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Solid-phase synthesis of branched oligoribonucleotides related to messenger RNA splicing intermediates

Masad J.Damha*, Kanjana Ganeshan, Robert H.E.Hudson and Steven V.Zabarylo
Department of Chemistry, Erindale College, University of Toronto, Mississauga, Ontario L5L 1C6, Canada

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ABSTRACT

The chemical synthesis of oligoribonucleotides containing vicinal (2′-5′)- and (3′-5′)-phosphodiester linkages is described. The solid-phase method, based on silyl-phosphoramidite chemistry, was applied to the synthesis of a series of branched RNA [$(Xp)_nA2'(pN)_n3'(pN)_n$] related to the splicing intermediates derived from *Saccharomyces cerevisiae* rp51a pre-messenger RNA. The branched oligonucleotides have been thoroughly characterized by nucleoside and branched nucleotide composition analysis. Branched oligoribonucleotides will be useful in the study of messenger RNA splicing and in determining the biological role of RNA 'lariats' and 'forks' *in vivo*.

INTRODUCTION

Since the discovery of pre-messenger RNA splicing, remarkable progress has been made in identifying the components of the splicing machinery (spliceosome) and determining the general pathway of this ubiquitous reaction (1). Analysis of pre-messenger RNA splicing reactions in yeast and higher eukaryotes has led to the identification of branched RNA intermediates in quite novel 'lariat' (1,2) and 'Y-like' configurations (3) (Figure 1). A common structural feature of these intermediates is the vicinal (2′-5′)- and (3′-5′)-phosphodiester linkages at their branch point (Figure 2).

Little is known about the biological role of branched RNA (bRNA) and the nature of the participating spliceosomal groups which recognize it. *In vitro* studies have demonstrated that the efficiency of nuclear pre-mRNA splicing is influenced by the sequences at the 5′- and 3′-splice sites and the branch site (4–8). Mutation of the normal branch-accepting adenosine nucleoside to G (7) or C (8), for example, did not inhibit branch point formation (step I, Figure 1a) at the mutated positions, but the splicing reaction stopped at this stage with accumulation of the mutated IVS-3′-exon lariat intermediate. Mutation of the 5′-splice site residues, normally G-U to A-U, G-A, and G-G also prevented the second step (step II, Figure1a) while allowing the first step (7). These results suggest that sequence changes at either the branch or the 5′-splice site do not block recognition and cleavage of the 5′-splice site. There appears to be a 'proofreading' step, however, in which sequences at the branch point of the lariat IVS-3′-exon intermediate are examined before execution of the second step of the reaction (5, 7). Therefore, recognition of bRNA might be an essential step of messenger RNA biosynthesis. Alternatively, the three-dimensional structure and dynamics of the branch point sequences of bRNA may play an important role in the splicing process.

