A Universal Solid-phase Synthesis of Branched Oligoribonucleotides

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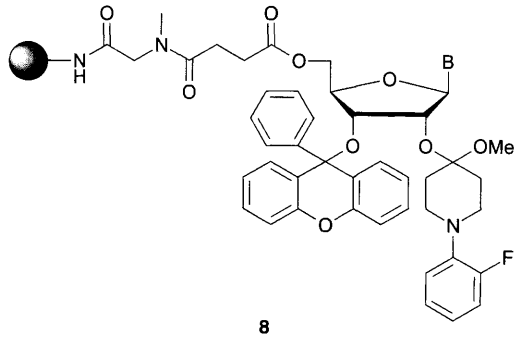
A novel branch-point adenosine monomer is synthesized which enables the routine solid-phase synthesis of branched oligoribonucleotides in the 5′ to 3′ direction, such that the sequences at the 2′ and 3′ positions of the branch-point adenosine can be of different lengths and base composition; the method can be easily scaled up to produce milligram amounts of branched oligoribonucleotides, which are readily purified by reversed-phase HPLC whilst partially protected.

Branched RNAs either in the form of lariats or Y-shaped molecules are generated in *cis*-[1,2] and *trans*-splicing[3], respectively as a result of the processing of pre-messenger RNAs. 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.[4] Small branched oligoribonucleotides and analogues thereof would be particularly useful for studying this so-called debranching enzyme. Pioneering work in the solution synthesis of branched oligoribonucleotides combining phosphotriester, *H*-phosphonate and phosphoramidite chemistry has been performed by the group of Chattopadhyaya,[5] who also demonstrated the full chemical synthesis of small lariat RNAs which in some cases are capable of self-cleavage.[6] Damha *et al.* were subsequently able to demonstrate the first automated solid-phase synthesis of branched oligoribonucleotides although the sequences attached at the 2′- and 3′-positions of the branch-point adenosine were identical.[7] We recently reported substantial improvements to the latter method[8] and now report the logical extension of this method enabling the routine synthesis of branched oligoribonucleotides which can have sequences of different length and base composition attached to the 2′- and 3′-hydroxy groups of the branch-point ribonucleoside. As our work was more or less complete, we heard that the Damha group[9] had submitted an article describing an alternative route to synthesizing branched oligoribonucleotides of any composition, always involving a difficult to scale up purification of four very similar structures present in more or less equimolar amounts.

To achieve our goal we developed a new branch-point adenosine monomer, compound **7** (see Scheme 1) bearing a 5′-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) moiety, 2′-*O*-(9-phenylxanthen-9-yl), *i.e.* pixyl,[10] protection and 3′-*O*-laevulinyl[11] protection.† Branched oligoribonucleotides are then synthesized in the 5′ to 3′ direction by phosphoramidite chemistry starting with a reversed sarcosine linked support, compound **8** (see Fig. 1) bearing a 2′-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl], *i.e.* Fpmp protecting group[12] and a 3′-*O*-pixyl protecting group. The pixyl group is removed and the liberated 3-hydroxy group is condensed with the appropriate reversed monomer, compound **9** *viz.* a protected ribonucleoside 5′-*O*-(methyl phosphoramidite) in the presence of tetrazole.‡ After capping and oxidation the cycle is repeated up to and including incorporation of the branch-point adenosine monomer, compound **7**, which bears 2′-*O*-pixyl and 3′-*O*-laevulinyl protection. At this point the 2′-*O*-pixyl group is removed,§ allowing chain extension from the 2′-hydroxy group of the branch-point adenosine, and condensation is performed with a reversed monomer in which R is β-cyanoethyl. Further chain extension is performed *y* times with the methyl phosphoramidites and finally the last pixyl group is removed and the liberated 3′-hydroxy group is capped with acetic anhydride to generate the support bound non-branched oligomer, *viz.* compound **10** (see Scheme 2). The β-cyanoethyl protecting group of the 2′-5′ phosphotriester moiety vicinal to the laevulinyl group is then selectively removed by treatment with anhydrous triethylamine in acetonitrile for 1 h prior to removal of the 3′-*O*-laevulinyl group by treatment with a fresh solution of 0.5 mol dm^{-3} hydrazine hydrate in pyridine-acetic acid[11] (4:1 *v*/*v*) for 15 min. The sarcosine linked support[13] prevents cleavage of the succinate moiety during the triethylamine treatment. The column is washed thoroughly with anhydrous acetonitrile and then the branch is extended *z* times by condensation of reversed monomers¶ followed by capping, oxidation and depixylation to generate the fully protected support-bound branched oligoribonucleotide, *viz.* compound **11**.

