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# Solid-phase Synthesis of Branched RNA and Branched DNA/RNA Chimeras

**Morten Grøtli*, Ramon Eritja and Brian Sproat[a]**

EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany.

[a]Innovir, Olenhuser Landstrasse 20 a, D-37124 Rosdorf, Germany.

**Abstract**: An effective method for synthesising branched oligonucleotides on solid phase in the 5' to 3' direction has been developed. Special branch-point monomers enable the synthesis of branched oligonucleotides which can have sequences of different length and base composition attached to the 2'- and 3'-hydroxyl groups of the branch point ribonucleoside. The branched oligonucleotides are assembled on commercial DNA synthesisers, the crude products are readily purified by reversed phase HPLC and the fully deprotected products are conveniently analysed by mass spectrometry. © 1997 Elsevier Science Ltd.

## INTRODUCTION

The correct expression of eukaryotic genes depends on the processing (splicing) of pre-mRNA, which involves the accurate excision of introns and ligation of exons.[1] The splicing of nuclear polyadenylated RNA occurs with the formation of either a single-stranded circular RNA with a tail originating from a branch point (the lariat structure in *cis*-splicing reactions)[2, 3] or branches between two linear molecules (the Y structures observed in *trans*-splicing reactions)[4]. Unlike normal RNA, these structures have vicinal 2'-5' and 3'-5' internucleotidic phosphodiester linkages. The lariat structures are subsequently debranched, prior to degradation, by a specific endonuclease that cleaves on the 2'-side of the 2', 5'-phosphodiester linkage thus generating a linear molecule.[5]

Branched oligonucleotides and analogues thereof would be particularly useful for probing the structural requirements of branched RNA (bRNA) recognition in the splicing reaction and for studying the so-called debranching enzyme. In this context, considerable attention has been directed towards the synthesis of branched oligoribonucleotides.[6] Most reported syntheses have been conducted in solution. While solution-phase strategies can provide branched oligonucleotides in good yields, they involve excessive consumption of reagents and time consuming chromatographic purifications. The first automated solid phase synthesis of branched oligoribonucleotides was developed by Damha *et al.*[7] A reasonable yield of branched oligonucleotides with identical sequences at the 2' and 3' positions of the branch point adenosine, could be formed by reaction of a low concentration of an adenosine 2',3'-*O*-bis(phosphoramidite) with the free 5'-hydroxyl groups of two adjacent support bound oligonucleotide chains present on a highly loaded support. Synthesis could then be continued in the normal fashion from the 5'-hydroxyl group of the adenosine branch point, using 2'-*O*-(*tert.*

butyldimethylsilyl)-5'-*O*-dimethoxytritylribonucleoside-3'-*O*-phosphoramidites. Using a similar strategy, the same group reported the synthesis of branched oligonucleotides of any composition.[8] However, the synthesis resulted in a very difficult purification of four very similar structures present in more or less equimolar amounts. Using a different approach, Sproat *et al.*[9] described an efficient solid phase method for branched RNA involving synthesis from the 5' to 3' end with 5'-*O*-phosphoramidites and special branch point nucleosides, enabling simultaneous extension from the 2'- and 3'-hydroxyl groups.

In this report we describe a universal solid phase method for the synthesis of branched oligonucleotides. Special branch-point monomers, *viz.* protected nucleoside 5'-*O*-phosphoramidites bearing 2'-*O*-(9-phenylxanthene-9-yl) (Pix)[10] and 3'-*O*-laevulinyl[11] protecting groups, enable the synthesis of branched oligonucleotides in the 5' to 3' direction which can have sequences of different length and base composition attached to the 2'- and 3'-hydroxyl groups of the branch point ribonucleoside. The procedure is carried out on an automatic DNA synthesiser using phosphoramidite chemistry, and affords, upon deprotection and purification, branched sequences in good yield. A preliminary report of some of these results has appeared.[12]

## RESULTS AND DISCUSSION

*General considerations*

The synthesis of branched oligonucleotides with sequences of different length and base composition attached to the 2'- and 3'-hydroxyl groups of the branch point ribonucleoside excludes the possibility to introduce both 2'-5' and 3'-5' vicinal phosphate linkages simultaneously. A special branch point monomer carrying different protecting groups on the 2'- and 3'-hydroxyl groups, which can be removed independently of each other is required. The basis of this strategy is outlined in **Fig. 1**. Chemical constraints dictated that the branched

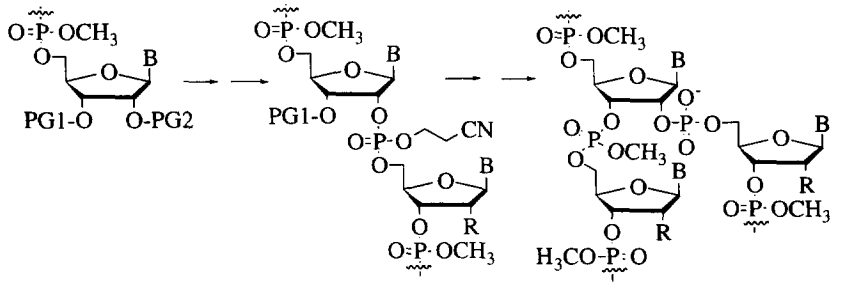



**Fig. 1**. The strategy for the introduction of the branched framework. PG1 and PG2 represent the two different protecting groups.

oligonucleotides should be synthesised from the 5' to 3' direction, that is in the opposite direction to that of standard automated DNA or RNA synthesis. After introduction of the branch point monomer one of the protecting groups is removed to allow chain extension, and condensation is continued with 5'-*O*-phosphoramidites ("reversed monomers"). Upon completion of the first arm and capping of its terminal hydroxyl function the second protecting group at the branch point is removed and then the second branch is extended by

condensation of reversed monomers. To prevent strand cleavage or migration following removal of the PG1 the vicinal phosphotriester must be converted to the more stable phosphodiester. One the other hand, it can not be recommended to deprotect all the phosphate moieties along the polymer, since the phosphates are potentially reactive sites that can interfere with the coupling reaction. Thus, two compatible phosphate protecting groups had to be used. We reasoned that this could be done by using reversed monomers, *viz.* protected deoxyribonucleosides or ribonucleosides in the form of 5'-*O*-(methyl *N,N*-diisopropylphosphoramidite)s bearing 3'-*O*-Pixyl protection and 2'-*O*-[1-(-2-fluorophenyl)-4-methoxypiperidin-4-yl] (Fpmp)[13] protection for the ribonucleosides **9**, and only one single reversed monomer in the form of a 5'-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) **10** for coupling at the branch-point (**Fig. 2**). The 2-cyanoethyl group is reasonably stable in the phosphoramidite monomer, but can rapidly be removed from the phosphotriester by treatment with base (by a β-elimination mechanism), for example 1,8-diazabicyclo[5.5.0]undec-7-ene (DBU). However, a side-reaction can occur when unprotected thymidines or uridines are exposed to DBU.[14] Deprotonation of the lactam function occurs, giving rise to a rapid reaction with the adjacent phosphotriester and the formation of an 3-N methyl thymidine or uridine residue. Thus, thymidine and uridine base protection should clearly be employed in the synthesis protocol.

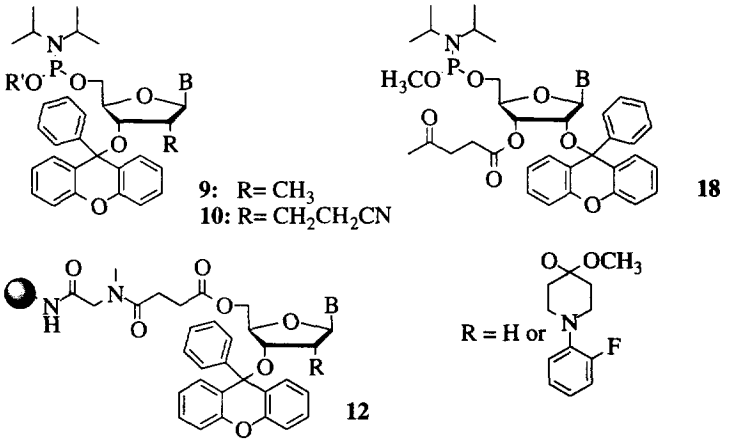
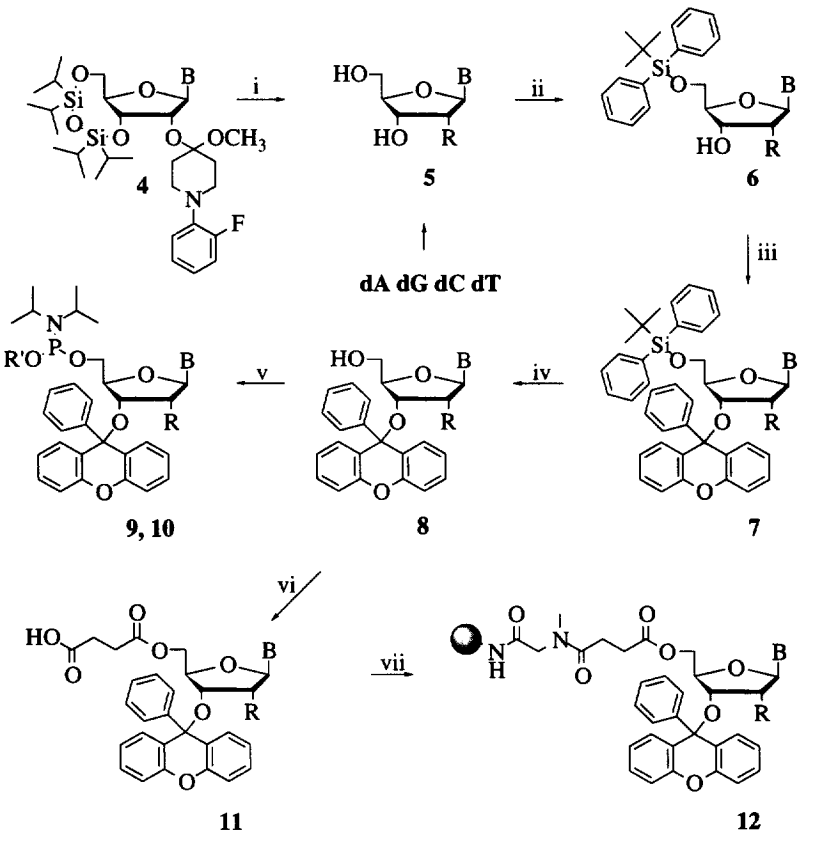



**Fig. 2.** Structures of the reversed monomers **9** and **10**, the branch-point monomers **18** and the reversed supports **12**. The stippled circle represents the long chain alkylamine-controlled pore glass (LCAA-CPG); R' is methyl or 2-cyanoethyl; B is a protected nucleobase, *viz.* 6-*N*-pivaloyladenin-9-yl, 6-*N*-benzoyladenin-9-yl, 4-*N*-benzoylcytosin-1-yl, 2-*N*-isobutyrylguanin-9-yl, 2-*N*-(dimethylaminomethylidene)guanin-9-yl, 3-*N*-(pivaloyloxymethyl)uracil-1-yl or 3-*N*-(pivaloyloxymethyl)thymin-1-yl.

Then the PG1 group is cleaved and chain extension to give the desired branched oligonucleotide is then continued with reversed monomers. For the special branch point nucleoside **18** the combination of 2'-*O*-Pixyl and 3'-*O*-laevulinyl protection (**Fig. 2**) seemed very attractive since the laevulinyl group[11] can be selectively removed under neutral conditions by a brief treatment with hydrazine hydrate in buffered pyridine-acetic acid. Unfortunately, the linker conventionally used to attach the oligonucleotide to the support matrix, the succinyl

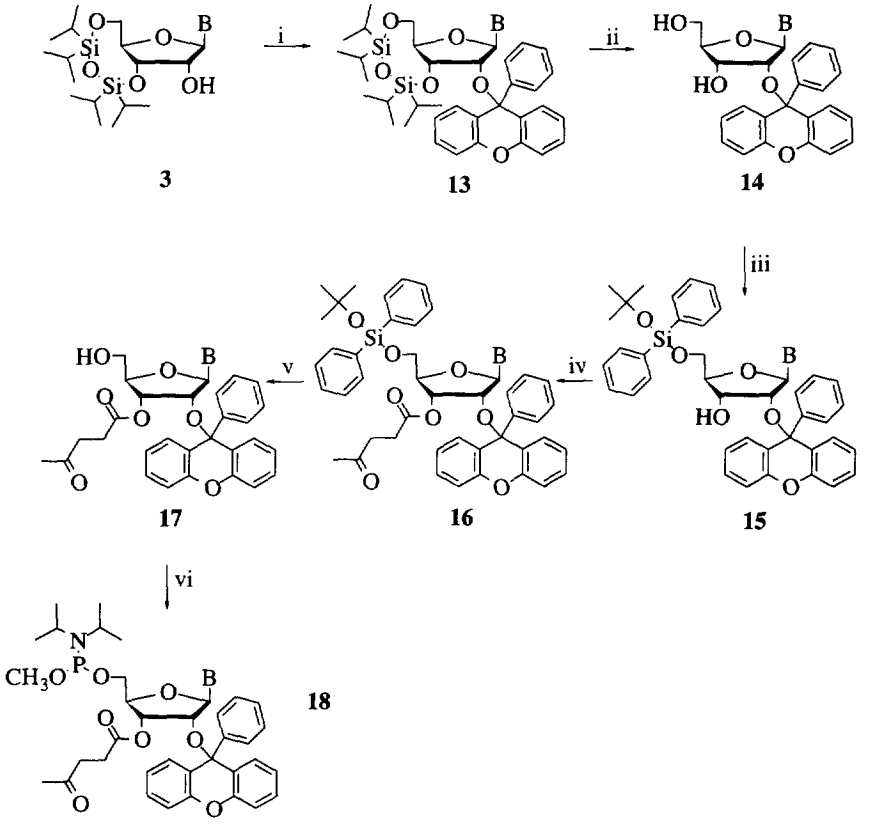
linker, is unstable to DBU. Scission is fast, presumably due to a mechanism involving deprotonation of the amide nitrogen followed by intramolecular nucleophilic displacement at the ester carbonyl group. However, this problem is readily solved by using a sarcosine linked reversed support[15] **12** (**Fig. 2**) which prevents cleavage of the oligonucleotide from the support during the base treatment.