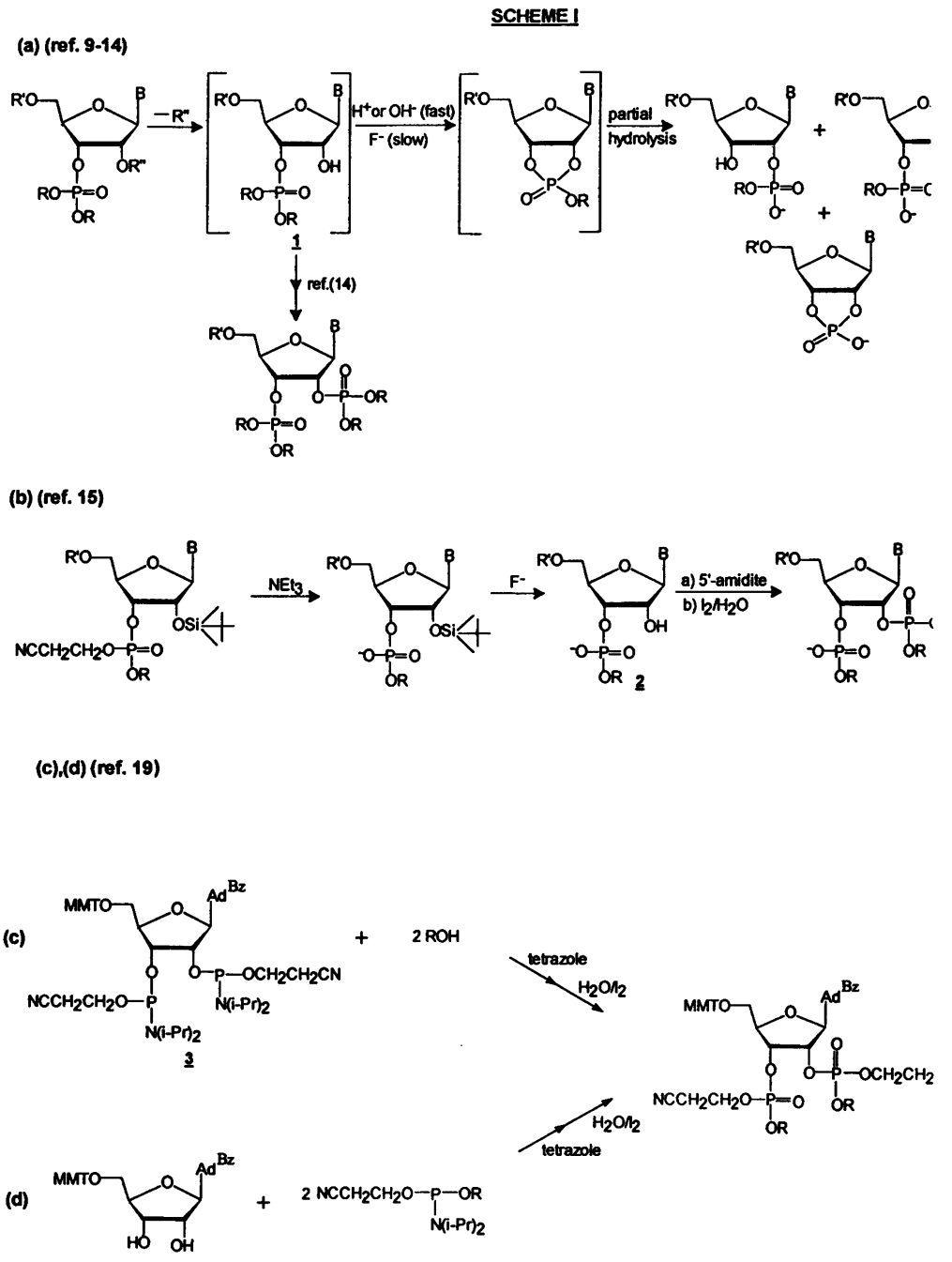
Our current research program is directed toward the development of a practical method for the chemical synthesis of branched nucleic acids. The availability of bRNA in large quantities will allow systematic study of this novel structure as well as probing the structural requirement of bRNA recognition in the splicing reaction.

The stepwise assembly of the vicinal (2′-5′)- and (3′-5′)-internucleotidic linkages present in bRNA via traditional 'phosphate triester' methods can be difficult, as cyclic phosphates may be formed (Scheme 1a). For example, acid (9–11) or base-promoted (12, 13) deblocking of a 2′-O-protecting group vicinal to a (3′-5′)-phophotriester linkage leads to chain cleavage and phosphoryl migration (Scheme 1a). Fourrey *et al.* (14) dealt with this problem by blocking the 2′-OH function with the *t*-butyldimethylsilyl (TBDMS) group. This protecting group could be removed under conditions which were mild (F^-, 0°C) and prevented subsequent attack of the released 2′-hydroxy function on the vicinal phosphotriester linkage (Scheme 1a). The labile 2′-OH, 3′-phosphotriester intermediate (**1** was then phosphorylated in situ to generate a branched trinucleoside diphosphotriester in moderate yield.

