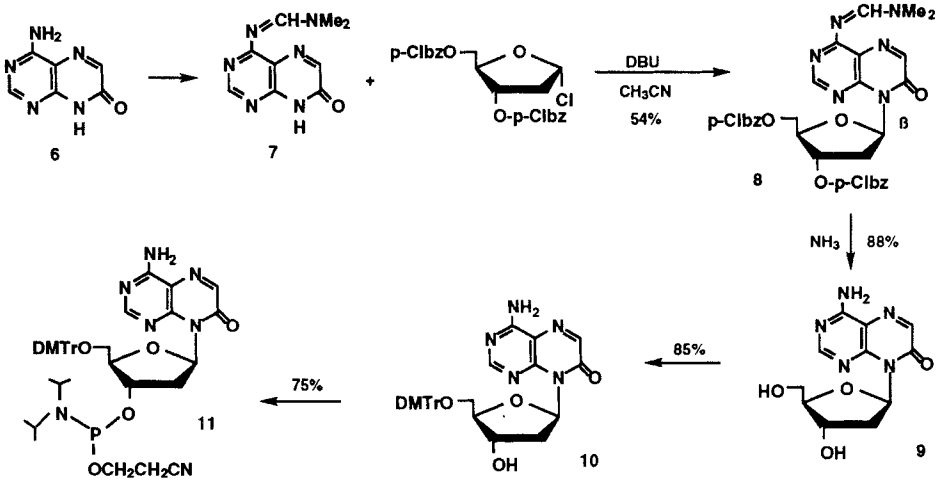


dimethylformamide dimethylacetal leads to the better soluble dimethylaminomethylene derivative **7** which could be glycosylated with 2-deoxy-3,5-di-O-p-chlorobenzoyl-α-D-ribofuranosyl chloride in a stereospecific manner in presence of DBU and CH_3CN as a solvent yielding 54% of **8.** Treatment with ammonia led to **9** and subsequent dimethoxytritylation to **10** and phos-phitylation to **11** could be achieved without further blocking of the amino function which possesses low nucleophilic reactivity due to its resonance with the 7-oxo group.

Most difficulties in pteridine nucleoside synthesis have so far been encountered during the anticipated approaches to prepare 8-(2-deoxy-β-D-ribofuranosyl)isoxanthopterin. The extremely insoluble starting isoxanthopterin did not react in the expected manner even in its trimethylsilylated form under a variety of reaction conditions with various halo-sugars. In order to get into the isoxanthopterin series at all 3-methyl-2-methylthio-4(3H),7(8H)pteridinedione (**12**) was treated in form of its sodium salt with

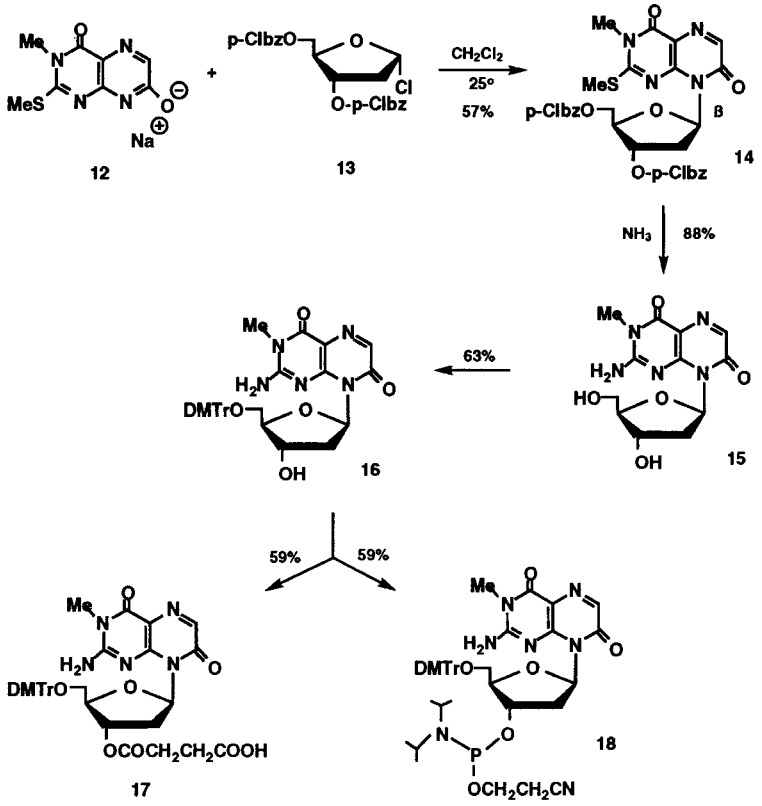
TABLE 3. Duplex formation and T_m values of oligonucleotides modified with the nucleobase 6,7-dimethylisopterin (**F**)