**Scheme 1** Reaction scheme for the preparation of reversed monomers and supports: adenosine series (**A4-A12**), B= 6-*N*-pivaloyladenin-9-yl; deoxyadenosine series (**dA5-dA12**), B=6-*N*-benzoyladenin-9-yl; cytidine (**C4-C12**) and deoxycytidine series (**dC5-dC12**), B=4-*N*-benzoylcytosin-1-yl; guanosine series (**G4-G12**), B=2-*N*-isobutyrylguanin-9-yl and deoxyguanosine series (**dG5-dG12**), B=2-*N*-(dimethylaminomethylidene)-guanin-9-yl; uridine series (**U4-U12**), B=3-*N*-(pivaloyloxymethyl)uracil-1-yl; thymidine series (**dT5-dT12**); B= 3-*N*-(pivaloyloxymethyl)thymin-1-yl. R= H or OFpmp. Reagents: i) TBAF in THF; ii) TBDPSCl and imidazole in DMF; iii) PixCl in pyridine; iv) TBAF in THF; v) methoxy *N*,*N*-diisopropylaminochlorophosphine or 2-cyanoethoxy *N*,*N*-diisopropylaminochlorophosphine and *N*,*N*-diisopropylethylamine in dichloromethane; vi) succinic anhydride and DMAP in dichloromethane; vii) 2,2'-dithio-bis(5-nitropyridine), DMAP, triphenylphosphine and sarcosine linked long chain alkylamine-controlled pore glass.

*Monomer synthesis*

The syntheses of the reversed monomers are illustrated in **Scheme 1**. Compounds **A4**, **C4**, and **G4** were synthesised according to Sproat *et al.*[9] In order to protect the lactam function of the uracil moiety from side reactions during the oligoribonucleotide synthesis we decided to block the 3-N-position with the ammonia labile pivaloyloxymethyl (Pom) group. Uridine was reacted with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane[16]. The POM protection group[17] was then introduced via the transient protection procedure to give compound **U3** in 79 % yield. The 2'-hydroxyl group in compound **U3** was then protected with the Fpmp group by reaction with 1-(2-fluorophenyl)-4-methoxy-1,2,5,6-tetrahydropyridine in the presence of trifluoroacetic acid to give compound **U4** in 76 % yield. Desilylation to give compound **5** (A-U) proceeded smoothly in almost quantitative yield.




**Scheme 2** Reaction scheme for the preparation of branch-point monomers: adenosine series (**A3-A18**), B= 6-*N*-pivaloyladenin-9-yl; cytidine (**C3-C18**), B=4-*N*-benzoylcytosin-1-yl; guanosine (**G3-G18**), B=2-*N*-isobutyrylguanin-9-yl and uridine series (**U3-U18**), B=3-*N*-(pivaloyloxymethyl)uracil-1-yl; Reagents: i) PixCl in pyridine; ii) TBAF in THF; iii) TBODPSCl and imidazole in DMF; iv) laevulinic anhydride and DMAP in dichloromethane; v) TBAF in THF; vi) methoxy *N*,*N*-diisopropylaminochlorophosphine and *N*,*N*-diisopropylethylamine in dichloromethane.

The exocyclic amino protection for deoxyadenosine and deoxycytidine was introduced via per-acylation[18] to give compounds **dA5** and **dC5**. Deoxyguanosine was protected on the exocyclic amino group by reaction with *N,N*-dimethylformamide (DMF) dimethyl acetal[19] to give compound **dG5**. Subsequent silylation with *tert*-butyldiphenylchlorosilane followed by treatment with pixyl chloride and desilylation afforded compound **8** (**A-U** and **dA-dT**) in excellent yields. In the case of compound **dT7** the POM protection group was introduced before desilylation to afford compound **dT8**. Finally, 5'-hydroxyl group phosphitylation with methoxy *N,N*-diisopropylaminochlorophosphine afforded the desired reversed monomers, *viz.* compound **9** (**A-U** and **dA-dT**) as foams in yields between 89 and 96 %.

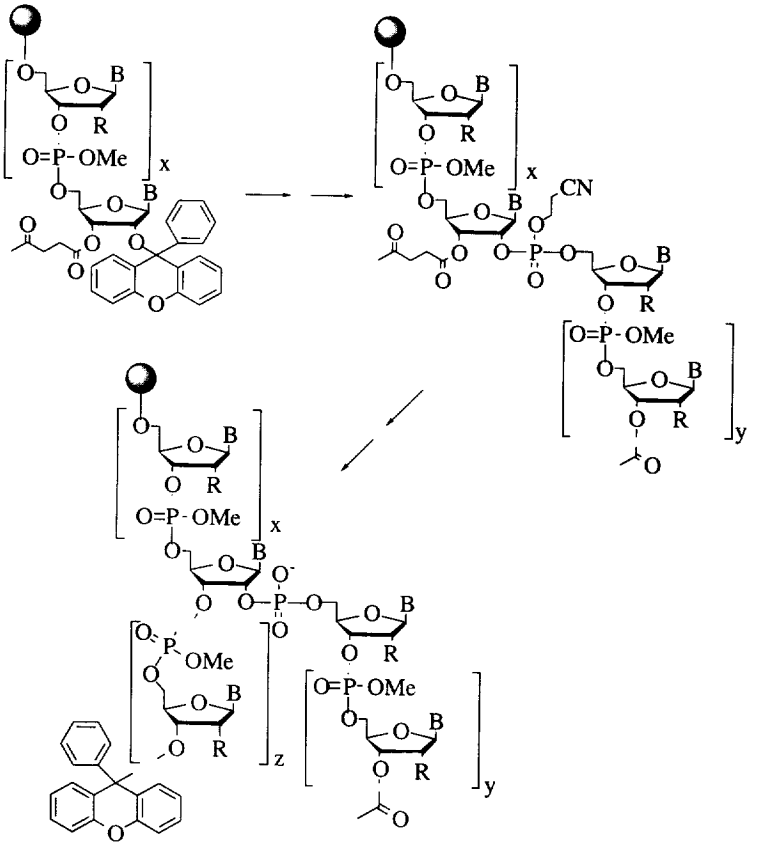
The syntheses of the solid supports are illustrated in **Scheme 1**. FMOC protected sarcosine was anchored to LCAA-CPG using triphenylphosphine 2,2'-dithio-bis(5-nitropyridine)[20] as coupling reagent. After capping and cleavage of the FMOC group, the succinyl-esters of compound **8** (**A-U** and **dA-dT**) were reacted with the sarcosine containing support using the same coupling reagent as above.

The synthesis of the branch-point monomers is illustrated in **Scheme 2**. The branch-point monomer was first designed with the laevulinyl group on the 2'-hydroxyl function and the acid labile pixyl group on the 3'-hydroxyl function. Thus, **A3** was laevulinylated on the 2'-hydroxyl group. However, subsequent desilylation yielded two products in 1:1 ratio. The $^{13}C$ NMR spectrum showed one product that was consistent with the anticipated 2'-*O*-laevulinyl derivative while the other product was consistent with migration of the 2'-*O*-laevulinyl group to the 3'-hydroxyl function. The branch-point monomers were therefore redesigned to carry the laevulinyl group on the 3'-hydroxyl function and pixyl group on the 2'-hydroxyl function. Pixylation of compounds **3** (**A-U**) followed by desilylation afforded compounds **14** ( **A-U**) in high yields (83-93 %). In order to introduce the laevulinyl group on the 3'-hydroxyl moiety it was necessary to selectively protect the 5'-hydroxyl function. Initially, the bulky *tert*-butyldiphenylsilyl group was used. Removal of this protecting group, after introduction of the laevulinyl group, with tetrabutylammonium fluoride (TBAF) was rather slow, requiring 6-7 hours. During this prolonged reaction time the laevulinyl group was partially cleaved. To avoid the long treatment with TBAF, the 5'-hydroxyl function was protected with the *tert*-butoxydiphenylsilyl group[21]. This alkoxysilyl ether offers high fluoride reactivity due to the electron-withdrawing oxygen atom. And indeed, the desilylation of **16** (**A-U**) was complete within 10 min without any observable side reactions in good isolated yield (91-98 %). Finally, phosphitylation of the 5'-hydroxyl group of **17** gave the desired monomers, compounds **A18**, **C18**, **G18** and **U18** as foams in yields between 80 and 97 %.

*Assembly of branched oligonucleotides*

Solid-phase synthesis of branched oligonucleotides were carried out on an automatic DNA synthesiser. The strategy is illustrated in **Scheme 3**. The 5'-end of the oligonucleotides were assembled using the reversed monomers **9** on the reversed supports **12**. The branch-point monomers **18** were then coupled to the 3' hydroxyl function of the growing chain. The 2'-*O*-pixyl group was then removed, allowing chain extension from the 2'-hydroxyl group of the branch point monomer, and condensation was first continued with a single reversed monomer in the form of a 5'-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) **10**. Further chain extension was then performed with the appropriate normal reversed monomers **9** to complete one arm. The 3'-*O*-Pixyl group was then removed and the resultant hydroxyl group capped by acetylation to prevent further chain extension from this part of the branch. To prevent strand cleavage or migration following removal of the laevulinyl group the vicinal 2-cyanoethyl group was first removed from the 2'-5' phosphotriester moiety by

treatment with 0.5 M anhydrous DBU in acetonitrile for 1 min.[22] Then the 3'-*O*-laevulinyl group was cleaved with hydrazine hydrate in pyridine-acetic acid. Chain extension to give the desired branched oligonucleotide was then continued with the reversed monomers **9**.



**Scheme 3**. Synthesis of branched oligonucleotides.

*tert*-Butyl hydroperoxide (TBHP) can be used as an alternative to iodine for oxidation of the P(III) species to P(V) species during oligonucleotide chain propagation.[23-29] The reagent was prepared by combining 20 ml of the TBHP solution in isooctane with 40 ml of dichloromethane. The time of oxidation per cycle was extended to 1 min compared with 15 sec. for iodine at the 0.2 μmol scale. The use of TBHP has the advantage that moisture is completely excluded from the system. Removal of phosphate protecting groups, heterocyclic base protecting groups, and cleavage of the oligonucleotide from the solid support was achieved by use of a 3:1 mixture of concentrated aqueous ammonia and ethanol at 55 °C overnight. The Fpmp protecting groups are completely stable under the ammonolysis conditions.[24] As indicated above, this allows the synthetic branched oligonucleotides to be purified by reversed phase HPLC without any risk of their being digested by contaminating traces of ribonucleases. Moreover, reversed phase HPLC could be used to accurately assess the success or failure of a synthesis or deprotection protocol. Together with spectrophotometric quantification this provided accurate

measurements of the full-length product generated. After the stabilised RNA sequences had been purified, the 2'-*O*-Fpmp groups and the 3'-terminal pixyl group were removed by treatment with 0.5 M Tris acetate buffer (pH 3.03) at room temperature.[30] The unprotected RNA sequences were then, if necessary, further purified and isolated by precipitation.

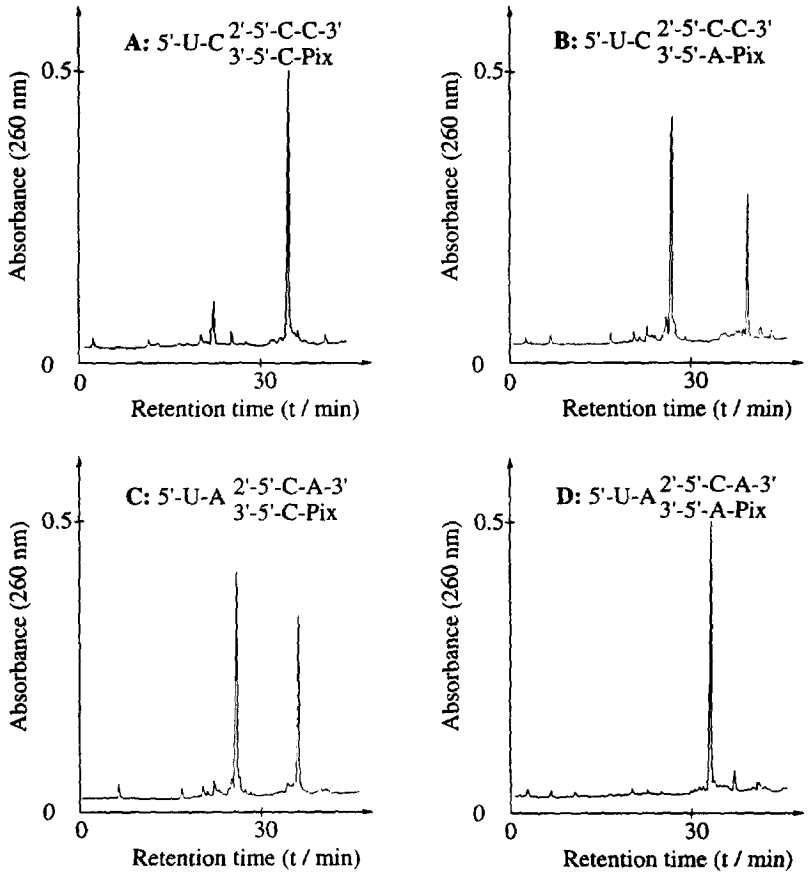



**Fig.3** Sequence dependent coupling at the branch-point. Analytical reversed-phase HPLC profiles of crude 2'-*O*-Fpmp, pixyl-protected branched oligonucleotides on a 4 mm x 250 mm Nucleosil C18 steel column (10 μ); buffer A: 95 % 0.1 M triethylammonium acetate (pH 6.5) and 5 % acetonitrile; buffer B: 30 % 0.1 M triethylammonium acetate (pH 6.5) and 70 % acetonitrile; gradient: 20-100 % buffer B during 40 min, flow rate, 1 ml $min^{-1}$.