In a similar approach, Kierzek *et al.* (15) removed the 2′-O-TBDMS group by treatment with tetra-*n*-butylammonium fluoride (TBAF, r.t.), after conversion of the internucleotidic phosphotriester to the more stable diester (Scheme 1b). The resulting 2′-OH, 3′-phosphodiester **2**, unlike **1**, could be purified by silica gel column chromatography. Evidently, the negative charge of the 3′-phosphodiester group present in **2**, greatly diminished the rate of nucleophilic attack by the vicinal 2′-hydroxyl group. Condensation of **2** with a nucleoside 5′-phosphoramidite was then carried out for introducing the second phosphate function at the branch point. The same basic 'phosphotriester–phosphoramidite' strategy for introducing vicinal internucleotide linkages was

*To whom correspondence should be addressed at: Department of Chemistry, McGill University, Montreal, P.Q., H3A 2K6, Canada

SCHEME I



applied by others to the synthesis of medium size (3–9-mers) branched ribonucleotides (16–18). Two other approaches to the assembly of symmetrical (2′-5′)- and (3′-5′)-internucleotidic phosphate linkages were proposed by Damha *et al.* (11, 19). One approach involved the reaction of an appropriately 5′-O-, 6-N-protected, 2′,3′-O-bisphosphoramidite branch-point adenosine with a suitably protected 5′-hydroxy nucleoside block (Scheme 1c). The other approach involved condensation of a 5′-O-, 6-N-protected branch-point adenosine with an excess of a nucleoside-5′-O-phosphoramidite (Scheme 1d). Subsequent treatment with iodine/water in both cases generated the vicinal phosphotriester linkages. The major advantages of these methods were speed, efficiency, and the two-step introduction of the vicinal (2′-5′)- and (3′-5′)-linked framework. In addition, for syntheses involving bisamidite 3, internucleotide chain cleavage was completely avoided. Similar methodologies were applied by others to the synthesis of myo-inositol-4,5-bisphosphates (20, 21). Detailed conformational studies of short (3–7-mers) branched oligomers in aqueous solution have appeared (22, 23).

While the above solution-phase strategies can provide branched oligoribonucleotides in good yields, they involve high consumption of reagents and time-consuming chromatographic purifications. They are, therefore, only practical for the assembly of medium size (3–9 mers) bRNA sequences. In this report we describe a solid-phase approach for the synthesis of high-molecular weight branched oligoribonucleotides. The procedure is simple to use, amenable to automation in any synthesizer that uses the phosphoramidite chemistry, and affords, upon deprotection and purification, branched sequences in good yields. A preliminary account of some of these results has appeared (24).

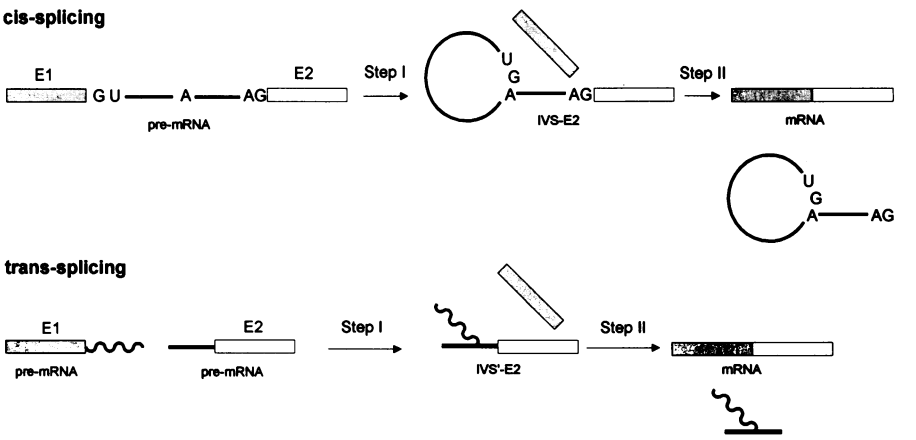


Figure 1. Schematic representation of cis- and trans-splicing reactions of pre-mRNA. (Monomolecular) cis-splicing proceeds through a lariat intermediate (IVS-E2) while (biomolecular) trans-splicing proceeds through a 'Y'-like or forked intermediate (IVS'-E2). (From ref. 1, 3).