5'-d(GTG-TGG-AAA-ATC-TCT-AGC-AGT)-3' 3'-d(CAC-ACC- TTT-TAG-AGA-TCG-TCA)-5'	**46.6**
5'-d(GTG-TGG-AAA-AT**F**-TCT-AGC-AGT)-3' 3'-d(CAC-ACC- TTT-TAG-AGA-TCG-TCA)-5'	44.2
5'-d(GTG-TGG-AAA-ATC-T**F**T-AGC-AGT)-3' 3'-d(CAC-ACC- TTT-TAG-AGA-TCG-TCA)-5'	44.7
5'-d(GTG-TGG-AAA-ATC-TCT-AG**F**-AGT)-3' 3'-d(CAC-ACC- TTT-TAG-AGA-TCG-TCA)-5'	43.6
5'-d(GTG-TGG-AAA-AT**F**-T**F**T-AGC-AGT)-3' 3'-d(CAC-ACC- TTT-TAG-AGA-TCG-TCA)-5'	41.5
5'-d(GTG-TGG-AAA-ATC-TCT-AGC-AGT)-3' 3'-d(CA**F**-ACC- TTT-TAG-AGA-TCG-TCA)-5'	44.3
5'-d(GTG-TGG-AAA-**ATC**-TCT-AGC-AGT)-3' 3'-d(CAC-ACC- TTT-**CFA**-AGA-TCG-TCA)-5	> 25



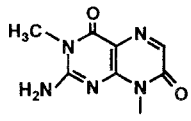
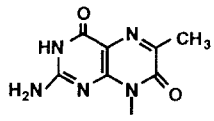
2-deoxy-3,5-di-O-p-chlorobenzoyl-α-D-ribofuranosyl chloride(**13**) in CH_2Cl_2 at room temperature forming in 57% yield the anticipated β-D-2'-deoxyribofuranoside **14**. Treatment with ammonia afforded not only deprotection at the sugar moiety but led also to a smooth nucleophilic substitution of the methylthio group ending up in 88%

yield with 3-methyl-8-(2-deoxy-β-D-ribofuranosyl)isoxanthopterin (**15**). The reduced reactivity of the 2-amino group has not to be blocked during dimethoxytritylation to **16** and subsequent acylations to the 3'-O-succinoyl derivative **17** and the corresponding phosphoramidite **18**.



The 3-methyl-isoxanthopterin-3'-phosphoramidite (**18**) turned out to be a valuable component for oligonucleotide syntheses but the presence of the 3-methyl group prohibits intermolecular hydrogen-bonding which seems to be a potentially strong stabilizing factor similar to the G-C pair combination. In order to overcome this disadvantage the synthesis of 8-(2-deoxy-5-O-dimethoxytrityl-β-D-ribofuranosyl)-6-methyl-O^4-2-(4-nitrophenyl)-ethyl-isoxanthopterin (**26**) was performed starting from 6-methyl-2-methylthio-4(3H), 7(8H)-pteridinedione (**19**). Glycosylation with **13** and DBU in CH_3CN led in this case to an α,β-anomeric mixture from which **20** was separated chromatographically in 31%. The next steps consisted of a Mitsunobu reaction to introduce the 2-(4-nitrophenyl)-ethyl group to O^4 (**21**), an oxidation by m-chloroperbenzoic acid to to form the 2-methyl-sulfonyl function (**22**) which could easily displaced by ammonia with simultaneous cleavage of the sugar protecting groups yielding **23** in high yield. Finally dimethoxy-tritylation and