To establish the necessary criteria for the efficient introduction of the branch framework, the syntheses of several small branched oligonucleotide sequences were conducted. During this work, it was found that the coupling between the 3'-hydroxyl function at the branch-point and the activated 5'-phosphoramidite is dependent upon the base at the branch point. When a 0.1 M solution of **C9** was allowed to react with the 3'-hydroxyl group of a cytidine branch-point for 15 min with tetrazole as activator, the full-length product was obtained in good

yield. HPLC analysis of the crude material (**Fig. 3A**) revealed two major peaks which were identified as the linear oligonucleotide 5'-UC(2'-CC)-3' and the desired branched oligonucleotide (**Fig. 3A**), still carrying a 3'-*O*-pixyl group and 2'-*O*-Fpmp protection. Changing the 5'-phosphoramidite from cytidine to adenosine resulted

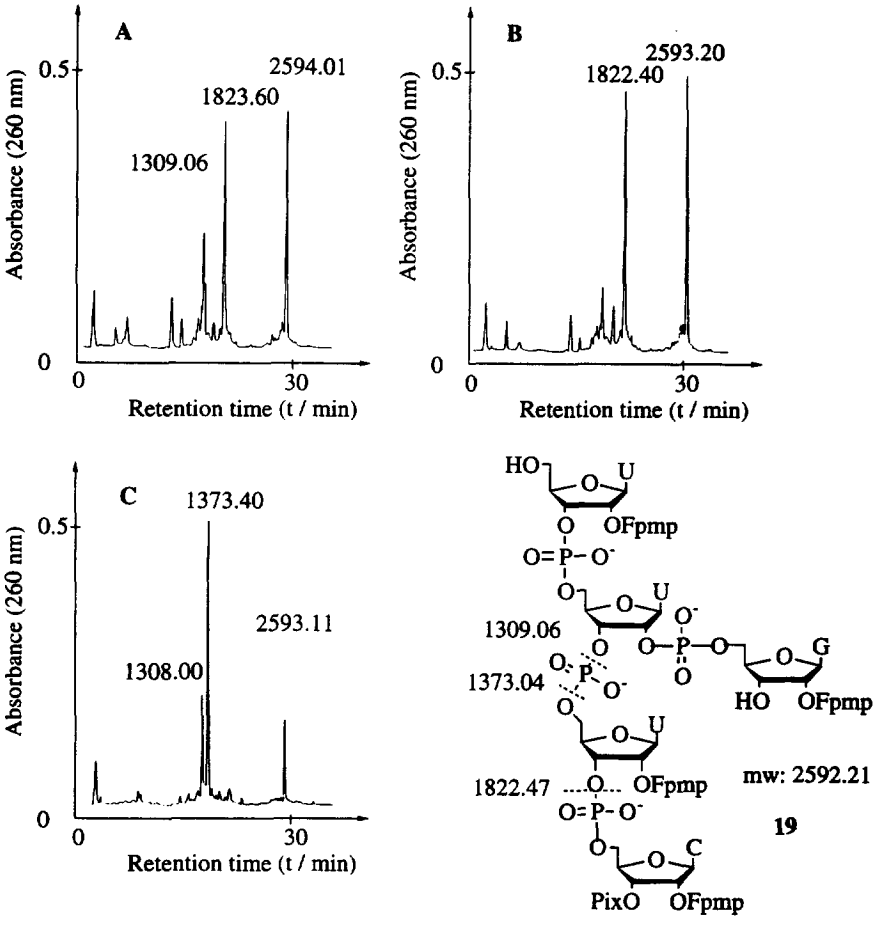



**Fig.4** The use of different activators for the synthesis of compound **19**. Coupling time 900 sec. Panel A: 0.5 M 5-ethylthio-1*H*-tetrazole; panel B: 5-(2-nitrophenyl)tetrazole; panel C: 0.5 M pyridine-HCl; the profiles were obtained on an 4 mm x 250 mm Nucleosil C18 steel column (10 µ); buffer A: 95 % 0.1 M triethylammonium acetate (pH 6.5) and 5 % acetonitrile; buffer B: 30 % 0.1 M triethylammonium acetate (pH 6.5) and 70 % acetonitrile; gradient: 20-100 % buffer B during 40 min, flow rate 1 ml $min^{-1}$.

in the preferential formation of the linear tetramer rather than the desired branched oligonucleotide (**Fig. 3B**). The same result was obtained with a adenosine branch-point and **C9** (**Fig. 3C**) under otherwise identical conditions. The coupling between **A9** and the 3'-hydroxyl group of a adenosine branch-point produced hardly any of the desired branched oligonucleotide. Changing the concentration of the 5'-phosphoramidite from 0.1 M to

0.2 M improved the latter synthesis dramatically (**Fig. 3D**). On the other hand, doubling the coupling time only resulted in marginal improvements. A higher concentration of the 5'-phosphoramidite is therefore essential for obtaining good yields in a purine-pyrimidine or a purine-purine coupling at the branch-point. All other syntheses referred to in this manuscript were carried out with 0.2 M solutions for coupling at the branch-point. This result also reflects, in part, the marked difference in steric environment of the 3'-hydroxyl group of the branch-point and the 5'-phosphoramidite for purines and pyrimidines.

The key step in the automated synthesis of an oligonucleotide by the phosphoramidite approach is the 1-*H*-tetrazole mediated coupling reaction of a phosphoramidite with a hydroxyl group of the growing chain anchored to the polymeric support.[31, 32] Activation of phosphoramidites by tetrazole creates a highly electrophilic intermediate that reacts with the nucleophilic hydroxyl group of the oligonucleotide. To further improve the coupling between the 3'-hydroxyl function at the branch-point and the activated 5'-phosphoramidite other activators were tested out. Recently the use of substituted tetrazoles[33] as activators for DNA and RNA synthesis has been reported[34-37]. 5-Ethylthio-1-*H*-tetrazole[36, 37] has been found to be a more effective activator, due to its excellent solubility properties and greater acidity. This reagent was tested by synthesising a small branched oligoribonucleotide using both tetrazole and 5-ethylthio-1-*H*-tetrazole, varying only the activator and the coupling time. The coupling through the 3'-position, following removal of the cyanoethyl group and delaevulinylation as measured via the depixylation yield after coupling phosphoramidite **U9** was 90.1 and 91.5 % respectively. However, the analytical reversed-phase HPLC profile of compound **19**, synthesised with 5-ethylthio-1-*H*-tetrazole as activator (**Fig 4A**) showed three main products. The three products were identified by electrospray mass spectrometry as the unbranched product (mw = 1309.06), the n-1 product (mw = 1822.47) and the full length product carrying a pixyl group (mw = 2592.21). Similar results were obtained with 5-(4-nitrophenyl)-1*H*-tetrazole[38] (**Fig 4B**). These results indicate that the last coupling proceeds with low coupling efficiency due to steric hindrance. The G residue attached to the 2' hydroxyl group at the branch point reduces the accessibility of the 3'-hydroxy group on the neighbouring U residue. The activator reacts with the incoming amidite to produce a reactive intermediate that can couple to this 3'-hydroxyl group. The size or the bulkiness of the reactive phosphoramidite-intermediate is important for the coupling efficiency, since the use of tetrazole did not produce significant amounts of any of these side-products (data not shown). When pyridine hydrochloride[39, 40] was used as activator hardly any of the desired branched oligonucleotide was obtained. The main product was identified as the unbranched oligonucleotide with an additional phosphate group attached to the 3'-hydroxy funtion at the branch-point (**Fig 4C**). This result can not be explained by steric hindrance, but is more likely a result of an unknown sidereaction.

We have also performed the oligonucleotide synthesis on an optimised type of polystyrene support[41]. These were prepared by coupling the 2'-*O*-Fpmp-3'-*O*-pixyl-5'-*O*-succinate nucleoside esters with activated non-swelling aminomethyl-polystyrene sarcosine beads. The rigid non-swelling polystyrene beads of 50-70 micron particle size retain less water and wet more thoroughly with organic solvents than CPG. The drier environment provides for a more efficient synthesis. However, in every instance where a comparison was made with CPG support, with all other variables controlled, the polystyrene support gave identical results (data not shown).

After establishing the necessary criteria for the efficient introduction of the branched framework, the syntheses of several branched oligonucleotide sequences were conducted (**Table 1**). All syntheses were performed on an automated DNA/ RNA synthesiser (Applied Biosystems 394) on a 0.2 μmol scale. A typical reversed phase HPLC analysis of the crude compound **23** (see **Table 1**) is shown in **Fig. 5A**. The crude

sample consisted of a major peak accounting for the full length product separated from several smaller peaks. The main by-products are failure sequences from the synthesis of the arm attached to the 3'-hydroxyl function at the branch point. The same pattern was observed for most branched oligonucleotides prepared. The coupling at

**Table 1**. Branched oligonucleotides

| Compound. Sequence | Coupling yield: bp[a] | Yield[b] Crude / RP-HPLC | Yield[b] Unprotected | MS (MALDI or ES) Calculated / Found |
|---|---|---|---|---|
| 20. 2'GCA UACAC 3'UCAGA | 89 | 22.8 / 5.0 | 4.3 | 4108.57 / 4109.34 |
| 21. 2'G UGUUG 3'UACA | 85 | 21.2 / 4.1 | 3.8 | 3161.91 / 3163.02 |
| 22. 2'GCCG UAGGACU 3'CCG | 87 | 26.6 / 4.4 | 4.0 | 4460.73 / 4462.71 |
| 23. 2'AUC CUCUA 3'UCG | 88 | 21.6 / 4.5 | 4.0 | 3387.03 / 3388.43 |
| 24. 2'GdTdCdC dCdCdTdTA 3'dTdCdCdT | 83 | 23.5 / 5.5 | 5.1 | 3868.49 / 3870.13 |
| 25. 2'UGCGAC dTdTdCA 3'C | 52 | 18.0 / 1.2 | 0.9 | 3405.09 / 3407.01 |
| 26. 2'CCGCAC GUCCA 3'UC | 49 | 16.6 / 0.9 | 0.7 | 4035.46 / 4036.51 |
| 27. 2'GCUCCA UG 3'dTdAdC | 58 | 16.9 / 0.9 | 0.45 [c] | 3392.11 / 3394.01 |

a) The coupling through the 3' position as measured via the depixylation yield after coupling the reversed monomer.

b) $A_{260}$ units

c) The low yield is at least partly due to depurination during the cleavage of the Fpmp groups.

the 3'-position of the branch point measured via the depixylation yield was in the range from 79-90 % ( **Table 1**). However, when the length of the 2'-arm is increased the coupling efficiency at the 3'-hydroxyl function goes down. For example, in the synthesis of compound **25** the coupling yield at the branch-point as measured by depixylation was only 52 %. Changes in concentration and or coupling time did not result in increased coupling yields. This trend was observed for several "long" sequences (compounds **25, 26** and **27**). Despite this

limitation, the desired product could be isolated although in low yield (**Table 1**).

In each case, the desired product was purified by reverse-phase HPLC. Isolated yields of the branched oligonucleotides carrying pixyl and in the case of ribomonomers Fpmp protection were generally in the range of 1- 6 $A_{260}$ units and are reported in **Table 1**. As a check on the purity the samples were analysed by ion exchange chromatograpy. A typical example is shown in **Fig 5B**. The identity of the fully deprotected branched oligonucleotides was confirmed by MALDI or ES mass spectrometry (**Table 1**).

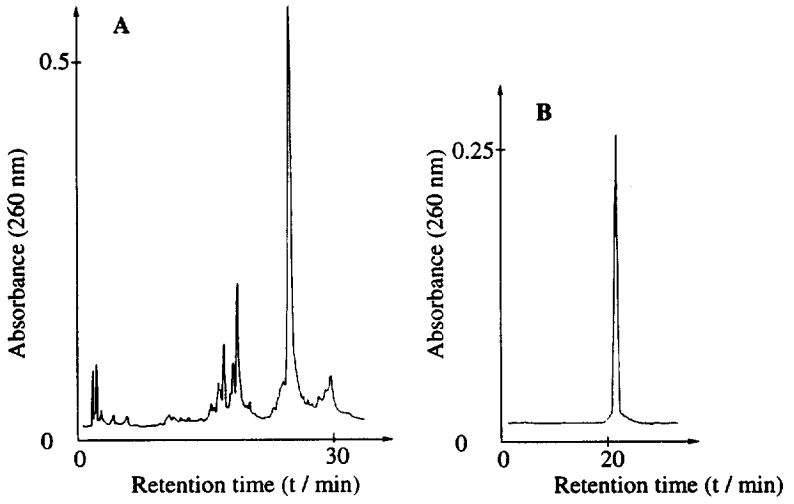



**Fig.5. A)** Chromatogram of crude 0.2 μmol synthesis of oligonucleotide **23** analysed on an 4 mm x 250 mm Nucleosil C18 steel column (10 μ); buffer A: 95 % 0.1 M triethylammonium acetate (pH 6.5) and 5 % acetonitrile; buffer B: 30 % 0.1 M triethylammonium acetate (pH 6.5) and 70 % acetonitrile; gradient: 20-100 % buffer B in 40 min, flow rate 1 ml $min^{-1}$. **B)** Chromatogram of oligonucleotide **23** purified by a two step process, reversed phase, pixyl-on, followed by cleavage of the pixyl and the 2'-*O*-Fpmp groups and then ethanol pecipitation; analysed on a Mono Q HR 5/5 column; buffer A: 0.3 M NaCl and 10 mM NaOH (pH 12.5); buffer B: 0.9 M NaCl and 10 mM NaOH (pH 12.5); gradient 20-70 % buffer B in 40 min, flow rate 0.5 ml $min^{-1}$.

## CONCLUSIONS

The method presented in this paper provides an efficient and controlled protocol for the synthesis and purification of branched oligonucleotides which can have sequences of different length and base composition attached to the 2'-, 3' and 5'-hydroxyl groups of the branch point ribonucleoside. The chemistry is applicable to syntheses of bRNA as well as branched RNA / DNA chimeras. The branched oligonucleotides are assembled on commercial synthesisers and obtained in high yield. We believe this procedure is the method of choice for preparing a range of branched oligonucleotides. Several synthetic branched oligomers are currently being used to study substrate requirements for the RNA-debranching enzyme.

## EXPERIMENTAL SECTION

Ribonucleosides were purchased from Pharma Waldhof GmbH (Düsseldorf, Germany), 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane was obtained from Ifotam (Lodz, Poland), N,N-diisopropylmethylphosphonamidic chloride was obtained from Aldrich, and 9-chloro-9-phenylxanthene was obtained from Fluka GmbH. All other reagents used were of the highest available purity. Anhydrous solvents were purchased from Romil Chemicals Ltd. and SDS. 1-(2-Fluorophenyl)-4-methoxy-1,2,5,6-tetrahydropyridine was prepared according to the method of Reese and Thompson.[12] Compounds **A3**, **C3**, **G3**, **A4**, **C4**, **G4**, **A10**, **G10** and **C10** were prepared as described previously.[9] Compounds **11**(A-U and dA-dT) were prepared by a standard procedure and were used in the preparation of activated esters for coupling to sarcosine-long chain alkylamine-controlled pore glass using triphenylphosphine 2,2'-dithio-bis(5-nitropyridine) as coupling reagent[19]. Column chromatograpy was performed on Kieselgel 60H (SDS) and ascending mode TLC was performed on aluminium-foil-supported silica gel 60 $F_{254}$. $^{13}C$ and $^{31}P$ NMR spectra were recorded on a Bruker AM250 spectrometer, using tetramethylsilane and external trimethyl phosphate as the respective references. $^{13}C$ NMR spectral data are reported below with broad-band proton-noise decoupling; however, assignments were done with the aid of the off-resonance data. The oligoribonucleotides were synthesized on an Applied Biosystems synthesizer model 394 (Foster City, California).

