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AUTOMATED SOLID-PHASE SYNTHESIS OF BRANCHED OLIGONUCLEOTIDES

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Summary: A general procedure for the synthesis of branched RNA and DNA oligonucleotides has been developed. The synthetic strategy involves the 3'->5' synthesis of a DNA or RNA oligomer on a solid-phase, controlled-pore glass support with an automated DNA synthesizer. The branch point nucleoside is introduced by coupling of two adjacent polymer bound nucleotide chains with a tetrazole-activated adenosine 2',3'-bisphosphoramidite derivative.

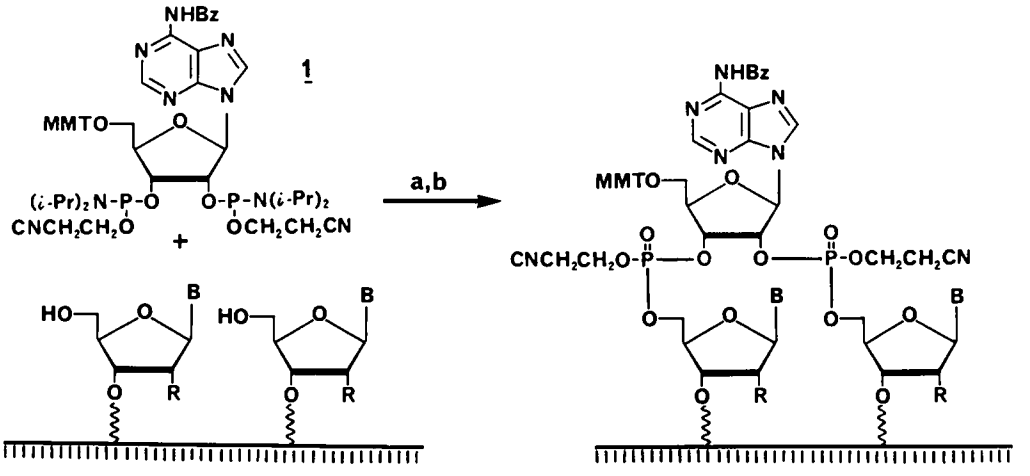
Branched ribonucleic acids containing vicinal 2'-5' and 3'-5' phosphodiester linkages were first detected in nuclear polyadenylated RNA from HeLa cells by Wallace and Edmons[1] and were subsequently shown by others to form an integral part of messenger RNA splicing[2]. Two unique branched RNA configurations have been detected: circles with a tail containing a branch point (*i.e.*, a "lariat" configuration) in *cis*-splicing reactions[2], or branches between two linear RNAs (*i.e.*, a "Y"-like structure) in the case of *trans*-splicing reactions[3]. The exact mechanism of branch point selection in RNA splicing is unknown as are the role and three-dimensional structure of branched RNAs. The availability of specific branched oligonucleotides in large amounts could be of considerable value as an aid to understanding the properties and biological role of branched RNA. To date there is no general procedure for the solid-phase synthesis of branched oligonucleotides. In this report we wish to describe such a procedure.

We have recently demonstrated a method for the high yield conversion of adenosine (the central core nucleoside at which all branches occur) to its 2',3'-bisphosphoramidite derivative (**1**)[4]. This allows for the ready introduction of vicinal 2',5'- and 3',5'-phosphodiester linkages at the branchpoint nucleoside. In principle, the reaction of **1** with two adjacent polymer-bound nucleotide chains in the presence of tetrazole should give a branched phosphite triester product that can be readily oxidized with an iodine/water solution to the more stable branched phosphate triester derivative (Scheme 1). After removal of the 5'-monomethoxytrityl (MMT) protecting group, the protected branched sequence can be extended in the 5'-direction to yield "Y"-like branched structures similar to those detected in *trans*-splicing reactions.

The extent of branching is expected to be an inverse function of the distance between neighbouring polymer-bound nucleotide chains *(i.e.*, as the distance between adjacent polymer bound nucleotides decreases, the probability of coupling of **1** with terminal 5'-hydroxyl groups of two adjacent nucleotide chains increases). The distance between terminal 5'-hydroxyl groups on the solid support is a direct function of the number of polymer-bound oligomers per surface area of support (*i.e.*, the nucleotide loading). Therefore, the branching reaction would be favoured when solid supports with high-nucleotide loadings are employed.