In this way we synthesized support-bound fully protected 5′-UpCpCpA(2′-5′pCpC, 3′-5′pApA) and 5′-UpApCpA(2′-5′pCpA, 3′-5′pApCpG).** Upon completion of the assembly the support-bound branched oligoribonucleotides were treated with ethanol–30% aq. ammonia (1:3 v/v) for 12 h at 60 °C to cleave the succinate linkage and remove all protecting groups except for the Fpmp groups and the single pixyl group. The crude partially protected products were purified by reversed-phase HPLC,†† and analysed by ESMS[14] as described previously.[8] The relative molecular masses of the purified, partially protected branched oligoribonucleotides were found to be 4157.2 (calc. *M*, for $C_{178}H_{206}F_7N_{36}O_{60}P_7$ is 4159.62) and 4735.0 (calc. *M*, for $C_{201}H_{232}F_8N_{44}O_{67}P_8$ is 4736.10) respectively, by ESMS. Finally, the acid labile blocking groups



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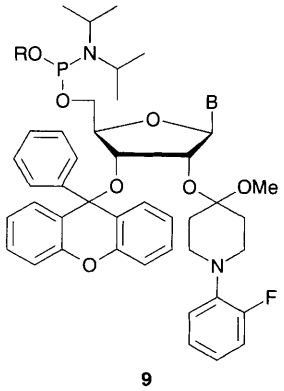

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Fig. 1 Structures of the reversed sarcosine support, compound **8** and the reversed monomers, compound **9**. The stippled circle represents the aminopropyl controlled pore glass support; R is methyl or 2-cyanoethyl; B is a protected nucleobase, *viz.* uracil-1-yl, 4-*N*-benzoylcytosin-1-yl, 6-*N*-pivaloyladenosin-9-yl or 2-*N*-(dimethylaminomethylene)guanin-9-yl.

were removed under sterile conditions[15] to give 2.3 and 4.1 A_{260} units respectively of pure branched oligoribonucleotides, corresponding to overall yields of 13 and 20% based on the amount of support. The lower yield was for the branched octaribonucleotide in which the decyanoethylation was performed manually. In the case of the branched nonaribonucleotide the decyanoethylation was performed on the synthesizer. It should be noted that the method has not yet been optimized.