*Synthesis of monomers*

**3-*N*-Pivaloyloxymethyl-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)uridine (U3)**

3',5'-*O*-(Tetraisopropyldisiloxane-1,3-diyl)uridine (14.9 g, 30.61 mmol) was dissolved in dry dichloromethane (300 ml) and triethylamine (15 ml, 107.62 mmol) and trimethylsilyl chloride (10 ml, 79.07 mmol) were added dropwise to the stirred solution with exclusion of moisture. The reaction mixture was left at room temperature for 15 min. Silica gel TLC showed complete reaction. The reaction mixture was poured into vigorously stirred 1M aq. sodium hydrogen carbonate (300 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The resulting foam was dissolved in dry dimethylformamide (100 ml) and potassium carbonate (21.5 g, 155.56 mmol) followed by chloromethyl pivalate (11 ml, 76.32 mmol) were added with stirring and exclusion of moisture, and the reaction mixture left over night at room temperature. TLC showed complete reaction. The solvent was removed under reduced pressure, and the residue was mixed with ethyl acetate (300 ml) and filtered. The solution was washed with 1M aq. sodium hydrogen carbonate (300 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. In order to remove the 2'-*O*-TMS group the residue was dissolved in dichloromethane (200 ml) and treated with a solution of *p*-toluenesulfonic acid (PTSA) monohydrate (15 g, 78.85 mmol) in tetrahydrofuran (25 ml). After 2 min the reaction was quenched by addition of triethylamine (12 ml, 86.16 mmol). The reaction mixture was poured into vigorously stirred 1M aq. sodium hydrogen carbonate (300 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The crude product was purified by chromatography on silica gel (400 g) and eluted with hexane-ethyl acetate (3:1 and 2:1 v/v). Pure title compound was obtained as a solid white foam (14.17 g, 77.1 %) of $R_f$ 0.31 on TLC in hexane-ethyl acetate (2:1 v/v); Analysis of $C_{27}H_{48}N_2O_{10}Si_2$ requires C, 53.97; H, 8.07; N, 4.66; found C, 54.02; H, 8.10; N, 4.67. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 176.99 (POM C=O), 161.35 (C-4), 149.73 (C-2), 136.84 (C-6), 100.99 (C-5), 91.45 (C-1'), 81.76 (C-4'), 74.91 (C-2'), 69.03( C-3'), 64.31 (C-5'), 60.23 (POM $CH_2$), 38.52 (POM tBu, q), 26.73 (POM $CH_3$'s), 17.19-16.58 (isopropyl $CH_3$'s) and 13.13-12.31 (isopropyl CH's).

**3-*N*-Pivaloyloxymethyl-2'-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl]uridine (U5)**

Compound **U3** (9.01 g, 15.00 mmol) was dried by evaporation of anhydrous acetonitrile (50 ml) and dissolved in dry THF (100 ml). 1-(2-Fluorophenyl)-4-methoxy-1,2,3,6-tetrahydropyridine (9.10 g, 43.91 mmol) was added followed by trifluoroacetic acid (3.10 ml, 40.4 mmol). After 24 h at room temperature, an additional quantity of 1-(2-fluorophenyl)-4-methoxy-1,2,3,6-tetrahydropyridine (2.00 g, 9.65 mmol) was added and after a further period of 4 h the reaction was quenched with triethylamine (4 ml, 28.70 mmol). The reaction mixture was evaporated *in vacuo*. The dark oily residue was dissolved in ethyl acetate (250 ml), washed with 1M aq. sodium hydrogen carbonate (250 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The crude material was dissolved in dry tetrahydrofuran (75 ml) and treated with 1.1 M tetrabutylammonium fluoride in dry tetrahydrofuran (12 ml) for 15 min at room temperature. TLC showed complete reaction. The reaction mixture was quenched with pyridine-methanol-water (25 ml; 3:1:1 by volume) and pyridinium Dowex 50 W x 2 (100-200 mesh) resin (70 g) suspended in pyridine-methanol-water (100 ml) was added. The reaction mixture was stirred for 30 min at room temperature, then the resin was filtered off. The filtrate and washings were pooled, and evaporated to dryness under reduced pressure. Residual pyridine was removed by coevaporation with toluene (2x 50 ml). The crude product was purified by chromatography on silica gel (200 g) and eluted with a gradient of ethanol (0-5%) in dichloromethane. Pure title compound was obtained as a solid white foam (6.53 g, 76.9 %, calculated from **U3**) of $R_f$ 0.19 on TLC in dichloromethane-ethanol (95:5 v/v); Analysis of $C_{39}H_{62}FN_3O_{11}Si_2$ requires C, 57.96; H, 7.75; N, 5.20; found C, 58.00; H, 7.76; N, 5.21. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 177.44 (POM C=O), 161.53 (C-4), 157.59 and 153.68 (fluorophenyl C-2), 150.67 (C-2), 140.76 (C-6), 139.65 and 139.51 (fluorophenyl C-1), 124.37 (fluorophenyl C-5), 122.85 and 122.73 (fluorophenyl C-4), 119.38 (fluorophenyl C-6), 116.16 and 115.83 (fluorophenyl C-3), 102.19 (C-5), 100.24 (piperidine C-4), 89.37(C-1'), 85.85 (C-4'), 72.30 (C-2'), 71.36( C-3'), 64.70 (C-5'), 62.39 (POM $CH_2$), 47.96 ($OCH_3$), 47.75 (piperidine C-2, C-6), 38.71 (POM tBu, q), 34.22 and 32.75 (piperidine C-3, C-5) and 26.82 (POM $CH_3$'s).

**3-*N*-Pivaloyloxymethyl-2'-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl]-3'-*O*-(9-phenylxanthen-9-yl)uridine (U8)**

Compound **U5** (6.40 g, 11.32 mmol) was dried by evaporation of dimethylformamide (50 ml) under reduced pressure and the residue was dissolved in stirred, anhydrous dimethylformamide (100 ml). Imidazole (1.5 g, 22.03 mmol) and *tert*-butyl(chloro)diphenylsilane (3.2 ml, 12.31 mmol) were added to the stirred solution with exclusion of moisture, and the reaction mixture left for 4 h at room temperature. TLC showed complete reaction, and excess of reagent was quenched by addition of ethanol (5 ml). Solvent was removed under reduced pressure and the residue dissolved in ethyl acetate (200 ml), washed with 1M aq. sodium hydrogen carbonate (200 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The crude product was dried by evaporation of dry pyridine (50 ml) under reduced pressure. The residue was dissolved in stirred, anhydrous pyridine (80 ml), 9-chloro-9-phenylxanthene (4.1 g, 14.00 mmol) was added with exclusion of moisture, and the reaction mixture was left overnight at room temperature. TLC showed complete reaction, the solvent was removed under reduced pressure, and the residue was dissolved in dichloromethane (200 ml). The solution was washed with 1M aq. sodium hydrogen carbonate (200 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The resulting foam was redissolved in dry tetrahydrofuran (50 ml) and treated with 1.1 M tetrabutylammonium fluoride in dry

tetrahydrofuran (8 ml) for 15 min at room temperature. TLC showed complete reaction. The reaction mixture was quenched with pyridine-methanol-water (20 ml; 3:1:1 by volume) and pyridinium Dowex 50 W x 2 (100-200 mesh) resin (25 g) suspended in pyridine-methanol-water (50 ml) was added. The reaction mixture was stirred for 20 min at room temperature, then the resin was filtered off, the filtrate and washings were pooled, and evaporated to dryness under reduced pressure. Residual pyridine was removed by coevaporation with toluene. The crude product was purified by chromatography on silica gel (200 g) and eluted with hexane-ethyl acetate (2:1 and 1:1 v/v) containing 2% triethylamine. Pure title compound was obtained as a solid white foam (7.49 g, 80.5 %, calculated from **U5**) of $R_f$ 0.47 on TLC (pre-incubated with triethylamine) in dichloromethane-ethanol (95:5 v/v); Analysis of $C_{46}H_{48}FN_3O_{10}$ requires C, 67.22; H, 6.42; N, 5.11; found C, 67.27; H, 6.44; N, 5.12. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 177.08 (POM C=O), 161.31 (C-4), 157.43 and 153.53 (fluorophenyl C-2), 151.49 and 151.40 (xanthene C-4a, C-10a), 150.69 (C-2), 146.89 (pixyl phenyl C-1), 140.39 (C-6), 140.04 and 139.90 (fluorophenyl C-1), 131.70 and 131.56 (xanthene C-1, C-8), 129.89 and 129.56 (xanthene C-3, C-6), 127.56 (pixyl phenyl C-3, C-5), 127.42 (pixyl phenyl C-2, C-6), 126.71 (pixyl phenyl C-4), 124.49 and 124.12 (xanthene C-2, C-7), 123.03 (fluorophenyl C-5), 123.03 and 122.63 (xanthene C-8a, C-9a), 122.63 and 122.02 (fluorophenyl C-4), 119.04 (fluorophenyl C-6), 116.23 and 115.61 (xanthene C-4, C-5), 115.95 and 115.48 (fluorophenyl C-3), 101.60 (C-5), 99.54 (piperidine C-4), 87.62 (C-1'), 84.58 (C-4'), 76.50 (xanthene C-9), 73.47 (C-2'), 71.06 ( C-3'), 64.50 (C-5'), 61.92 (POM $CH_2$), 47.73 and 47.14 (piperidine C-2, C-6), 46.87 ($OCH_3$), 38.49 (POM tBu, q), 34.95 and 31.17 (piperidine C-3, C-5) and 26.73 (POM $CH_3$'s).

**3-*N*-Pivaloyloxymethyl-2'-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl]-3'-*O*-(9-phenylxanthen-9-yl)uridine-5'-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (U9)**

Compound **U8** (5.99 g, 7.27 mmol) was dried by evaporation of anhydrous acetonitrile under reduced pressure at room temperature. The residue was dissolved in dry 1,2-dichloroethane (60 ml) containing *N*,*N*-diisopropylethylamine (3.3 ml, 18.38 mmol) under argon, cooled to 0°C, and methoxy *N*,*N*-diisopropylaminochlorophosphine (2.2 ml, 11.33 mmol) was added to the stirred solution with exclusion of moisture. The reaction mixture was stirred at room temperature. TLC showed complete reaction after 50 min. The reaction mixture was diluted with dichloromethane (140 ml), washed with 1M aq. sodium hydrogen carbonate (200 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The crude product was purified by chromatography on silica gel (150 g) eluting with hexane-dichloromethane (3:1 v/v) containing 3 % triethylamine. Pure title compound was obtained as a solid white foam (6.40 g, 89.4 % ) of $R_f$ 0.37 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (2:1 v/v); Analysis of $C_{53}H_{64}FN_4O_{11}P$ requires C, 64.75; H, 6.58; N, 5.70; found C, 64.79; H, 6.59; N, 5.72. $^{31}P$ NMR, $\delta_P(CH_2Cl_2$, concentric external $D_2O$ lock): 147.02 and 146.91.

**4-*N*-Benzoyl-2'-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl]-3'-*O*-(9-phenylxanthen-9-yl)cytidine-5'-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (C9)**

Compound **C9** was prepared from **C8** (5.94 g, 7.45 mmol) in the same manner as described for **U9**. The product was purified by chromatography on silica gel (200 g) eluting with hexane-dichloromethane (2:1 and 1:1 v/v) containing 3% triethylamine. Pure title compound was obtained as a solid white foam (7.04 g, 97.2 %) of $R_f$ 0.15 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (1:1 v/v); Analysis of $C_{54}H_{59}FN_5O_9P$

requires C, 66.72; H, 6.13; N, 7.21; found C, 66.78; H, 6.16; N, 7.23. $^{31}P$ NMR, $\delta_P$ ($CH_2Cl_2$, concentric external $D_2O$ lock): 146.86 and 146.65.

**6-*N*-Pivaloyl-2'-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl]-3'-*O*-(9-phenylxanthen-9-yl)adenosine-5'-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (A9)**

Compound **A9** was prepared from **A8** (4.51 g, 5.53 mmol) in the same manner as described for **U9**. The crude product was purified by chromatography on silica gel eluting with hexane-dichloromethane (1:1 v/v) containing 3% triethylamine. Pure title compound was obtained as a solid white foam (5.15 g, 95.4 %) of $R_f$ 0.11 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (2:1 v/v); Analysis of $C_{53}H_{63}FN_7O_8P$ requires C, 65.21; H, 6.52; N, 10.05; found C, 65.24; H, 6.54; N, 10.06. $^{31}P$ NMR, $\delta_P$($CH_2Cl_2$, concentric external $D_2O$ lock): 146.49 and 146.21.

**2-*N*-Dimethylaminomethylidene-2'-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl]-3'-*O*-(9-phenylxanthen-9-yl)adenosine-5'-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (G9)**

Compound **G9** was prepared from **G8** (4.85 g, 7.25 mmol) in the same manner as described for **U9**. The crude product was purified by chromatography on silica gel (200 g) eluting with a gradient of dichloromethane (50-80%) in hexane containing 3% triethylamine. Pure title compound was obtained as a solid white foam (4.80 g, 79.7 %) of $R_f$ 0.55 on TLC (pre-incubated with triethylamine) in dichloromethane-ethanol (95:5 v/v); Analysis of $C_{51}H_{61}FN_8O_8P$ requires C, 63.53; H, 6.39; N, 11.63; found C, 63.57; H, 6.42; N, 11.65. $^{31}P$ NMR, $\delta_P$($CH_2Cl_2$, concentric external $D_2O$ lock): 146.91 and 146.38.

**3-*N*-Pivaloyloxymethyl-3'-*O*-(9-phenylxanthen-9-yl)-2'-deoxythymidine (dT8)**

Thymidine ( 4.8 g, 19.82 mmol) was dried by evaporation of dry pyridine under reduced pressure. The residue was dissolved in stirred, anhydrous pyridine (80 ml), *tert*-butyl(chloro)diphenylsilane (5.75 ml, 22.11 mmol) was added with exclusion of moisture, and the reaction mixture was left overnight at room temperature. TLC showed complete reaction. 9-Chloro-9-phenylxanthene (8.5 g, 29.03 mmol) was added with exclusion of moisture. TLC showed complete reaction after 1h at room temperature. Solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate (400 ml). The solution was washed with 1M aq. sodium hydrogen carbonate (400 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The resulting foam was redissolved in dry dimethylformamide (50 ml) and potassium carbonate (21.5 g, 155.56 mmol) followed by chloromethyl pivalate (11 ml, 76.32 mmol) were added with stirring and exclusion of moisture, and the reaction mixture left over night at room temperature. TLC showed complete reaction, the solvent was removed under reduced pressure, and the residue was mixed with ethyl acetate (400 ml) and filtered. The resulting foam was redissolved in dry tetrahydrofuran (50 ml) and treated with 1.1 M tetrabutylammonium fluoride in dry tetrahydrofuran (20 ml) for 15 min at room temperature. TLC showed complete reaction. The reaction mixture was quenched with pyridine-methanol-water (25 ml; 3:1:1 by volume) and pyridinium Dowex 50 W x 2 (100-200 mesh) resin (70 g) suspended in pyridine-methanol-water (100 ml) was added. The reaction mixture was stirred for 20 min at room temperature, then the resin was filtered off. The filtrate and washings were pooled, and evaporated to dryness under reduced pressure. Residual pyridine was removed by coevaporation with toluene. The crude product was then purified by chromatography on silica gel (300g) and eluted with hexane-ethyl acetate (2:1 and 1:1 v/v) containing 1 % triethylamine. The title compound

was obtained as a white foam (9.81 g, 80.9 %) of $R_f$ 0.16 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (1:1 v/v); Analysis of $C_{35}H_{36}N_2O_8$ requires C, 68.61; H, 5.93; N, 4.57; found C, 68.65; H, 5.94; N, 4.58. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 177.43 (POM C=O), 162.37 (C-4), 151.47 (C-2), 151.05 and 150.25 (xanthene C-4a and C-10a), 147.55 (C-1 pixyl phenyl), 134.98 (C-6), 130.87 and 130.60 (xanthene C-1, C-8), 129.70 (xanthene C-3, C-6), 127.75 (pixyl phenyl C-3, C-5), 127.08 (pixyl phenyl C-2, C-6), 126.89 (pixyl phenyl C-4), 123.54 and 123.43 (xanthene C-2, C-7), 123.18 and 122.90 (xanthene C-8a, C-9a), 116.37 (xanthene C-4, C-5), 110.13 (C-5), 86.03 (C-4'), 85.55 (C-1'), 76.55 (xanthene C-9), 72.07( C-3'), 64.97 (C-5'), 61.47 (POM $CH_2$), 38.82 (C-2'), 38.49 (POM tBu, q) and 26.95 (POM $CH_3$'s).