Another important consideration is the molar concentration of bisamidite derivative **1** employed in the branching reaction. If one assumes that the vicinal 2',5'- and 3',5'-phosphodiester linkages are not formed simultaneously but are formed in two kinetically distinct steps, [*i.e.*, (i) chain extension followed by (ii) branching (Scheme 2a)], a dilute solution of bisamidite **1** should be employed for optimum branching. The use of a high solution concentration of **1** would yield predominantly (n+1) sequences which are unable to undergo branching (Scheme 2b).

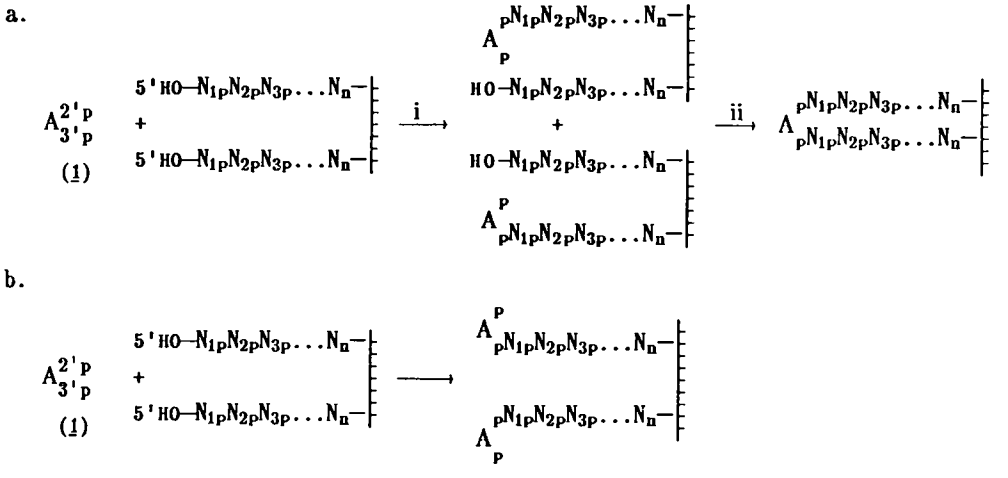
SCHEME 1



a, tetrazole; b, I_2/H_2O

R = H or OSi(Me)$_2$tBu; Bz = benzoyl; MMT = monomethoxytrityl

SCHEME 2



The branched trinucleotide A^{T}_{T} [*i.e.*, A(2'T)(3'T)] was prepared first to test the methodology. Thus, 5'-O-MMT-thymidine-3'-O-succinate was attached onto long-chain alkylamine controlled-pore glass (LCAA-CPG, Pierce Chemical Co.) according to Pon *et al.*[5] using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (DEC) as coupling reagent. The loading obtained after a 24 h reaction was 47 *u*mol g^{-1}. By decreasing the coupling time, LCAA-CPG with thymidine loadings of 26 and 7 *u*mol g^{-1} were also obtained. For each of these LCAA-CPG supports, the synthesis of A^{T}_{T} was tested using (effective) bisamidite **1** concentrations of 0.010M, 0.040M and 0.075M, and the synthesis cycle shown in Table 1.

The coupling yields of these syntheses were determined by spectrophotometric quantitation of the released monomethoxytrityl cation at 478 nm and are presented in Table 2. They ranged from 97 % (7 *u*mol g^{-1} CPG/0.075M **1**) to 43% (47 *u*mol g^{-1} CPG/ 0.010M **1**), and since 100% branch formation should give a yield of 50%, it appeared that a significant amount of A^{T}_{T} had formed in the latter case. The LCAA-CPG beads were then treated with 29% aqueous ammonia at 20 °C for 24 h to simultaneously cleave the

Table 1. Synthesis cycle for branchpoint insertion in the preparation of branched oligonucleotides.[a]