TABLE 4. Duplex formation and T_m values

5'-d(GTG-TGG-AAA-ATC-TCT-AGC-AGT)-3' 3'-d(CAC-ACC-TTT-TAG-AGA-TCG-TCA)-5'		**63.2**
5'-d(GT**F**-TGG-AAA-ATC-TCT-AGC-AGT)-3' 3'-d(CAC-ACC-TTT-TAG-AGA-TCG-TCA)-5'	56	
5'-d(GTG-T**F**G-AAA-ATC-TCT-AGC-AGT)-3' 3'-d(CAC-ACC-TTT-TAG-AGA-TCG-TCA)-5'	55.6	
5'-d(GTG-TG**F**-AAA-ATC-TCT-AGC-AGT)-3' 3'-d(CAC-ACC-TTT-TAG-AGA-TCG-TCA)-5'	53.8	63.6
5'-d(GTG-TGG-AAA-ATC-TCT-A**F**C-AGT)-3' 3'-d(CAC-ACC-TTT-TAG-AGA-TCG-TCA)-5'	52	
5'-d(GTG-TGG-AAA-ATC-TCT-AGC-A**F**T)-3' 3'-d(CAC-ACC-TTT-TAG-AGA-TCG-TCA)-5'	58.8	62.6
5'-d(GTG-TGG-AAA-ATC-TCT-AGC-AGT)-3' 3'-d(CAC-ACC-TTT- TA**F**-AGA-TCG-TCA)-5'	58.8	
5'-d(GTG-TGG-AAA-ATC-TCT-AGC-AGT)-3' 3'-d(CAC-ACC-TTT-TAG- A**F**A-TCG-TCA)-5'	50.4	61.6
5'-d(GTG-TGG-AAA-ATC-TCT-AGC-AGT)-3' 3'-d(CAC-ACC-TTT- TAG-AGA-TC**F**-TCA)-5'	54.6	

Buffer pH 7 ; Na+ conc. 0.1 M ; Wavelength 260 nm

subsequent reaction with succinic acid anhydride and β-cyano-ethoxy-bis-diisopropyl-aminophosphane, respectively, provided the two components **25** and **26** for solid-support syntheses in a DNA-synthesizer.

A series of modified oligonucleotides containing the 3- and 6-methylisoxanthoterin moiety instead of guanine in the chain have been synthesized and hybdridized with with their complementary sequence to form duplexes. As expected the 3-methylisoxantho-pterin residue caused an decrease of the reference T_m whereas the 6-methylisoxantho-pterin turned out to be a good substitute for labelling experiments (Tabel 4). The impor-tance of pteridine labelling in oligonucleotide chains has also been demonstrated in the HIV-1 integrase 3'-processing reaction[16] in which the two terminal nucleotide units at the