RESULTS AND DISCUSSION

General considerations and synthesis of A(2'pT)3'pT

The solid-phase method based on the phosphoramidite chemistry appears to be the most promising approach for preparing high-molecular weight oligonucleotide sequences (25). Several routes towards RNA chemical synthesis have recently been developed (26). We have utilized the Ogilvie's silyl−phosphoramidite method (27) since it is fully compatible, in terms of equipment and reagents, with DNA synthesis permitting the use of commercially available DNA synthesizers. In addition, we find that the synthesis of RNA oligomers using this method can be achieved as efficiently as is the case for DNA oligomers. Scheme 2 outlines the pathway for the assembly of bRNA sequences, based on the silyl−phosphoramidite chemistry and the reaction of bisphosphoramidite **3** with LCAA-CPG bound nucleosides or oligoribonucleotide chains. Following branching, the 'V'-like branched sequences were either cleaved from the support and deprotected, or extended in the 3'→5' direction to yield 'Y'-like branched structures.

The synthetic strategy was proposed on the assumption that nucleosidic units on the CPG surface were in close proximity to appropriately branch with the bisphosphoramidite reagent. Simple geometric calculations showed that the maximum lateral separation between neighbouring nucleosidic units on the surface of LCAA-CPG with a nucleoside attachment of 50 μmol/g was *ca.* 25 Å. This distance, along with the 18 Å-long alkyl spacer which joins nucleoside units to the support surface, insured an appropriate distance between the reactive 5'-OH end groups (Scheme 2). Indeed, a dilute (0.02 M) solution of bisamidite **3** reacted efficiently with solid-phase bound deoxythymidine (47 μmol/g LCAA-CPG) in the presence of 1H-tetrazole to generate, after deprotection (conc. NH_4OH/ethanol, 3:1, r.t., 16 h), the branched trinucleoside diphosphate A(2'pT)3'pT. HPLC analysis of the crude material (Figure 3.1) revealed four major components which have been identified (see below) as unreacted dT, the isomeric linear dinucleoside diphosphates A(2'pT)3'p and A(2'p)3'pT, and the desired trimer A(2'pT)3'pT (19). Integration of the peaks in Figure 3.1 revealed that dT and A(2'pT)3'pT account for 18 and 68%, respectively, of the total absorbance at 254 nm, and that A(2'pT)3'p and A(2'p)3'pT combined account for a further 13%. As expected, the yield of A(2'pT)3'pT

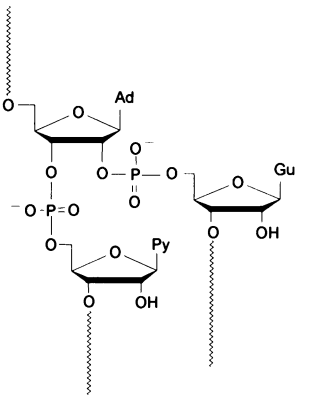
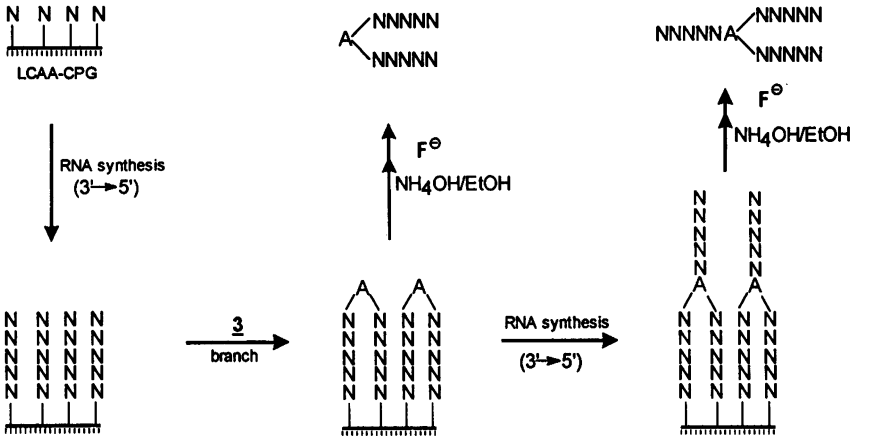


