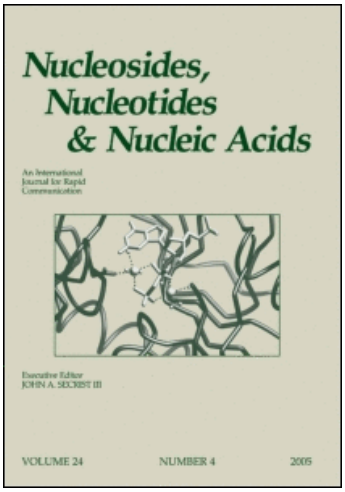


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N. Balgobin[a]; X-X. Zhou[a]; J-M. Vial[a]; A. Nyilas[a]; A. Földesi[a]; J. Chattopadhyaya[a]
[a] Department of Bioorganic Chemistry Biomedical Center, University of Uppsala, Uppsala, Sweden

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# SYNTHESIS OF BRANCHED OLIGORIBONUCLEOTIDES ("LARIAT")

N. Balgobin, X-X. Zhou, J-M. Vial, A. Nyilas, A. Földesi &
J. Chattopadhyaya*

*Department of Bioorganic Chemistry, Biomedical Center, University of Uppsala, Box 581, S-751 23 Uppsala, Sweden*



***Abstract:*** *Four different strategies for the synthesis of branched oligoribonucleotides, from trimers to heptamer, are described.*

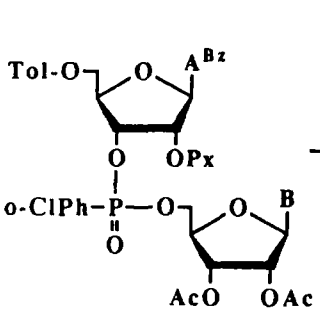
The synthesis of branched oligoribonucleotides ("lariat")[1] imposes a formidable problem as sequential introduction of nucleotides at the vicinal 2′- and 3′- hydroxyls at the branch-point nucleoside are required. A major consideration is the stability of the 2′- or 3′-phosphate on removal of the vicinal hydroxyl protecting group. The nucleophilic reactivity of the vicinal hydroxyl function towards the phosphate is dictated by the nature of the phosphate. While a phosphotriester[2], hydrogen-phosphonate[4] and phosphoramidate[3] are prone to attack by the vicinal hydroxyl group, the phophite-triester[5] and phosphodiester are relatively stable under mild acidic conditions and certain neutral conditions. Upon these observations, we have developed four different strategies[6-11] for the synthesis of branched oligonucleotides.

***Route I***: We first prepared the fully protected dinucleotides **1** by the condensation of 5′-toluoyl-2′-O-[9-(phenylxanthen)-9-yl] (Px) $N^6$-benzoyl adenosine 3′-O-triethyl-ammonium (o-chlorophenyl)phosphate and the 5′-hydroxy blocks using standard phosphotriester methodology. The o-chlorophenyl group was then smoothly removed to give 3′→ 5′ phosphodiester with tetrabutylammonium fluoride (TBAF) in THF-pyridine-water. Subsequently, the 2′-O-Px was removed by 80% aqueous acetic treatment at RT. The freed 2′-OH group was then coupled with an appropriately protected 5′-phosphor-amidite block in presence of tetrazole in dry acetonitrile. The uridine residue was protected

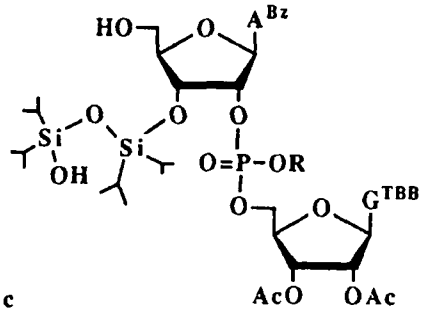
with the 2-nitrophenyl group at the $O^4$- position, since such an intermediate can be converted either to cytosine or uracil moieties depending upon the different deprotection procedures chosen. Using this approach, four branched trimers $A^G_U$, $A^G_C$, $A^U_G$, $A^C_G$ were synthesized[6,7].

***Route II***: The key intermediate dimer **2** were prepared by the condensation of the 3′,5′ -O-(1,1,3,3-tetraisopropyl-1,3-disiloxane-1,3-diyl)adenosine 2′-phosphoramidites with the appropriately protected 5′-hydroxy blocks and subsequently opening the 5′ -silyl selectively. The reasons for the employment of the disilyl group are that (i) it can protect both the 3′- and 5′-hydroxyl functions simultaneously, and subsequently, after functionalization of the 2′-OH, the 5′-hydroxyl group can be released specifically using 0.2 M aqueous HCl in dioxane at 20 °C, allowing protection or condensation of the 5′-OH selectively, (ii) the residual silyl-protected 3′-hydroxyl group can be freed under neutral condition without any isomerization. We employed the 9-fluorenylmethyl (FM) and 2-phenylsulfonylethyl (PSE) groups, which can be selectively removed *via* β-elimination reactions, for the protection of internucleotidic phosphates at the 2′ of the branch-site residue. The 5′-hydroxyl function of the dimers were coupled with the appropriately protected nucleoside 3′-phosphodiester in the synthesis of branched tetramers. Alternatively, they were acetylated for the synthesis of branched trimers. After removal of the FM and PSE with $Et_3N$ in dry pyridine, the 3′-silyl group were removed by TBAF treatment. The freed hydroxyls were then condensed with $O^4$-protected uridine 5′-phosphoramidite. We have synthezised six branched trimers $U^G_U$, $U^G_C$, $C^G_U$, $C^G_C$, $G^G_U$, $G^G_C$, and four branched tetramers $UA^G_U$, $UA^G_C$, $AA^G_U$, $AA^G_C$ *via* this method[8,9].