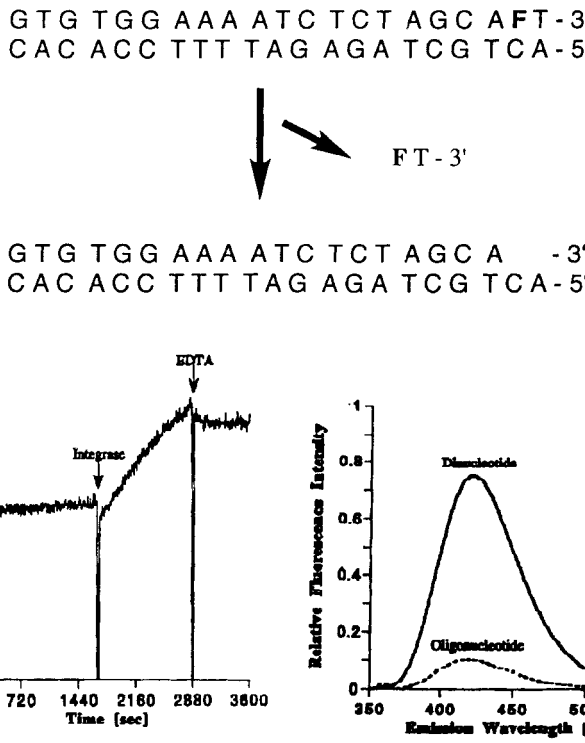
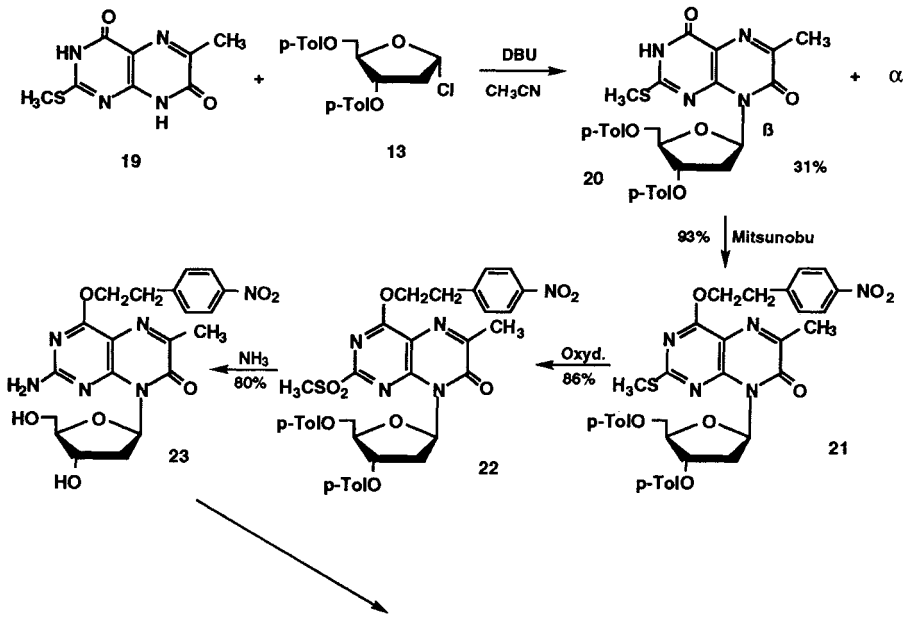
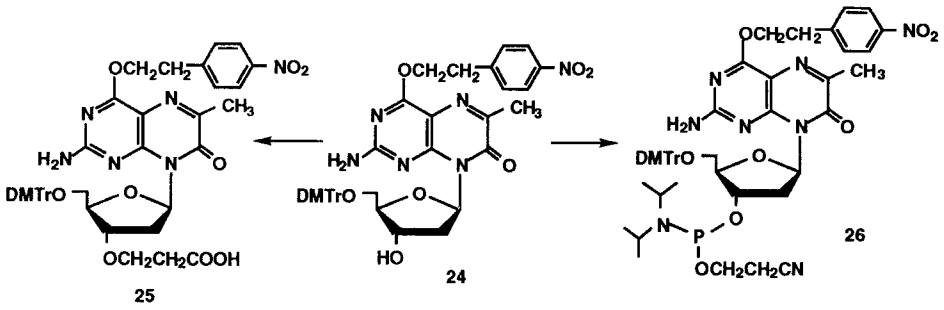


FIGURE 1. HIV-1 Integrase 3'- Processing Reaction





3'-end are cleaved off and can be detected very effectively by the increase of fluorescence resulting from higher conformational freedom in the dinucleotide compared to the duplex arrangement revealing a strong fluorescence quenching by inter- and intromolecular interactions with the fluorophor (Fig.1).

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