**3-*N*-Pivaloyloxymethyl-3'-*O*-(9-phenylxanthen-9-yl)-2'-deoxythymidine-5'-*O*-(methyl-*N*,*N*-diisopropylphosphoramidite) (dT9)**

Compound **dT8** (5.00 g, 8.17 mmol) was dried by evaporation of anhydrous acetonitrile under reduced pressure at room temperature. The residue was dissolved in dry 1,2-dichloroethane (25 ml) containing *N*,*N*-diisopropylethylamine (3.6 ml, 20.06 mmol) under argon, cooled to 0°C, and methoxy *N*,*N*-diisopropylaminochlorophosphine (2.38 ml, 12.26 mmol) was added to the stirred solution with exclusion of moisture. The reaction mixture was stirred at room temperature. TLC showed complete reaction after 1 h. The reaction mixture was diluted with dichloromethane (150 ml), washed with 1M aq. sodium hydrogen carbonate (200 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The crude product was then purified by chromatography on silica gel (130 g) and eluted with hexane-dichloromethane (5:1 v/v) containing 3 % triethylamine. The title compound was obtained as a white foam (5.75 g, 91.1 %) of $R_f$ 0.37 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (95:5 v/v); Analysis of $C_{42}H_{52}N_3O_9P$ requires C, 65.18; H, 6.79; N, 5.43; found C, 65.23; H, 6.81; N, 5.44. $^{31}P$ NMR, $\delta_P(CH_2Cl_2$, concentric external $D_2O$ lock): 149.19 and 149.96.

**3-*N*-Pivaloyloxymethyl-3'-*O*-(9-phenylxanthen-9-yl)-2'-deoxythymidine-5'-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (dT10)**

Compound **dT8** (1.00 g, 1.63 mmol) was dried by evaporation of anhydrous acetonitrile under reduced pressure at room temperature. The reside was dissolved in dry 1,2-dichloroethane (10 ml) containing *N*,*N*-diisopropylethylamine (0.8 ml, 4.46 mmol) under argon, cooled to 0°C, and cyanoethoxy *N*,*N*-diisopropylaminochlorophosphine (0.6 ml, 2.54 mmol) was added to the stirred solution with exclusion of moisture. The reaction mixture was stirred at room temperature. TLC showed complete reaction after 2 h. The reaction mixture was diluted with dichloromethane (90 ml), washed with 1M aq. sodium hydrogen carbonate (100 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The crude product was then purified by chromatography on silica gel (40 g) and eluted with hexane-dichloromethane (5:1 v/v) containing 3 % triethylamine. The title compound was obtained as a white foam (1.18 g, 89.0 %) of $R_f$ 0.46 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (95:5 v/v); Analysis of $C_{44}H_{53}N_4O_{10}P$ requires C, 65.01; H, 6.59; N, 6.89; found C, 65.06; H, 6.60; N, 6.91. $^{31}P$ NMR, $\delta_P(CH_2Cl_2$, concentric external $D_2O$ lock): 148.78 and 148.13.

**4-*N*-Benzoyl-3'-*O*-(9-phenylxanthen-9-yl)-2'-deoxycytidine (dC8)**

Deoxycytidine (5.03 g, 22.14 mmol) was dried by evaporation of anhydrous pyridine and mixed with dry pyridine (100 ml) under argon. The mixture was cooled on ice and trimethylsilyl chloride (10 ml, 79.06 mmol) in pyridine (20 ml) was added dropwise over 15 min. TLC showed complete reaction. Benzoyl chloride (8 ml, 53.76 mmol) in pyridine (20 ml) was added dropwise over 20 min. After 1 h at room temperature the solution was cooled on ice. Water (20 ml) and a 30% aqueous solution of $NH_3$ (20 ml) were added with stirring. After 30 min at room temperature the solvent was evaporated *in vacuo* and residual water removed by coevaporation of dry pyridine (2x25 ml). The resulting foam was dissolved in dioxane (100 ml) and filtered. The solution was evaporated *in vacuo* and the residue redissolved in dry pyridine (100 ml). *tert*-Butyl(chloro)diphenylsilane (9 ml, 34.61 mmol) was added with exclusion of moisture, and the reaction mixture was left overnight at room temperature. TLC showed complete reaction. 9-Chloro-9-phenylxanthene (8.0 g, 27.32 mmol) was added with exclusion of moisture. TLC showed complete reaction after 1h at room temperature. Solvent was removed under reduced pressure and residual pyridine removed by coevaporation of toluene (2x40 ml). The residue was dissolved in ethyl acetate (400 ml), washed with 1M aq. sodium hydrogen carbonate (400 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The compound was then disilylated and worked up according to the procedure used to prepare compound **dT1**. The crude product was then purified by chromatography on silica gel (250g) and eluted with a gradient of ethanol (0-5 %) in dichloromethane containing 1 % triethylamine. The title compound was obtained as a white foam (9.54 g, 75.1 %) of $R_f$ 0.39 on TLC (pre-incubated with triethylamine) in ethanol/dichloromethane (5:95 v/v); Analysis of $C_{35}H_{29}N_3O_6P$ requires C, 71.53; H, 4.98; N, 7.15; found C, 71.57; H, 4.99; N, 7.17. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 167.45 (benzoyl C=O), 162.09 (C-4), 154.75 (C-2), 151.33 and 150.88 (xanthene C-4a and C-10a), 147.83 (C-1 pixyl phenyl), 145.19 (C-6), 132.92 (benzoyl C-4), 130.80 and 130.53 (xanthene C-1, C-8), 129.58 (xanthene C-3, C-6), 128.79 (benzoyl C-3, C-5), 127.70 (pixyl phenyl C-3, C-5), 127.56 (bezoyl C-2, C-6), 127.01 (pixyl phenyl C-2, C-6), 126.77 (pixyl phenyl C-4), 123.57 and 123.27 (xanthene C-2, C-7), 123.16 and 122.71 (xanthene C-8a, C-9a), 116.42 and 116.32 (xanthene C-4, C-5), 96.68 (C-5), 87.59 (C-4'), 86.56 (C-1'), 77.19 (xanthene C-9), 72.17( C-3'), 61.27 (C-5') and 40.25 (C-2').

**4-*N*-Benzoyl-3'-*O*-(9-phenylxanthen-9-yl)-2'-deoxycytidine-5'-*O*-(methyl-*N*,*N*-diisopropylphosphoramidite) (dC9)**

Compound **dC8** (2.99 g, 5.21 mmol) was phosphitylated according to the procedure used to synthesise compound **dT9** above. The crude product was purified by chromatography on silica gel (80 g) and eluted with hexane-dichloromethane (2:1-1:2 v/v) containing 3 % triethylamine. The title compound was obtained as a white foam (3.39 g, 88.5 %) of $R_f$ 0.32 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (1:1 v/v); Analysis of $C_{42}H_{46}N_4O_7P$ requires C, 67.36; H, 6.20; N, 7.48; found C, 67.38; H, 6.22; N, 7.49. $^{31}P$ NMR, $\delta_P(CH_2Cl_2$, concentric external $D_2O$ lock): 149.21 and 148.12.

**4-*N*-Benzoyl-3'-*O*-(9-phenylxanthen-9-yl)-2'-deoxycytidine-5'-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (dC10)**

Compound **dC8** (1.05 g, 1.79 mmol) was phosphitylated according to the procedure used to synthesise compound **dT10** above. The crude product was purified by chromatography on silica gel (40 g) and eluted with

hexane-dichloromethane (2:1-1:2 v/v) containing 3 % triethylamine. The title compound was obtained as a white foam (1.24 g, 87.9 %) of $R_f$ 0.38 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (1:1 v/v); Analysis of $C_{44}H_{47}N_5O_7P$ requires C, 67.07; H, 6.03; N, 8.89; found C, 67.07; H, 6.06; N, 8.93. $^{31}P$ NMR, $\delta_P(CH_2Cl_2$, concentric external $D_2O$ lock): 148.70 and 148.02.

**6-*N*-Benzoyl-3'-*O*-(9-phenylxanthen-9-yl)-2'-deoxyadenosine (dA8)**

Compound **dA8** was prepared from deoxyadenosine (5.09 g, 22.26 mmol) according to the procedure used to synthesise compound **dC8** above. The crude product was then purified by chromatography on silica gel (250g) and eluted with a gradient of ethanol (0-5 %) in dichloromethane containing 1 % triethylamine. The title compound was obtained as a white foam (9.01 g, 74.4 %) of $R_f$ 0.34 on TLC (pre-incubated with triethylamine) in ethanol/dichloromethane (5:95 v/v); Analysis of $C_{36}H_{29}N_5O_5$ requires C, 70.69; H, 4.79; N, 11.45; found C, 70.74; H, 4.81; N, 11.47. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 164.62 (benzoyl C=O), 151.91 (C-2), 151.30 and 150.14 (xanthene C-4a and C-10a), 150.64 (C-6), 150.02 (C-4), 147.86 (C-1 pixyl phenyl), 142.23 (C-8), 133.48 (benzoyl C-1), 132.68 (benzoyl C-4), 130.45 (xanthene C-1, C-8), 129.68 and 129.56 (xanthene C-3, C-6), 128.69 (benzoyl C-3, C-5), 127.86 (pixyl phenyl C-3, C-5), 127.72 (benzoyl C-2, C-6), 127.02 (pixyl phenyl C-2, C-6), 126.82 (pixyl phenyl C-4), 124.17 (C-5), 123.36 (xanthene C-2, C-7), 122.86 (xanthene C-8a, C-9a), 116.63 (xanthene C-4, C-5), 88.06 (C-4'), 87.12 (C-1'), 76.79 (xanthene C-9), 74.05 ( C-3'), 62.46 (C-5') and 39.38 (C-2').

**6-*N*-Benzoyl-3'-*O*-(9-phenylxanthen-9-yl)-2'-deoxyadenosine-5'-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (dA9)**

Compound **dA8** (2.98 g, 4.98 mmol) was phosphitylated according to the procedure used to synthesise compound **dT9** above. The crude product was purified by chromatography on silica gel (80 g) and eluted with hexane-dichloromethane (2:1-1:2 v/v) containing 3 % triethylamine. The title compound was obtained as a white foam (3.42 g, 90.5 %) of $R_f$ 0.28 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (1:1 v/v); Analysis of $C_{43}H_{45}N_6O_6P$ requires C, 66.82; H, 5.88; N, 10.88; found C, 66.85; H, 5.89; N, 10.90. $^{31}P$ NMR, $\delta_P(CH_2Cl_2$, concentric external $D_2O$ lock): 148.56 and 147.80.

**6-*N*-Benzoyl-3'-*O*-(9-phenylxanthen-9-yl)-2'-deoxyadenosine-5'-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (dA10)**

Compound **dA8** (1.11 g, 1.81 mmol) was phosphitylated according to the procedure used to synthesise compound **dT10** above. The crude product was purified by chromatography on silica gel (40 g) and eluted with hexane-dichloromethane (2:1-1:2 v/v) containing 3 % triethylamine. The title compound was obtained as a white foam (1.28 g, 87.1 %) of $R_f$ 0.34 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (1:1 v/v); Analysis of $C_{45}H_{46}N_7O_6P$ requires C, 65.58; H, 5.72; N, 12.08; found C, 65.64; H, 5.75; N, 12.10. $^{31}P$ NMR, $\delta_P(CH_2Cl_2$, concentric external $D_2O$ lock): 149.01 and 148.11.

**2-*N*-Dimethylaminomethylidene-3'-*O*-(9-phenylxanthen-9-yl)-2'-deoxyguanosine (dG8)**

Deoxyguanosine (5.0 g, 18.71 mmol) was dissolved in dry methanol (100 ml) under argon and dimethylformamide dimethyl acetal (10 ml, 75.02 mmol) was added. The mixture was stirred overnight at room temperature. TLC showed complete reaction. The solvent was evaporated in vacuo. The resulting white solid

compound was then converted to compound **dG8** according to the procedure used to synthesise compound **dC8** above. The crude product was then purified by chromatography on silica gel (200g) and eluted with ethanol/dichloromethane (5:95 v/v) in containing 2 % triethylamine. The title compound was obtained as a white foam (8.51 g, 78.6 %) of $R_f$ 0.09 on TLC (pre-incubated with triethylamine) in ethanol/dichloromethane (5:95 v/v); Analysis of $C_{32}H_{31}N_6O_5$ requires C, 66.30; H, 5.40; N, 14.50; found C, 66.34; H, 5.41; N, 14.53. $^{13}C$ NMR, $\delta_C$(DMSO); 157.97 (amidine CH), 157.49 (C-6), 157.26 (C-2), 150.59 and 150.38 (xanthene C-4a and C-10a), 149.47 (C-4), 148.26 (C-1 pixyl phenyl), 136.47 (C-8), 130.42 and 130.30 (xanthene C-1, C-8), 129.81 (xanthene C-3, C-6), 127.88 (pixyl phenyl C-3, C-5), 126.74 (pixyl phenyl C-4), 126.56 (pixyl phenyl C-2, C-6), 123.56 (xanthene C-2, C-7), 122.75 and 122.66 (xanthene C-8a, C-9a), 119.68 (C-5), 116.30 (xanthene C-4, C-5), 86.31 (C-4'), 83.07 (C-1'), 75.93 (xanthene C-9), 73.83 ( C-3'), 61.05 (C-5') 40.64 and 34.59 (amidine $CH_3$'s) and 34.59 (C-2').