Figure 2. Branch point structure of RNA 'lariats' and generated during (cis) splicing of nuclear pre-mRNA. The adenosine and guanosine residues are highly conserved in yeast and higher eukaryotes.

was highly dependent upon the CPG's nucleosidic concentration (Table 1). A change in the nucleosidic concentration on CPG from 47 to 7 μmol/g, for instance, resulted in the preferential formation of the linear dimers (*ca.* 44%) rather than the desired branched trimer A(2'pT)3'pT (28%) (Figure 3.2 and 4, Table 1). This result was expected since the lateral distance between the reactive 5'-OH groups on the solid support's surface increases with decreasing nucleosidic concentration. It is noteworthy that nucleoside loadings in the more 'conventional' range of 25−30 μmol/g afforded, under the same conditions, the branched trimer in a good yield (Table 1).

The yield of A(2'pT)3'pT was also dependent upon the molar concentration of bisamidite derivative employed in the branching reaction. Dilute solutions of **3** (0.02 M) gave the best results since under these conditions only a fraction of the chain end 5'-OH groups became phosphitylated, favouring branching between the CPG-bound unreacted dT and the CPG-bound intermediates A(2'p*)3'pT and A(3'p*)2'pT (where * = tetrazole-activated phosphoramidite moiety). Increasing the concentration of **3** while keeping the nucleosidic concentration on CPG constant led to a gradual decrease in the yield of A(2'pT)3'pT (Table 1). We

SCHEME II



have found that 0.02M-0.03M solutions of 3 and CPG's of nucleosides loadings in the range of 25−50 μmol/g gives the best results for the synthesis of medium size bRNA oligomers (i.e., A2'(pN)$_n$3'(pN)$_n$, where 10 < n < 1, *vide infra*).

Synthesis of branched oligonucleotides

After establishing the necessary criteria for the efficient introduction of the branch framework, the syntheses of several 'V'- and 'Y'-like branched oligonucleotide sequences were conducted (Table 2). Structures H and I, branched octadecaribonucleotides, possess a base sequence similar to that of RNA lariats generated in the splicing of *S.cerevisiae* rp51a pre-mRNA (4).

All syntheses were performed on an automated DNA synthesizer (Applied Biosystem 381A). The readily available building blocks, 5'-dimethoxytritylated, 2'-*tert*-butyldimethylsilylated N-protected [benzoyl (Bz) for cytidine and adenosine, isobutyryl (*i*-Bu) or dimethylformamidine (Dmf) for guanosine] ribonucleoside 3'-cyanoethylphosphoramidites were employed for chain assembly. The bisphosphoramidite reagent 3 was prepared by the procedure described by Damha and Ogilvie (19) and was added through the extra base port on the synthesizer. Typically, oligomers were prepared on a 1 μmol scale using a modification of the pulsed-delivery protocol provided by the Applied Biosystems software (see Experimental Section). In all cases, 0.15 M solutions of ribonucleoside 3'-phosphoramidites were used to assemble 'linear' chains, and a 0.02−0.03 M solution of 3 to introduce the branch point. Average coupling yields for 3'-phosphoramite and 2',3'-bisphosphoramidite additions were 95−98% and 40−60%, respectively, as determined by UV quantitation of the released trityl cation. Removal of phosphate 2-cyanoethyl 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 room temperature. These conditions have been found to minimize the loss of 2'-*tert*-butyldimethysilyl groups and subsequent cleavage of the ribonucleotide chain (28). Heating is not recommended since a substantial increase in the number of shorter fragments occured when this treatment was performed at 55°C (16 h) instead of at room temperature (24 h) (PAGE, data not shown). The milder ammonia/ethanol treatment (r.t., 24 h) was sufficient to deprotect rC^{iBu}, rA^{Bz} and rG^{Dmf} units but insufficient to deacylate rG^{iBu} units. Therefore for branched oligomers assembled with (commercially available) rG^{iBu} phosphoramidite units it was necessary to prolong the ammonia/ethanol treatment to 2−3 days (r.t.) (29).