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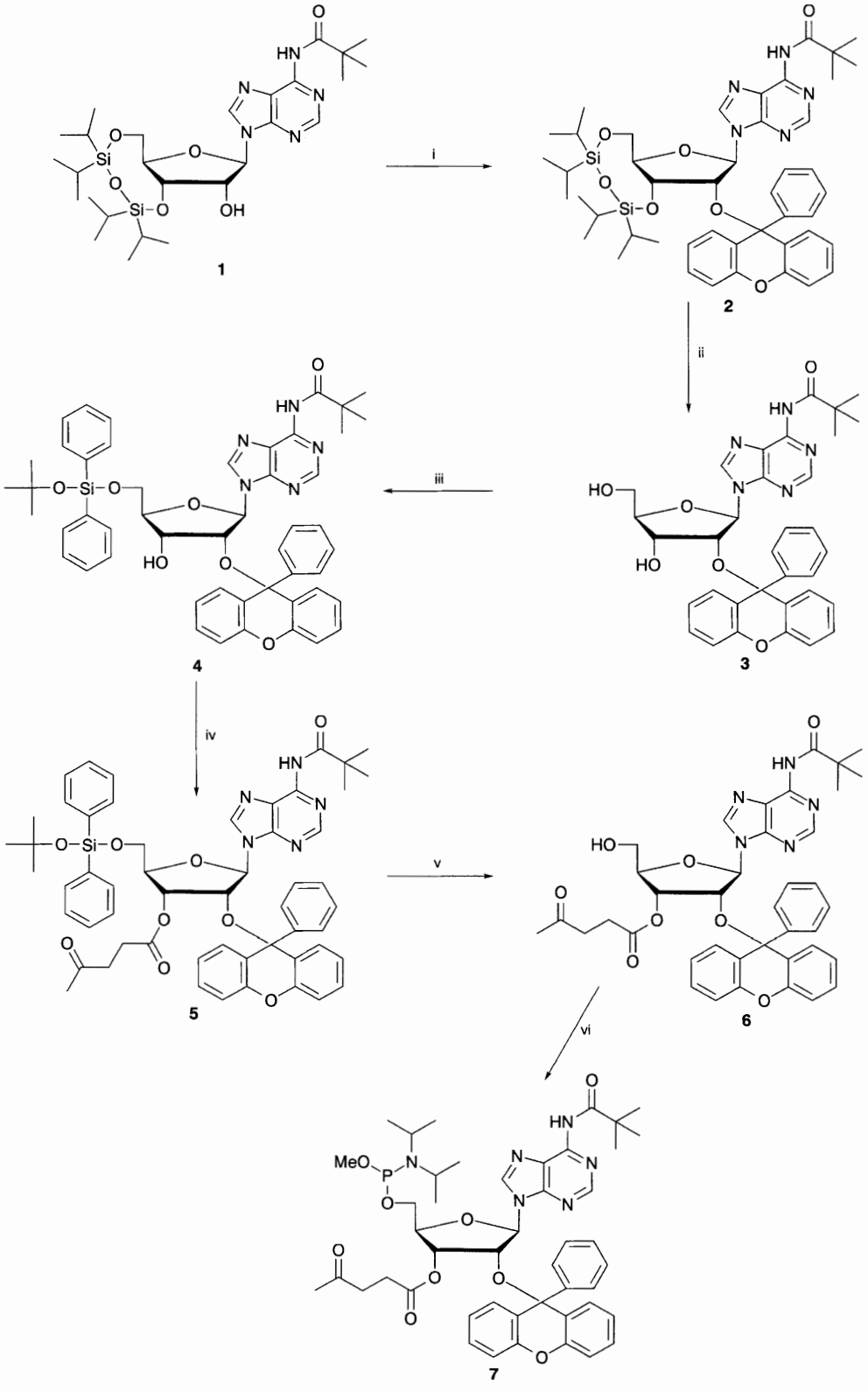
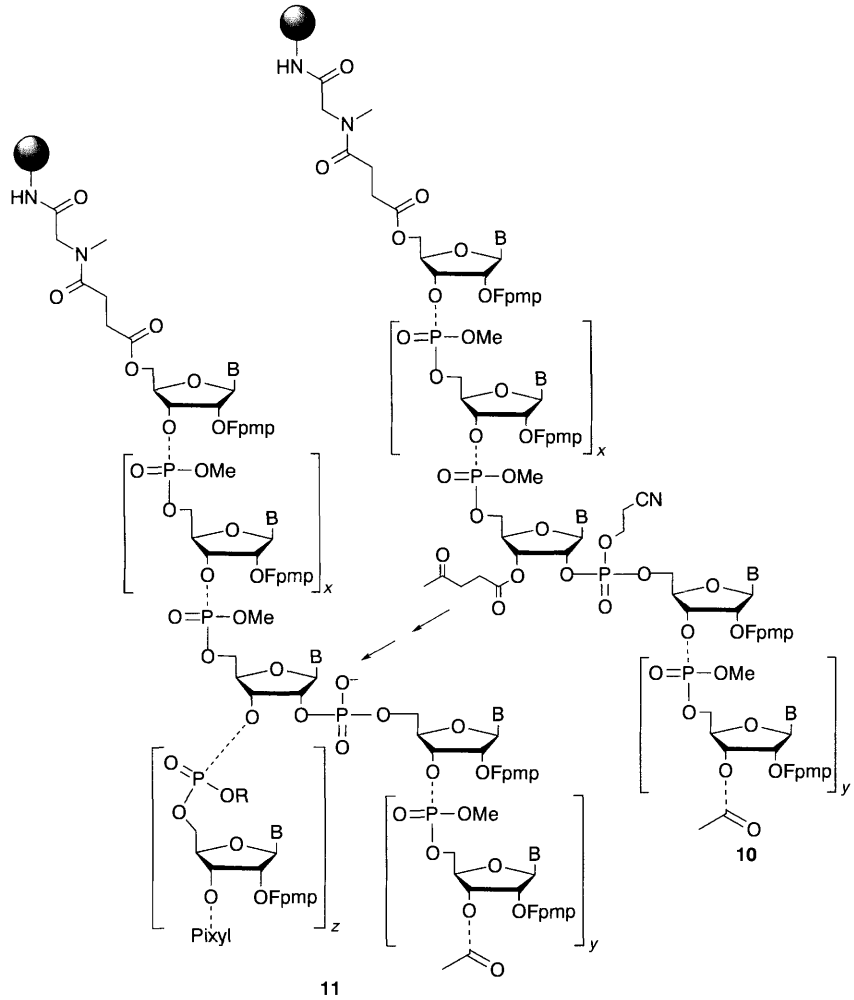