**2-*N*-Dimethylaminomethylidene-3'-*O*-(9-phenylxanthen-9-yl)-2'-deoxyguanosine-5'-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (dG9)**

Compound **dG8** (0.95 g, 4.98 mmol) was phosphitylated according to the procedure used to synthesise compound **dT9** above. The crude product was purified by chromatography on silica gel (80 g) and eluted with hexane-dichloromethane (2:3-1:3 v/v) containing 3 % triethylamine. The title compound was obtained as a white foam (3.26 g, 87.2 %) of $R_f$ 0.50 on TLC (pre-incubated with triethylamine) in ethanol/dichloromethane (5:95 v/v); Analysis of $C_{39}H_{47}N_7O_6P$ requires C, 63.22; H, 6.41; N, 13.24; found C, 63.25; H, 6.44; N, 13.25. $^{31}P$ NMR, $\delta_P$($CH_2Cl_2$, concentric external $D_2O$ lock): 148.97 and 147.87.

**2-*N*-Dimethylaminomethylidene-3'-*O*-(9-phenylxanthen-9-yl)-2'-deoxyguanosine-5'-*O*-(2'-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (dG10)**

Compound **dG8** (2.98 g, 4.98 mmol) was phosphitylated according to the procedure used to synthesise compound **dT10** above. The crude product was purified by chromatography on silica gel (80 g) and eluted with hexane-dichloromethane (2:3-1:3 v/v) containing 3 % triethylamine. The title compound was obtained as a white foam (3.26 g, 87.2 %) of $R_f$ 0.50 on TLC (pre-incubated with triethylamine) in ethanol/dichloromethane (5:95 v/v); Analysis of $C_{41}H_{48}N_8O_6P$ requires C, 63.14; H, 6.22; N, 14.37; found C, 63.17; H, 6.24; N, 14.39. $^{31}P$ NMR, $\delta_P$($CH_2Cl_2$, concentric external $D_2O$ lock): 149.34 and 148.58.

**3-*N*-Pivaloyloxymethyl-2'-*O*-(9-phenylxanthen-9-yl)uridine (U14)**

Compound **U3** ( 4.9 g, 8.16 mmol) was dried by evaporation of dry pyridine under reduced pressure. The residue was dissolved in stirred, anhydrous pyridine (80 ml), 9-chloro-9-phenylxanthene (4.1 g, 14.00 mmol) was added with exclusion of moisture, and the reaction mixture was left overnight at room temperature. TLC showed complete reaction, solvent was removed under reduced pressure, and the residue was dissolved in dichloromethane (200 ml). The solution was washed with 1M aq. sodium hydrogen carbonate (200 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The resulting foam was redissolved in dry tetrahydrofuran (50 ml) and treated with 1.1 M tetrabutylammonium fluoride in dry tetrahydrofuran (8 ml) for 15 min at room temperature. TLC showed complete reaction. The reaction mixture was quenched with pyridine-methanol-water (20 ml; 3:1:1 by volume) and pyridinium Dowex 50 W x 2 (100-200 mesh) resin (25 g) suspended in pyridine-methanol-water (50 ml) was added. The reaction mixture was stirred

for 20 min at room temperature, then the resin was filtered of, the filtrate and washings were pooled, and evaporated to dryness under reduced pressure. Residual pyridine was removed by coevaporation with toluene. The crude product was then purified by chromatography on silica gel (100 g) and eluted with hexane-ethyl acetate (1:1 v/v) containing 1 % triethylamine. The title compound was obtained as a white foam (4.19 g, 83.5 %) of $R_f$ 0.16 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (1:1 v/v); Analysis of $C_{34}H_{36}N_2O_9$ requires C, 66.44; H, 6.44; N, 4.56; found C, 66.48; H, 6.45; N, 4.57. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 177.50 (POM C=O), 161.53 (C-4), 151.19 and 151.10 (xanthene C-4a, C-10a), 150.12 (C-2), 146.24 (pixyl phenyl C-1), 139.06 (C-6), 130.34 and 129.81 (xanthene C-1, C-8), 129.81 and 129.68 (xanthene C-3, C-6), 127.92 (pixyl phenyl C-3, C-5), 127.16 (pixyl phenyl C-4), 127.05 (pixyl phenyl C-2, C-6), 123.89 and 123.69 (xanthene C-2, C-7), 122.32 and 121.93 (xanthene C-8a, C-9a), 116.73 and 116.35 (xanthene C-4, C-5), 102.25 (C-5), 88.72 (C-1'), 85.79 (C-4'), 77.51 (xanthene C-9), 75.07 (C-2'), 70.66 ( C-3'), 64.90 (C-5'), 62.83 (POM $CH_2$), 38.76 (POM tBu, q) and 26.97 (POM $CH_3$'s).

### 3-*N*-Pivaloyloxymethyl-2'-*O*-(9-phenylxanthen-9-yl)-5'-*O*-(*tert*-butoxydiphenylsilyl)uridine (U15)

Compound **U14** (4.10 g, 6.67 mmol) was dried by evaporation of dimethylformamide (50 ml) under reduced pressure and the residue was dissolved in stirred, anhydrous dimethylformamide (100 ml). Imidazole (1.5 g, 22.03 mmol) and *tert*-butoxy(chloro)diphenylsilane (2.2 ml, 7.03 mmol) were added to the stirred solution with exclusion of moisture, and the reaction mixture left for 4 h at room temperature. TLC showed complete reaction, so excess of reagent was quenched by addition of ethanol (1 ml). Solvent was removed under reduced pressure and the residue dissolved in ethyl acetate (200 ml), washed with 1M aq. sodium hydrogen carbonate (200 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The crude product was then purified by chromatography on silica gel (200 g) and eluted with hexane-ethyl acetate (3:1 and 2:1 v/v) containing 1 % triethylamine. The title compound was obtained as a white foam (4.85 g, 83.6 %) of $R_f$ 0.30 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (2:1 v/v); Analysis of $C_{50}H_{54}N_2O_{10}Si$ requires C, 69.10; H, 6.28; N, 3.22; found C, 69.13; H, 6.30; N, 3.23. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 177.00 (POM C=O), 161.11 (C-4), 150.79 (C-2), 150.23 and 150.14 (xanthene C-4a, C-10a), 146.73 (pixyl phenyl C-1), 137.29 (C-6), 134.40 (PhSi C-2's, C-6's), 133.45 and 132.92 (PhSi C-1's), 130.34 and 129.99 (xanthene C-1, C-8), 129.70 and 129.54 (xanthene C-3, C-6), 127.67 (pixyl phenyl C-3, C-5 and PhSi C-3's, C-5's), 126.76 (pixyl phenyl C-4), 126.50 (pixyl phenyl C-2, C-6), 123.57 (xanthene C-2, C-7), 121.98 and 121.84 (xanthene C-8a, C-9a), 116.54 and 116.20 (xanthene C-4, C-5), 101.87 (C-5), 85.98 (C-1'), 85.50 (C-4'), 77.18 (xanthene C-9), 76.02 (C-2'), 73.75 (tBuO, q), 70.33 ( C-3'), 64.92 (C-5'), 62.91 (POM $CH_2$), 38.45 (POM tBu, q), 31.62 (tBuO, $CH_3$'s) and 26.97 (POM $CH_3$'s).

### 3-*N*-Pivaloyloxymethyl-2'-*O*-(9-phenylxanthen-9-yl)-3'-*O*-laevulinyl-5'-*O*-(*tert*-butoxydiphenylsilyl)uridine (U16)

Compound **U15** (4.85 g, 5.58 mmol) was dissolved in dry dichloromethane (100 ml). 4-Dimethylaminopyridine (50 mg, 0.41 mmol), triethylamine (10 ml, 71.75 mmol) and laevulinic anhydride (3.0 ml, 14.00 mmol) were added to the stirred solution with exclusion of moisture, and the reaction mixture left at room temperature overnight. TLC showed complete reaction. The reaction mixture was diluted with dichloromethane (100 ml) and poured into vigorously stirred 1M aq. sodium hydrogen carbonate (200 ml). The organic layer was separated,

dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The crude product was then purified by chromatography on silica gel (120 g) and eluted with hexane-ethyl acetate (3:1, 2:1 and 1:1 v/v) containing 1 % triethylamine. The title compound was obtained as a white foam (4.56 g, 84.4 %) of $R_f$ 0.16 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (2:1 v/v); Analysis of $C_{55}H_{60}N_2O_{12}Si$ requires C, 70.71; H, 6.49; N, 3.00; found C, 70.75; H, 6.51; N, 3.02. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 205.76 (laevulinyl C=O), 177.35 (POM C=O), 171.71 (laevulinyl $O_2C$) 161.28 (C-4), 150.62 (C-2), 150.62 and 150.40 (xanthene C-4a, C-10a), 147.63 (pixyl phenyl C-1), 137.02 (C-6), 134.63 and 134.55 (PhSi C-2's, C-6's), 133.71 and 133.01 (PhSi C-1's), 130.62 and 129.97 (xanthene C-1, C-8), 129.75 and 129.53 (xanthene C-3, C-6), 127.63 (pixyl phenyl C-3, C-5 and PhSi C-3's, C-5's), 126.63 (pixyl phenyl C-2, C-4, C-6), 123.39 (xanthene C-2, C-7), 122.33 and 121.67 (xanthene C-8a, C-9a), 116.42 and 116.28 (xanthene C-4, C-5), 102.17(C-5), 85.92 (C-1'), 84.70 (C-4'), 76.90 (xanthene C-9), 74.30 (C-2'), 74.02 (tBuO, q), 72.62 ( C-3'), 65.02 (C-5'), 62.70 (POM $CH_2$), 38.69 (POM tBu, q), 37.66 (laevulinyl C-2), 31.83 (tBuO, $CH_3$'s), 29.60 (laevulinyl C-5), 28.02 (laevulinyl C-3) and 26.93 (POM $CH_3$'s).

**3-*N*-Pivaloyloxymethyl-2'-*O*-(9-phenylxanthen-9-yl)-3'-*O*-laevulinyluridine (U17)**

Compound **U16** (4.56 g, 4.71 mmol) was dissolved in dry tetrahydrofuran (50 ml) and treated with 1.1 M tetrabutylammonium fluoride in dry tetrahydrofuran (6 ml) for 10 min at room temperature. TLC showed complete reaction. The reaction mixture was quenched with pyridine-methanol-water (20 ml; 3:1:1 by volume) and pyridinium Dowex 50 W x 2 (100-200 mesh) resin (15 g) suspended in pyridine-methanol-water (30 ml) was added. The reaction mixture was stirred for 20 min at room temperature, then the resin was filtered off. The filtrate and washings were pooled, and evaporated to dryness under reduced pressure. Residual pyridine was removed by coevaporation with toluene. The crude product was then purified by chromatography on silica gel (100 g) and eluted with hexane-ethyl acetate (1:1 and 1:3 v/v) containing 1 % triethylamine. The title compound was obtained as a white foam (3.05 g, 90.8 %) of $R_f$ 0.29 on TLC (pre-incubated with triethylamine) in dichloromethane-ethanol (95:5 v/v); Analysis of $C_{39}H_{42}N_2O_{11}$ requires C, 68.91; H, 6.24; N, 4.12; found C, 68.94; H, 6.25; N, 4.13. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 205.97 (laevulinyl C=O), 177.42 (POM C=O), 171.79 (laevulinyl $O_2C$), 161.48 (C-4), 151.19 (C-2), 150.83 and 150.23 (xanthene C-4a, C-10a), 147.07 (pixyl phenyl C-1), 137.21 (C-6), 130.67 and 130.04 (xanthene C-1, C-8), 129.78 and 129.61 (xanthene C-3, C-6), 127.69 (pixyl phenyl C-3, C-5), 126.96 (pixyl phenyl C-2, C-6), 126.76 (pixyl phenyl C-4), 123.64 and 123.39 (xanthene C-2, C-7), 122.24 and 121.80 (xanthene C-8a, C-9a), 116.21 and 116.13 (xanthene C-4, C-5), 102.30 (C-5), 87.57 (C-1'), 84.83 (C-4'), 77.00 (xanthene C-9), 73.37 (C-2'), 73.01 ( C-3'), 64.87 (C-5'), 62.23 (POM $CH_2$), 38.70 (POM tBu, q), 37.63 (laevulinyl C-2), 29.66 (laevulinyl C-5), 28.00 (laevulinyl C-3) and 26.93 (POM $CH_3$'s).

**3-*N*-Pivaloyloxymethyl-2'-*O*-(9-phenylxanthen-9-yl)-3'-*O*-laevulinyluridine-5'-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (U18)**

Compound **U17** (2.08 g, 2.92 mmol) was dried by evaporation of anhydrous acetonitrile under reduced pressure at room temperature. The residue was dissolved in dry 1,2-dichloroethane (25 ml) containing *N*,*N*-diisopropylethylamine (1.3 ml, 7.24 mmol) under argon, cooled to 0°C, and methoxy *N*,*N*-diisopropylaminochlorophosphine (0.85 ml, 4.38 mmol) was added to the stirred solution with exclusion of moisture. The reaction mixture was stirred at room temperature. TLC showed complete reaction after 1 h. The

reaction mixture was diluted with dichloromethane (150 ml), washed with 1M aq. sodium hydrogen carbonate (200 ml), the organic layer separated, dried ($Na_2SO_4$), filtered and evaporated to dryness under reduced pressure. The crude product was then purified by chromatography on silica gel (60 g) and eluted with hexane-dichloromethane (2:1 v/v) containing 3 % triethylamine. The title compound was obtained as a white foam (2.49 g, 97.6 %) of $R_f$ 0.51 on TLC (pre-incubated with triethylamine) in dichloromethane-ethanol (95:5 v/v); Analysis of $C_{46}H_{59}N_3O_{12}P$ requires C, 65.69; H, 6.97; N, 5.00; found C, 65.74; H, 6.98; N, 5.03. $^{31}P$ NMR, $\delta_P(CH_2Cl_2$, concentric external $D_2O$ lock): 145.42 and 144.98.

**4-*N*-Benzoyl-2'-*O*-(9-phenylxanthen-9-yl)cytidine (C14)**

Compound **C3** ( 7.5 g, 12.72 mmol) was converted into **C14** according to the procedure used to synthesise compound **U14** above. The crude product was then purified by chromatography on silica gel (100 g) and eluted with hexane-ethyl acetate (1:1 v/v) containing 1 % triethylamine. The title compound was obtained as a white foam (7.16 g, 93.2 %) of $R_f$ 0.19 on TLC (pre-incubated with triethylamine) in ethanol-dichloromethane (5:95 v/v); Analysis of $C_{35}H_{29}N_3O_7$ requires C, 69.64; H, 4.85; N, 9.96; found C, 69.69; H, 4.86; N, 9.97. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 167.01 (benzoyl C=O), 161.75 (C-4), 152.91 (C-2), 151.11 and 150.45 (xanthene C-4a, C-10a), 146.71 (pixyl phenyl C-1), 142.34 (C-6), 133.69 (benzoyl C-1), 132.56 (benzoyl C-4), 130.41 (benzoyl C-3, C-5), 130.11 and 129.81 (xanthene C-1, C-8), 129.71 (xanthene C-3, C-6), 128.63 (benzoyl C-2, C-6), 127.98 (pixyl phenyl C-3, C-5 ), 126.81 (pixyl phenyl C-4), 126.54 (pixyl phenyl C-2, C-6), 124.01 and 123.75 (xanthene C-2, C-7), 122.09 and 121.69 (xanthene C-8a, C-9a), 116.89 and 115.91 (xanthene C-4, C-5), 98.45 (C-5), 86.13 (C-1'), 85.89 (C-4'), 77.44 (xanthene C-9), 76.91 (C-2'), 73.78 (tBuO, q), 70.31 ( C-3'), 62.90 (C-5') and 31.09 (tBuO, $CH_3$'s).