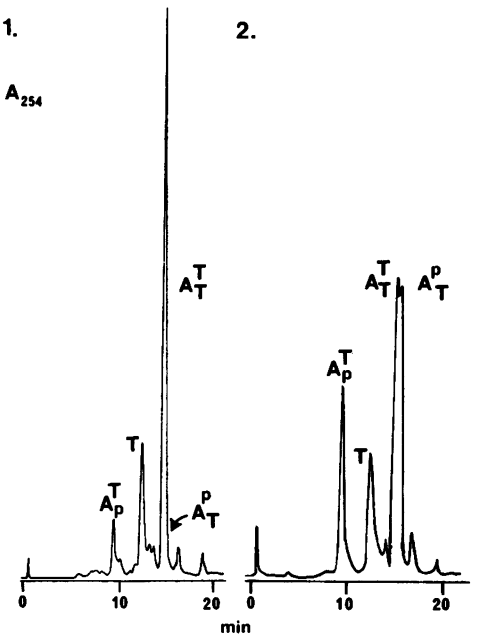


Figure 3. Reverse-phase HPLC analysis of the crude branched trimer A(2'pT)3'pT resulting from coupling of bisamidite 3 with (1) LCAA-CPG-dT (47 μmol/g) and (2)LCAA-CPG-dT (7 μmol/g). Specific assignments of the isomeric dimers A(2'pT)3'p and A(2'p)3'pT are tentative. HPLC conditions: ODS-2 column (250×4.6 mm); solvent A, 0.02 M KH_2PO_4 (pH 5.5); solvent B: methanol; gradient: 0−50% solvent B in 30 min.

Finally, the solutions were evaporated to dryness and treated with tetra-*n*-butylammonium fluoride (TBAF; 1 M in THF) for 24 h at room temperature to remove silyl groups from the ribonucleotide units in the chains. The crude bRNA oligomers were then analyzed and purified by polyacrylamide gel

Table 1. Effect of yield (%)[a] on LCAA-CPG-thymidine loading and bisphosphoramidite concentration in the synthesis of branched trinucleoside diphosphate A(2′pT)3′T

		LCAA-CPG (μmol/g)		
		7	26	47
	0.02 M	28	61	68
A^{p}_{p}(3)	0.08 M	13	32	44
	0.15 M	11	31	40

[a]Calculated from A(2′pT)3′T peak areas, HPLC analysis (Figure 1).

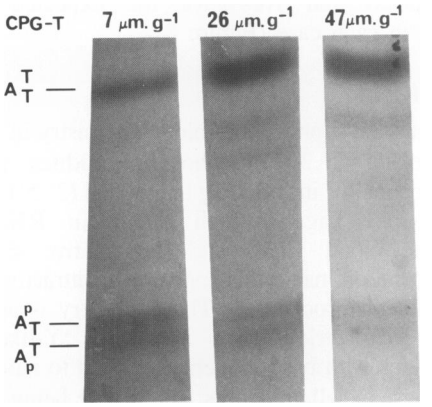


Figure 4. Effect of dT-LCAA-CPG loading on yield of trimer (A2′pT)3′pT. A 0.02 M solution of bisamidite **3** was allowed to react with LCAA-CPG of different dT concentrations. Following deprotection (see text) the crude mixtures were analyzed on 24% polyacrylamide/7 M urea gels. Gels were visualized by UV shadowing (see Experimental).

electrophoresis (PAGE). Because the molecular weight of the CPG-bound sequences more than doubles during the branching step, the desired products were easily identified. A typical gel analysis of the crude oligomer A(2′pUUUUU)3′pUUUUU (**B**) is shown in Figure 5. The mixture consisted of a major slow-moving band separated by a large gap from three fast closely-moving bands. The slow-moving band was identified as the desired branched oligomer **B** (*vide infra*). The fastest-moving band had an electrophoretic mobility identical to that of an authentic sample of linear $(Up)_4U$ and was assigned as such. The two other bands with intermediate mobility were tentatively assigned to the isomeric hexanucleotides by-products A(2′UUUUU)3′p and A(2′p)3′UUUUU. The same gel pattern was observed for most of the 'V'-liked sequences prepared. Despite the inherent limitations of the procedure (*i.e.*, incomplete branching to give linear $A2'(pX)_n3'p$ and $A2'p3'(pX_n)$), the desired products were generally the major components of the isolated crude mixtures (Figure 6).