Scheme 1 Preparation of the branch-point adenosine monomer. *Reagents and conditions:* i, 9-chloro-9-phenylxanthene in pyridine, 11 h; ii, tetrabutylammonium fluoride (TBAF) in THF, 15 min, 87% steps i and ii combined; iii, *tert*-butoxy(chloro)diphenylsilane and imidazole in DMF, 2.5 h, 84%; iv, laevulinic anhydride, 4-(dimethylamino)pyridine and triethylamine in dichloromethane, 20 h, 81%; v, TBAF in THF, 30 min, 94%; vi, chloro(*N*,*N*-diisopropylamino)methoxyphosphine and *N*,*N*-diisopropylethylamine in 1,2-dichloroethane, 40 min, 70%.



Scheme 2 Procedure for the solid-phase synthesis of a carrier-bound fully protected branched oligoribonucleotide, compound **11**. The stippled circle represents the aminopropyl controlled pore glass support; R is methyl or 2-cyanoethyl; B is a protected nucleobase, *viz.* uracil-1-yl, 4-*N*-benzoylcytosin-1-yl, 6-*N*-pivaloyladenosin-9-yl or 2-*N*-(dimethylaminomethylene)guanin-9-yl; *x*,*y* and *z* are integers.

Footnotes

† Compound **7** had R_f 0.24 on TLC (silica gel; eluent: ethyl acetate–light petroleum 2:1 containing 1% triethylamine). ^{31}P NMR (CH_2Cl_2, concentric external D_2O lock) δ 145.24 and 144.98.

‡ Syntheses were performed on a 0.2 μmol scale using a controlled pore glass support, compound **8**, which was loaded with 25.6 μmol of nucleoside g^{-1}. Couplings were performed with 0.1 mol dm^{-3} phosphoramidite in acetonitrile and 0.5 mol dm^{-3} 1*H*-tetrazole in acetonitrile for 15 min.

§ The coupling yield of **7** as measured by depixylation was 95%.

¶ The coupling through the 3′-position following removal of the cyanoethyl group and delaevulinylation as measured *via* the depixylation yield after coupling phosphoramidite **9** was between 60 and 75%.

** Overall coupling yields were 68 and 70% respectively and the crude yields were 20.8 and 15.7 A_{260} units respectively.

†† Yields of HPLC purified branched oligoribonucleotides carrying pixyl and Fpmp protection were 2.8 and 4.8 A_{260} units respectively.

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