**4-*N*-Benzoyl-2'-*O*-(9-phenylxanthen-9-yl)-5'-*O*-(*tert*-butoxydiphenylsilyl)cytidine (C15)**

Compound **C14** (7.16 g, 11.87 mmol) was silylated according to the procedure used to synthesise compound **U15** above. The crude product was purified by chromatography on silica gel (250 g) and eluted with hexane-ethyl acetate (2:1 v/v) and ethyl acetate-ethanol (97:3 v/v) containing 1 % triethylamine. The title compound was obtained as a white foam (7.65 g, 75.7 %) of $R_f$ 0.32 on TLC (pre-incubated with triethylamine) in ethanol-dichloromethane (5:95 v/v); Analysis of $C_{51}H_{47}N_3O_8Si$ requires C, 79.31; H, 5.53; N, 4.90; found C, 79.33; H, 5.54; N, 4.93. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 166.90 (benzoyl C=O), 161.56 (C-4), 153.79 (C-2), 151.03 and 150.25 (xanthene C-4a, C-10a), 146.82 (pixyl phenyl C-1), 142.68 (C-6), 134.61 (PhSi C-2's, C-6's), 133.65 (benzoyl C-1), 133.65 and 133.32 (PhSi C-1's), 132.87 (benzoyl C-4), 130.19 (benzoyl C-3, C-5), 130.05 and 129.98 (xanthene C-1, C-8), 129.75 (xanthene C-3, C-6), 129.53 (phenyl Si C-4), 128.71 (benzoyl C-2, C-6), 127.86 (pixyl phenyl C-3, C-5 and PhSi C-3's, C-5's), 126.90 (pixyl phenyl C-4), 126.80 (pixyl phenyl C-2, C-6), 124.06 and 123.62 (xanthene C-2, C-7), 122.17 and 121.80 (xanthene C-8a, C-9a), 116.70 and 115.98 (xanthene C-4, C-5), 98.40 (C-5), 86.09 (C-1'), 85.73 (C-4'), 77.50 (xanthene C-9), 76.89 (C-2'), 73.95 (tBuO, q), 70.44 ( C-3'), 62.96 (C-5') and 31.83 (tBuO, $CH_3$'s).

**4-*N*-Benzoyl-2'-*O*-(9-phenylxanthen-9-yl)-3'-*O*-laevulinyl-5'-*O*-(*tert*-butoxydiphenylsilyl)cytidine (C16)**

Compound **C15** (7.65 g, 8.92 mmol) was treated with laevulinic anhydride according to the procedure used to synthesise compound **U16** above. The crude product was purified by chromatography on silica gel (300 g) and

eluted with a gradient of ethanol (0-5 %) in dichloromethane containing 1 % triethylamine. The title compound was obtained as a white foam (8.41 g, 98.6 %) of $R_f$ 0.29 on TLC (pre-incubated with triethylamine) in ethanol-dichloromethane (5:95 v/v); Analysis of $C_{56}H_{53}N_3O_{10}Si$ requires C, 70.34; H, 5.60; N, 4.40; found C, 70.37; H, 5.62; N, 4.41. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 205.57 (laevulinyl C=O), 171.47 (laevulinyl $CO_2$), 161.63 (C-4), 153.74 (C-2), 150.40 and 150.22 (xanthene C-4a, C-10a), 147.47 (pixyl phenyl C-1), 141.99 (C-6), 134.37 (PhSi C-2's, C-6's), 133.48 and 133.36 (PhSi C-1's), 133.03 (benzoyl C-1), 132.58 (benzoyl C-4), 130.46 and 129.94 (xanthene C-1, C-8), 129.94 and 129.63 (benzoyl C-3, C-5), 129.63 and 129.17 (xanthene C-3, C-6), 128.42 (benzoyl C-2, C-6), 127.62 (PhSi C-3's, C-5's), 127.42 (pixyl phenyl C-3, C-5), 126.50 (pixyl phenyl C-4), 126.50 and 126.37 (pixyl phenyl C-2, C-6), 123.75 and 123.03 (xanthene C-2, C-7), 121.92 and 121.56 (xanthene C-8a, C-9a), 116.15 and 115.63 (xanthene C-4, C-5), 98.27 (C-5), 85.43 (C-1'), 84.50 (C-4'), 76.67 (xanthene C-9), 75.39 (C-2'), 73.72 (tBuO, q), 72.38 ( C-3'), 62.41 (C-5'), 37.41 (laevulinyl C-2), 31.62 (tBuO, $CH_3$'s), 29.35 (laevulinyl C-5), 27.81 (laevulinyl C-3).

**4-*N*-Benzoyl-2'-*O*-(9-phenylxanthen-9-yl)-3'-*O*-laevulinylcytidine (C17)**

Compound **C16** (8.41 g, 8.80 mmol) was desilylated according to the procedure used to synthesise compound **U17** above. The crude product was then purified by chromatography on silica gel (200 g) and eluted with a gradient of ethanol (0-2 %) in dichloromethane containing 2 % triethylamine. The title compound was obtained as a white foam (5.57 g, 90.3 %) of $R_f$ 0.61 on TLC (pre-incubated with triethylamine) in dichloromethane-ethanol (9:1 v/v); Analysis of $C_{40}H_{35}N_3O_9$ requires C, 68.46; H, 5.04; N, 5.99; found C, 68.51; H, 5.05; N, 6.01. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 207.14 (laevulinyl C=O), 172.61 (laevulinyl $O_2C$), 163.42 (C-4), 156.01 (C-2), 151.74 and 151.59 (xanthene C-4a, C-10a), 147.84 (pixyl phenyl C-1), 145.69 (C-6), 133.66 (benzoyl C-4), 131.27 and 130.60 (xanthene C-1, C-8), 130.60 and 130.33 (xanthene C-3, C-6), 130.23 (benzoyl C-3), 128.98 (benzoyl C-2, C-6), 128.56 (benzoyl C-3, C-5), 128.23 (pixyl phenyl C-3, C-5), 127.79 (pixyl phenyl C-2, C-6), 127.39 (pixyl phenyl C-4), 124.23 and 123.87 (xanthene C-2, C-7), 122.69 and 122.52 (xanthene C-8a, C-9a), 117.00 and 116.71 (xanthene C-4, C-5), 98.98 (C-5), 90.01 (C-1'), 86.25 (C-4'), 77.72 (xanthene C-9), 74.60 (C-2'), 74.02 ( C-3'), 62.62 (C-5'), 38.33 (laevulinyl C-2), 30.27 (laevulinyl C-5), 28.69 (laevulinyl C-3).

**4-*N*-Benzoyl-2'-*O*-(9-phenylxanthen-9-yl)-3'-*O*-laevulinylcytidine-5'-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (C18)**

Compound **C17** (5.50 g, 7.85 mmol) was phosphitylated according to the procedure used to synthesise compound **U18** above. The crude product was then purified by chromatography on silica gel (200 g) and eluted with a gradient of dichloromethane (33-66 %) in hexane containing 4 % triethylamine. The title compound was obtained as a white foam (5.89 g, 87.0 %) of $R_f$ 0.45 on TLC (pre-incubated with triethylamine) in dichloromethane-ethanol (95:5 v/v); Analysis of $C_{47}H_{41}N_4O_{10}P$ requires C, 65.41; H, 4.88; N, 6.49; found C, 65.45; H, 4.89; N, 6.50. $^{31}P$ NMR, $\delta_P(CH_2Cl_2$, concentric external $D_2O$ lock): 146.39 and 146.04

**6-*N*-Pivaloyl-2'-*O*-(9-phenylxanthen-9-yl)adenosine (A14)**

Compound **A3** ( 5.86 g, 9.87 mmol) was pixylated and desilylated according to the procedure used to synthesise compound **U14** above. The crude product was then purified by chromatography on silica gel (120 g) and eluted with a gradient of ethanol (2-4 %) in dichloromethane containing 1 % triethylamine. The title compound was

obtained as a white foam (5.20 g, 86.8 %) of $R_f$ 0.15 on TLC (pre-incubated with triethylamine) in ethanol-dichloromethane (5:95 v/v); Analysis of $C_{34}H_{33}N_5O_6$ requires C, 67.20; H, 5.48; N, 11.53; found C, 67.25; H, 5.49; N, 11.55. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 175.19 (pivaloyl C=O), 150.19 and 150.77 (xanthene C-4a, C-10a), 150.43 (C-2), 149.48 (C-4), 149.24 (C-6), 145.83 (pixyl phenyl C-1), 142.33 (C-8), 129.92 and 129.07 (xanthene C-1, C-8), 128.45 and 127.43 (xanthene C-3, C-6), 127.42 (pixyl phenyl C-3, C-5), 126.77 (pixyl phenyl C-4), 126.67 (pixyl phenyl C-2, C-6), 124.19 (C-5), 123.27 and 122.15 (xanthene C-2, C-7), 121.81 and 120.81 (xanthene C-8a, C-9a), 116.73 and 115.08 (xanthene C-4, C-5), 88.63 (C-1'), 87.56 (C-4'), 77.13 (xanthene C-9), 74.28 (C-2'), 70.61 ( C-3'), 62.60 (C-5'), 40.15 (pivaloyl tBu, q) and 26.97 (pivaloyl $CH_3$'s).

**6-*N*-Pivaloyl-2'-*O*-(9-phenylxanthen-9-yl)-5'-*O*-(*tert*-butoxydiphenylsilyl)adenosine (A15)**

Compound **A14** (5.01 g, 8.25 mmol) was silylated according to the procedure used to synthesise compound **U15** above. The crude product was purified by chromatography on silica gel (200 g) and eluted with a gradient of ethyl acetate (33-90 %) in hexane containing 1 % triethylamine. The title compound was obtained as a white foam (5.98 g, 84.2 %) of $R_f$ 0.28 on TLC (pre-incubated with triethylamine) in hexane-ethyl acetate (1:1 v/v); Analysis of $C_{55}H_{51}N_5O_7Si$ requires C, 76.62; H, 5.98; N, 8.13; found C, 76.66; H, 5.99; N, 8.15. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 175.16 (pivaloyl C=O), 151.55 (C-2), 150.77 (C-4), 150.51 and 150.11 (xanthene C-4a, C-10a), 148.86 (C-6), 146.52 (pixyl phenyl C-1), 141.91 (C-8), 134.44 (PhSi C-2's, C-6's), 133.99 and 133.86 (PhSi C-1's), 129.85 and 129.54 (xanthene C-1, C-8), 129.54 (phenyl Si C-4), 128.88 and 128.16 (xanthene C-3, C-6), 127.86 (pixyl phenyl C-3, C-5), 127.29 and 127.22 (PhSi C-3's, C-5's), 126.73 (pixyl phenyl C-4), 126.47 (pixyl phenyl C-2, C-6), 123.39 (C-5), 123.30 and 122.28 (xanthene C-2, C-7), 121.82 and 121.20 (xanthene C-8a, C-9a), 116.50 and 115.37 (xanthene C-4, C-5), 87.38 (C-1'), 85.44 (C-4'), 77.12 (xanthene C-9), 73.93 (C-2'), 73.49 (tBuO, q), 70.07 ( C-3'), 61.64 (C-5'), 40.05 (pivaloyl tBu, q), 31.54 (tBuO, $CH_3$'s) and 27.02 (pivaloyl $CH_3$'s).

**6-*N*-Pivaloyl-2'-*O*-(9-phenylxanthen-9-yl)-3'-*O*-laevulinyl-5'-*O*-(*tert*-butoxydiphenylsilyl)adenosine (A16)**

Compound **A15** (5.98 g, 6.94 mmol) was treated with laevulinic anhydride according to the procedure used to synthesise compound **U16** above. The crude product was purified by chromatography on silica gel (150 g) and eluted with a gradient of hexane (50-25 %) in ethyl acetate containing 1 % triethylamine. The title compound was obtained as a white foam (5.40 g, 81.0 %) of $R_f$ 0.13 on TLC (pre-incubated with triethylamine) in ethyl acetate-hexane (1:1 v/v); Analysis of $C_{60}H_{57}N_5O_9Si$ requires C, 75.05; H, 6.00; N, 7.30; found C, 75.07; H, 6.02; N, 7.32. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 205.44 (laevulinyl C=O), 175.10 (pivaloyl C=O), 170.97 (laevulinyl $CO_2$), 151.72 (C-2), 150.74 (C-4), 150.42 and 150.16 (xanthene C-4a, C-10a), 148.83 (C-6), 147.36 (pixyl phenyl C-1), 141.48 (C-8), 134.53 (PhSi C-2's, C-6's), 133.95 and 133.78 (PhSi C-1's), 130.31 and 129.65 (xanthene C-1, C-8), 129.55 (phenyl Si C-4's), 128.78 and 128.44 (xanthene C-3, C-6), 127.31 (pixyl phenyl C-3, C-5 and PhSi C-3's, C-5's), 126.41 (pixyl phenyl C-2, C-4, C-6), 123.15 (C-5), 123.15 and 122.27 (xanthene C-2, C-7), 121.80 and 121.26 (xanthene C-8a, C-9a), 116.19 and 115.25 (xanthene C-4, C-5), 86.92 (C-1'), 84.41 (C-4'), 76.79 (xanthene C-9), 73.55 (tBuO, q), 72.72 (C-2'), 72.34 ( C-3'), 61.66 (C-5'), 40.10 (pivaloyl tBu, q), 37.46 (laevulinyl C-2), 31.56 (tBuO, $CH_3$'s), 29.40 (laevulinyl C-5), 27.84 (laevulinyl C-3) and 27.07 (pivaloyl $CH_3$'s).