In each case, the appropriate bands from the gels were excised under UV-shadow, extracted with water at 37°C, and desalted by Sephadex G-25F® or C18 SEP-PAK® chromatography. Isolated yields of purified oligoribonucleotides were generally in the range of 5−15 A_{260} units and are reported in Table 2. The purity of a few bRNA sequences is shown in Figures 7 and 9.

Table 2. Synthesis of branched oligoribonucleotides

No.	Oligomer (5′ → 3′)	Length of Oligomer	Nucleoside Loading on CPG (μmol/g)	Purification Method[a]	Isolated Yield (A_{260} units) Crude	Pure
A	$A^{2'}_{3'}$ TTTTT / TTTTT	11	47	X,Y	52	10
B	$A^{2'}_{3'}$ UUUUU / UUUUU	11	31	X,Y,Z	54	13
C	$A^{2'}_{3'}$ CCCCC / CCCCC	11	16	X,Y,Z	33	3.2
D	$A^{2'}_{3'}$ AAAAA / AAAAA	11	38	X,Z	35	7
E	$A^{2'}_{3'}$ CAAGUU / CAAGUU	13	38	X,Z	44	13
F	$A^{2'}_{3'}$ GUAUGU / GUAUGU	13	38	X,Z X,Z	44[b] 54[c]	15[b] 10[c]
G	TTTTT$A^{2'}_{3'}$ TTTTT / TTTTT	16	47	X,Z	78	10
H	UACUAA$^{2'}_{3'}$ CAAGUU / CAAGUU	18	38	X,Z	61	12
I	UACUAA$^{2'}_{3'}$ GUAUGU / GUAUGU	18	38	X,Z X,Z	50[b] 58[c]	15[b] 11[c]

[a]X, Preparative polyacrylamide gel electrophoresis; Y, Size exclusion chromatography (sephardex G-25); Z, Reversed-phase C_{18} SEP- PAK® chromatography (see Materials and Methods). [b]Oligomer prepared from A^{Bz}, C^{Bz} and G^{DMF} units. [c]Oligomer prepared from A^{Bz}, C^{Bz} and G^{iBu} units.

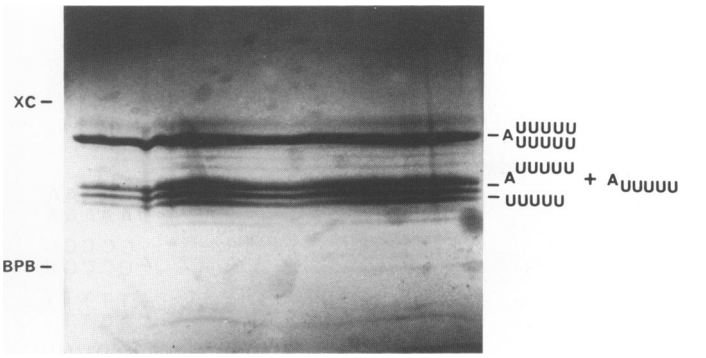


Figure 5. Preparative purification of branched A(2′pUUUUU)3′pUUUUU on a 24% polyacrylamide/7 M urea gel. The gel was visualized by UV shadowing. Positions of the dyes Bromophenol Blue (BPB) and xylene cyanol (XC) are indicated.