Step	Reagent	Time (sec)	
		(A^{T}_{T})[b]	(**2**,**3**)[c]
1	CH_3CN	60	60
2	0.5 M tetrazole	5	5
3	bisphosphoramidite **1**/0.5 M tetrazole, 1:1	3	5
4	0.5 M tetrazole	3	3
5	bisphosphoramidite **1**/0.5 M tetrazole, 1:1	3	5
6	0.5 M tetrazole	3	3
7	bisphosphoramidite **1**/0.5 M tetrazole, 1:1	3	5
8	0.5 M tetrazole	5	5
9	Couple	900	600
10	0.25 M Ac_2O/DMAP/collidine in THF	15	165
11	CH_3CN	–	60
12	0.10 M I_2 THF/pyridine/H_2O, 7:2:1	60	60
13	CH_3CN	140	140
14	5% trichloroacetic acid/dichloroethane	100	180
15	CH_3CN	120	60
Total		1420	1356

[a] syntheses were carried out using an Aplied Biosystems 381A DNA synthesizer; [b] 0.2 μmol-scale synthesis; [c] 1.0 μmol-scale synthesis; DMAP = 4-dimethylaminopyridine.

Table 2. Effect of coupling yield (%) on LCAA-CPG-thymidine loading and bisphosphoramidite **1** concentration in the synthesis of the branched trinucleotide A^{T}_{T}.

		LCAA-CPG (μm·g^{-1})		
		7	26	47
	0.075 M	97	74	74
A^{P}_{P}	0.040 M	98	80	74
(**1**)	0.010 M	61	55	43

product from the solid support, and remove the cyanoethyl and the adenine N^6-benzoyl (Bz) protecting groups. The residue resulting from the evaporation of the ammoniacal solution was checked by HPLC and electrophoresis on a 24% polyacrylamide gel by comparing it with an authentic sample of A^TT^4. The analyses showed that using the support with high-thymidine loading (47 *u*mol g^{-1}) and a bisamidite solution of low concentration (0.010M) leads to virtually exclusive formation of A^TT. In contrast, the use of a low-loading CPG (7 *u*mol g^{-1}) and high bisamidite concentration (0.075M) resulted in the formation of A^TT in low yield. In this case, the major products detected were two compounds moving very rapidly on a 24% polyacrylamide gel, indicative of the terminally phosphorylated dimers A(2'p)3'pT and A(2'pT)3'p.

After establishing the necessary criteria for the efficient synthesis of A^TT, we carried out the synthesis of the branched undecanucleotides A^{TTTTT}_{TTTTT} (2) and A^{UUUUU}_{UUUUU} (3). These branched oligomers were prepared on a 1 *u*mol scale with samples of CPG bearing protected pentathymidylic acid[6] (47 *u*mol g^{-1}) and pentauridylic acid[7] (31 *u*mol g^{-1}), respectively. In both cases, a bisamidite solution of 0.015M was used in the branching step. The sequences were deprotected in the usual way, except that 3 required an additional treatment with tetra-*n*-butylammonium fluoride (20 °C, 16 h) to cleave the *t*-butyldimethylsilyl protecting groups present at the 2'-position of uridine residues[7]. After deprotection, the crude products were purified by TLC cellulose (in the case of 2) or preparative polyacrylamide gel electrophoresis followed by size exclusion chromatography on a Sephadex G-25 column (in the case of 3). Typical isolated yields of 10-15 A_{260} units[8] of the branched sequences were obtained. Oligomers 2 and 3 had chromatographic (cellulose TLC) and electrophoretic mobilities similar to those of the linear undecanucleotide d(TTTTTATTTTT). These nucleotides were found to be resistant to spleen phosphodiesterase (16 h, 37 °C) but were completely degraded by a mixture of snake venom phosphodiesterase and alkaline phosphatase to the anticipated nucleoside products in the proper ratios (HPLC analysis).

This study shows that it is possible to synthesize branched oligonucleotides on a solid support. The procedure is simple to use, amenable to automation in any synthesizer that uses the phosphoramidite methodology, and affords, upon deprotection, branched oligonucleotide products in good yields. By using this synthesis methodology, it will be possible to systematically explore branched RNA and to probe the structural requirement of branch RNA recognition during RNA splicing.

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8. One A_{260} unit is the amount of material which will produce an absorbance of 1.0 at 260 nm, when dissolved in one milliliter of water, in a one centimeter cell.

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