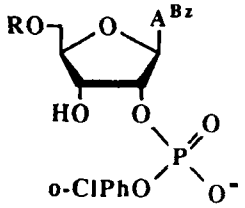
***Route III***: It is obvious that the synthesis of larger branched oligonucleotides extending from the branched core trimer in all three directions *via* the above-mentioned strategies requires at least two different internucleotidic phosphate protecting groups as well as two different 2′- hydroxyl protecting groups, all of which however should be complementary and removable in a regiospecific manner. These requirements impose a difficult problem for the manipulation of protecting groups. We prepared $N^6$-benzoyl-5′-O-(4-monomethoxytrityl) adenosine 2′-O-triethylammonium (o-chlorophenyl) phosphate and the 5′-O-[9-(p-anisoyl)xanthen-9-yl (MPx) analogue as the key intermediates **3** in order to circumvent the need for the two sets of complementary protecting groups. The 3′-hydroxyl of the key intermediate was condensed with an appropriately protected 5′-phosphoramidite to obtain a trinucleotide with a diester phosphate vicinal to the internucleotidic phosphate.



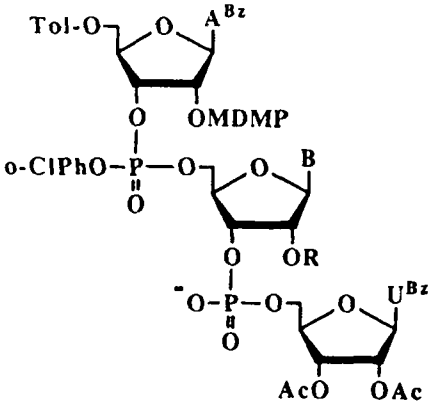

1



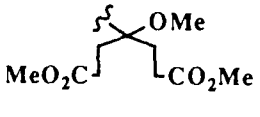

2: R = PSE or FM



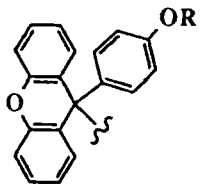

3: R = MMTr or MPx




4: R = Px
5: R = H




MDMP




R = H (Px)
R = $CH_3$ (MPx)

This phosphodiester was then directly condensed with the 5′-hydroxyl of a protected dinucleotide GpU to give a fully protected branched pentaribo-nucleotide, which was deprotected in two different ways to obtain the branched pentamers with uracil or cytosine. The 5′-O-MPx group was selectively removed by the treatment of trichloroacetic acid (TCA) in $CH_2Cl_2$ / MeOH at 0 $^0$C. The released hydroxyl group was condensed with a protected dinucleotides 3′-phosphodiester to give a fully protected branched hepta-nucleotide. We have synthesized two branched trimers $A^{G}_{U}$, $A^{C}_{U}$, two pentamers, $A^{GU}_{UC}$, $A^{GU}_{CC}$ and a heptamer $CUA^{GU}_{UC}$ by this method[10].

***Route IV***: An alternative strategy to the above, which is again independent of the compatability of two different phosphate protecting groups, is the use of H-phosphonate chemistry to generate the vicinal phosphodiester at the branch-point nucleotide as shown in **4** (R = Px)[11]. This has the further advantage of increasing the lability of the acid-sensitive 2′-protecting group by a factor of 40-75 fold. We therefore anticipated that if the branch-point nucleoside was protected at the 2′-hydroxyl with Px and the other 2′-hydroxyl with 3-methoxy-1,5-dicarbomethoxypentanyl (MDMP)[12], then this combination whereby the MDMP was stablizied by a vicinal phosphotriester and the pixyl was destablized by a vicinal phosphodiester, should allow the selective cleavage of the 2′-O-pixyl group under the correct choice of deprotection conditions. We therefore synthesized the trimers [**4**: B = $A^{Bz}$, U, $C^{Bz}$, $G^{TBB}$] and treated then with a ten fold excess of TCA at 0 °C. Within 4 min the 2′-O-pixyl group was cleaved, without any removal of the 2′-MDMP, and **5** (R = H; B = $A^{Bz}$, U, $C^{Bz}$, $G^{TBB}$) were obtained in good yields. These were then condensed with the guanosine phosphoramidite, to yield the partially protected tertramers, which were not isolated but deprotected as usual to yield the branched tetramers $AC^{G}_{U}$, $AG^{G}_{U}$, $AA^{G}_{U}$ and $AU^{G}_{U}$.

We have subsequently examined the conformations of these branched tri-, tetra-, penta-, and heptanucleotides by NMR spectroscopy[7,13-17] in order to understand why do the lariat structures form in the pre-mRNA processing reactions (splicing).

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