**6-*N*-Pivaloyl-2'-*O*-(9-phenylxanthen-9-yl)-3'-*O*-laevulinyladenosine (A17)**

Compound **A16** (5.33 g, 5.56 mmol) was desilylated according to the procedure used to synthesise compound **U17** above. The crude product was then purified by chromatography on silica gel (100 g) and eluted with a gradient of ethanol (0-5 %) in dichloromethane containing 2 % triethylamine. The title compound was obtained as a white foam (3.70 g, 94.4 %) of $R_f$ 0.25 on TLC (pre-incubated with triethylamine) in dichloromethane-ethanol (95:5 v/v); Analysis of $C_{34}H_{39}N_5O_8$ requires C, 57.86; H, 5.58; N, 9.93; found C, 57.91; H, 5.59; N, 9.95. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 205.50 (laevulinyl C=O), 175.14 (pivaloyl C=O), 171.21 (laevulinyl $CO_2$), 150.78 (C-2), 150.48 and 150.30 (xanthene C-4a, C-10a), 149.43 (C-4), 149.17 (C-6), 146.33 (pixyl phenyl C-1), 142.25 (C-8), 129.98 and 129.63 (xanthene C-1, C-8), 128.29 and 127.59 (xanthene C-3, C-6), 127.20 (pixyl phenyl C-3, C-5), 126.50 (pixyl phenyl C-2, C-4, C-6), 124.12 (C-5), 122.77 and 121.97 (xanthene C-2, C-7), 121.04 (xanthene C-8a, C-9a), 116.11 and 114.85 (xanthene C-4, C-5), 88.62 (C-1'), 86.58 (C-4'), 76.73 (xanthene C-9), 72.92 (C-2'), 72.17 (C-3'), 62.00 (C-5'), 40.03 (pivaloyl tBu, q), 37.23 (laevulinyl C-2), 29.24 (laevulinyl C-5), 27.53 (laevulinyl C-3) and 26.85 (pivaloyl $CH_3$'s).

**6-*N*-Pivaloyl-2'-*O*-(9-phenylxanthen-9-yl)-3'-*O*-laevulinyladenosine-5'-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (A18)**

Compound **A17** (1.55 g, 2.20 mmol) was phosphitylated according to the procedure used to synthesise compound **U18** above. The crude product was then purified by chromatography on silica gel (50 g) and eluted with a gradient of ethyl acetate (33-66 %) in hexane containing 2 % triethylamine. The title compound was obtained as a white foam (1.74 g, 90.2 %) of $R_f$ 0.24 on TLC (pre-incubated with triethylamine) in ethyl acetate-hexane (2:1 v/v); Analysis of $C_{41}H_{55}N_6O_9P$ requires C, 56.80; H, 6.41; N, 9.70; found C, 56.83; H, 6.44; N, 9.72. $^{31}P$ NMR, $\delta_P(CH_2Cl_2$, concentric external $D_2O$ lock): 145.24 and 144.98

**2-*N*-Dimethylaminomethylidene-2'-*O*-(9-phenylxanthen-9-yl)guanosine (G14)**

Compound **G3** ( 12.49 g, 21.50 mmol) was pixylated and desilylated according to the procedure used to synthesise compound **U14** above. The crude product was then purified by chromatography on silica gel (250 g) and eluted with a gradient of ethyl acetate (67-100 %) in hexane and then a gradient of ethanol (0-10 %) in ethyl acetate containing 1 % triethylamine. The title compound was obtained as a white foam (10.21 g, 83.2 %) of $R_f$ 0.16 on TLC (pre-incubated with triethylamine) in ethanol-dichloromethane (1:10 v/v); Analysis of $C_{32}H_{31}N_6O_6$ requires C, 64.52; H, 5.26; N, 14.11; found C, 64.56; H, 5.27; N, 14.13. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 158.99 (amidine CH), 157.61 (C-6), 156.83 (C-2), 151.14 and 150.63 (xanthene C-4a, C-10a), 148.35 (C-4), 146.05 (pixyl phenyl C-1), 138.40 (C-8), 130.28 and 129.85 (xanthene C-1, C-8), 129.26 and 128.78 (xanthene C-3, C-6), 127.82 (pixyl phenyl C-3, C-5), 127.12 (pixyl phenyl C-4), 126.89 (pixyl phenyl C-2, C-6), 123.87 and 123.66 (xanthene C-2, C-7), 122.65 and 121.54 (xanthene C-8a, C-9a), 122.24 (C-5), 116.40 and 115.06 (xanthene C-4, C-5), 88.44 (C-1'), 86.88 (C-4'), 76.99 (xanthene C-9), 73.70 (C-2'), 71.09 ( C-3'), 63.11 (C-5'), 41.27 and 35.03 (amidine $CH_3$'s).

**2-*N*-Dimethylaminomethylidene-2'-*O*-(9-phenylxanthen-9-yl)-5'-*O*-(*tert*-butoxydiphenylsilyl)guanosine (G15)**

Compound **G14** (5.01 g, 8.78 mmol) was silylated according to the procedure used to synthesise compound **U15** above. The crude product was purified by chromatography on silica gel (200 g) and eluted with a gradient

of ethanol (2-8 %) in dichloromethane containing 1 % triethylamine. The title compound was obtained as a white foam (7.04 g, 97.2 %) of $R_f$ 0.12 on TLC (pre-incubated with triethylamine) in ethanol-dichloromethane (5:95 v/v); Analysis of $C_{48}H_{49}N_6O_7Si$ requires C, 67.82; H, 5.82; N, 9.89; found C, 67.86; H, 5.83; N, 9.91. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 157.79 (C-6), 157.68 (amidine CH), 155.90 (C-2), 150.90 and 150.18 (xanthene C-4a, C-10a), 149.39 (C-4), 146.61 (pixyl phenyl C-1), 136.91 (C-8), 134.42 (PhSi C-2's, C-6's), 133.92 (PhSi C-1's), 129.83 and 129.67 (xanthene C-1, C-8), 129.67 (xanthene C-3, C-6), 128.81 (phenyl Si C-4), 127.51 (pixyl phenyl C-3, C-5), 127.38 and 127.33 (PhSi C-3's, C-5's), 126.77 (pixyl phenyl C-4), 126.60 (pixyl phenyl C-2, C-6), 123.46 and 123.29 (xanthene C-2, C-7), 122.30 and 121.24 (xanthene C-8a, C-9a), 120.81 (C-5), 116.21 and 115.46 (xanthene C-4, C-5), 86.53 (C-1'), 85.09 (C-4'), 77.22 (xanthene C-9), 74.16 (C-2'), 73.63 (tBuO, q), 70.05 ( C-3'), 62.40 (C-5'), 40.05 (pivaloyl tBu, q), 41.06 and 34.85 (amidine $CH_3$'s) and 31.57 (tBuO, $CH_3$'s).

**2-*N*-Dimethylaminomethylidene-2'-*O*-(9-phenylxanthen-9-yl)-3'-*O*-laevulinyl-5'-*O*-(*tert*-butoxydiphenylsilyl)guanosine (G16)**

Compound **G15** (7.04 g, 8.53 mmol) was treated with laevulinic anhydride according to the procedure used to synthesise compound **U16** above. The crude product was purified by chromatography on silica gel (300 g) and eluted with gradient of ethanol (2-8 %) in dichloromethane containing 2 % triethylamine. The title compound was obtained as a white foam (6.91 g, 87.8 %) of $R_f$ 0.10 on TLC (pre-incubated with triethylamine) in ethanol-dichloromethane (5:95 v/v); Analysis of $C_{53}H_{55}N_6O_9Si$ requires C, 67.13; H, 5.86; N, 8.87; found C, 67.16; H, 5.87; N, 8.89. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 205.52 (laevulinyl C=O), 171.03 (laevulinyl $CO_2$), 157.94 (amidine CH), 157.77 (C-6), 156.02 (C-2), 150.99 and 150.51 (xanthene C-4a, C-10a), 149.30 (C-4), 147.27 (pixyl phenyl C-1), 137.27 (C-8), 134.53 (PhSi C-2's, C-6's), 134.01 (PhSi C-1's), 130.60 and 129.60 (xanthene C-1, C-8), 129.60 (xanthene C-3, C-6), 129.32 (phenyl Si C-4), 127.44 (pixyl phenyl C-3, C-5), 127.32 (PhSi C-3's, C-5's), 126.78 (pixyl phenyl C-2, C-6), 126.65 (pixyl phenyl C-4), 123.15 (xanthene C-2, C-7), 121.98 and 121.76 (xanthene C-8a, C-9a), 120.91 (C-5), 116.02 and 115.73 (xanthene C-4, C-5), 87.77 (C-1'), 82.94 (C-4'), 76.99 (xanthene C-9), 73.65 (tBuO, q), 72.15 (C-2'), 71.50 ( C-3'), 62.01 (C-5'), 40.92 and 34.85 (amidine $CH_3$'s), 37.53 (laevulinyl C-2), 31.56 (tBuO, $CH_3$'s), 29.55 (laevulinyl C-5) and 27.78 (laevulinyl C-3).

**2-*N*-Dimethylaminomethylidene-2'-*O*-(9-phenylxanthen-9-yl)-3'-*O*-laevulinylguanosine (G17)**

Compound **G16** (6.91 g, 7.49 mmol) was desilylated according to the procedure used to synthesise compound **U17** above. The crude product was then purified by chromatography on silica gel (120 g) and eluted with ethanol-dichloromethane (4:96 v/v) containing 2 % triethylamine. The title compound was obtained as a white foam (4.92 g, 98.2 %) of $R_f$ 0.42 on TLC (pre-incubated with triethylamine) in dichloromethane-ethanol (9:1 v/v); Analysis of $C_{37}H_{37}N_6O_8$ requires C, 64.05; H, 5.39; N, 12.12; found C, 64.09; H, 5.41; N, 12.13. $^{13}C$ NMR, $\delta_C(CDCl_3)$: 205.82 (laevulinyl C=O), 171.58 (laevulinyl $CO_2$), 158.77 (amidine CH), 157.55 (C-6), 156.77 (C-2), 150.50 (xanthene C-4a, C-10a), 148.15 (C-4), 146.92 (pixyl phenyl C-1), 138.12 (C-8), 130.62 and 129.83 (xanthene C-1, C-8), 128.91 and 128.60 (xanthene C-3, C-6), 127.39 (pixyl phenyl C-3, C-5), 126.58 (pixyl phenyl C-2, C-4, C-6), 123.13 (xanthene C-2, C-7), 121.86 (C-5), 121.68 (xanthene C-8a, C-9a), 115.75 and 114.79 (xanthene C-4, C-5), 88.35 (C-1'), 85.79 (C-4'), 76.75 (xanthene C-9), 73.10 (C-2'),

71.47 ( C-3'), 62.30 (C-5'), 41.00 and 34.74 (amidine $CH_3$'s), 37.42 (laevulinyl C-2), 29.45 (laevulinyl C-5) and 27.74 (laevulinyl C-3).

**2-*N*-Dimethylaminomethylidene-2'-*O*-(9-phenylxanthen-9-yl)-3'-*O*-laevulinylguanosine-5'-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (G18)**

Compound **G17** (4.85 g, 7.25 mmol) was phosphitylated according to the procedure used to synthesise compound **U18** above. The crude product was then purified by chromatography on silica gel (200 g) and eluted with a gradient of dichloromethane (33-80 %) in hexane containing 4 % triethylamine. The title compound was obtained as a white foam (4.80 g, 79.7 %) of $R_f$ 0.36 on TLC (pre-incubated with triethylamine) in ethanol-dichloromethane (5:95 v/v); Analysis of $C_{44}H_{53}N_6O_9P$ requires C, 61.81; H, 6.26; N, 9.83; found C, 61.86; H, 6.28; N, 9.86. $^{31}P$ NMR, $\delta_P(CH_2Cl_2$, concentric external $D_2O$ lock): 145.68 and 144.92

*Synthesis of branched oligonucleotides*

The branched oligonucleotides (**Table 1**) were synthesised from the 5' to 3' end on a 0.2 μmol scale by using the reversed supports and the monomers described above. The carefully dried monomers were prepared as 0.1 M solutions in anhydrous acetonitrile. In the case of the monomer used for coupling at the branch-point (on the 3'-hydroxy function) a 0.2 M solution was prepared. A modified 0.2 μmol β–cyanoethyl phosphoramidite DNA assembly cycle was used with 0.5 M 1*H*-tetrazole in acetonitrile as activator: a) Coupling time: 900 sec. b) Capping: "cap to column" step , 15 sec. followed by a "wait" step, 60 sec. c) Oxidation: "oxidation solution to column" step, 15 sec. followed by a "wait" step, 60 sec. Upon completion of the first arm (manual end procedure: DMTr off) the 3'-hydroxy group was capped by acetylation ("cap to column" step , 20 sec. followed by a "wait" step, 900 sec.). In order to be able to chain extend from the 3'-hydroxyl group of the branch-point, the 2-cyanoethyl group was first removed from the 2'-5' phosphotriester moiety by treatment with 0.5 M anhydrous DBU in acetonitrile ("8 to column" step , 30 sec. followed by a "wait" step, 60 sec.). After washing the column was removed from the synthesiser. A fresh solution of 0.5 M hydrazine hydrate in pyridine-acetic acid (4:1 v/v) was slowly passed forwards and backwards for 20 min through the column, using two plastic syringes to remove the 3'-*O*-laevulinyl protecting group. The column was washed thoroughly with dry ethanol (5 ml) followed by anhydrous acetonitrile (5 ml) and was then reinstalled on the synthesiser. Chain extension to give the desired branched oligonucleotide was then continued with reversed monomers. The assembly cycle was modified so that the acid treatment of the support prior to the first coupling was avoided. The first coupling was carried out with a 0.2 M solution. In each synthetic cycle, the 9-phenylxanthene-9-yl cation released in the course of each step involving treatment with trichloroacetic acid was assayed spectrophotometrically. The average coupling yields for the reversed monomers and the branch-point monomers were 96-98 %. Coupling yields for the condensation of the 3' hydroxy function at the branch-point with a reversed monomer are shown in **Table 1**.

*Deprotection and purification of the branched oligonucleotides*

At the end of the assembly, the carrier bound oligonucleotide was treated with ethanol-30 % aq. ammonia (1:3 v/v,2 ml) for 12 h at 60 °C in a sealed, sterile vial. When cool the sample was lyophilised in a sterile Eppendorf tube and was then purified by reversed-phase HPLC on a 4 mm x 250 mm Nucleosil C18 steel column (10 μ) with a gradient of acetonitrile in 0.1 M triethylammonium acetate, pH 6.5 as eluent. The eluate containing the desired component was collected and transferred to a sterile Eppendorf tube. It was then evaporated under

reduced pressure in a vacuum centrifuge. The residue was redissolved in sterile, deionised water (2x1 ml) and re-evaporated. The purified samples were dissolved in 0.2 ml sterile 0.5 M tris acetate (pH 3.03) and kept at room temperature for 24 h. 0.3 ml of 2 M tris acetate (pH 7.4) was added. Pre-cooled (to -70 °C) butan-1-ol (0.75 ml) was added with thorough mixing. The resulting mixture was cooled to -70 °C for 5 min and was then centrifuged. The supernatant was carefully removed and the remaining pellet was resuspended in cold butan-1-ol, cooled to -70 °C and centrifuged. The small pellet was resuspended in absolute ethanol (0.4 ml) and re-evaporated. As a check on the purity the samples were analysed by ion exchange chromatography on a Mono Q HR 5/5 column using a gradient of sodium chloride in 10 mM sodium hydroxide, pH 12.5. The identity of the fully deprotected branched oligonucleotides was confirmed by MALDI or ES mass spectrometry (**Table 1**).

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