Characterization of branched oligoribonucleotides

As a check on the purity, branched structure, and base composition of the oligoribonucleotides, a small purified sample (0.2 A_{260}) was subjected to degradation by selected exo- and endonucleases. Specifically, nuclease P1 (*P.citrinum*), an endonuclease specific for single stranded DNA and RNA along with snake-venom phosphodiesterase (SVPDE, *C.durissus*), a 3′-exonuclease, were chosen for this study. A small amount of alkaline phosphatase (AP) was used in conjunction with the above

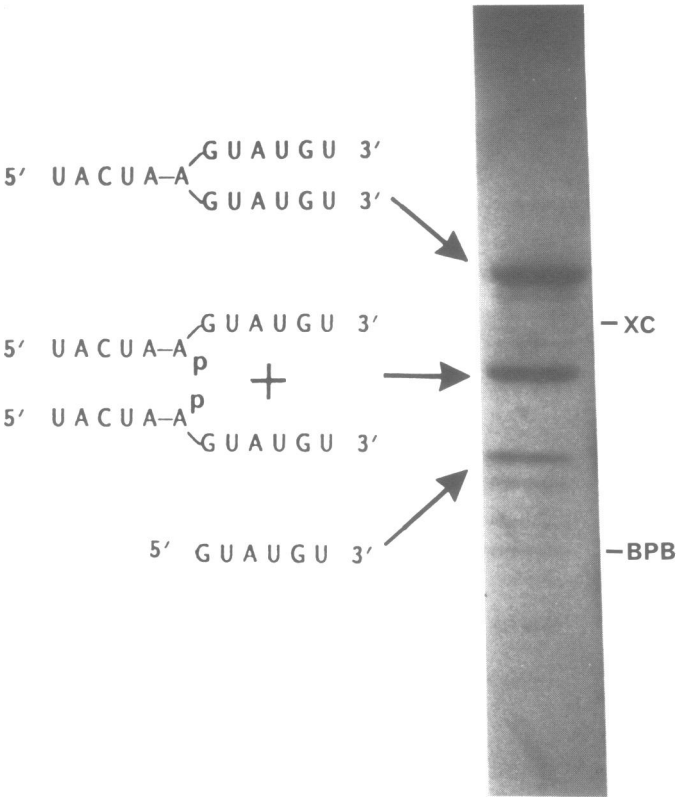


Figure 6. Electrophoresis of crude 'Y'-like oligomer I on a 24% polyacrylamide/7 M urea gel. Positions of Bromophenol Blue (BPB) and Xylene Cyanol (XC) are indicated.

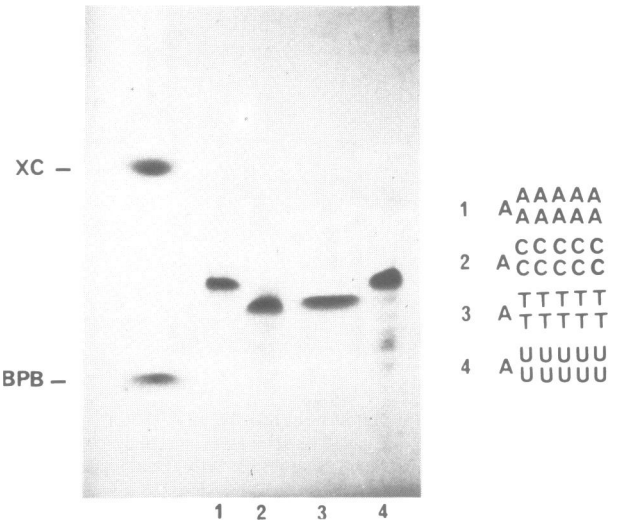


Figure 7. Electrophoresis of pure 'V'-like bRNA fragments on a 24% polyacrylamide/7 M urea gel. Positions of Bromophenol Blue (BPB) and Xylene Cyanol (XC) are indicated.

enzymes to convert the resulting nucleoside 5′-monophosphates into nucleosides. The digests were then analyzed by reversed-phase HPLC and PAGE (Figures 8 and 9).

Treatment of branched oligomers with a mixture of nuclease P1 and AP generated, in all cases, the branched core trinucleoside diphosphates, A(2′pX)3′pX, and nucleosides in the expected ratios (Figure 8). The branched products were identified by HPLC comparison (using a co-injection technique) and PAGE with authentic samples of the trimers prepared by the established solution-phase method (19). The resistance of the vicinal (2′-5′)- and (3′-5′)-phosphodiester linkages to nuclease P1 has also been observed for the branched cores A(2′pG)3′p(C or U) characteristic of most splicing intermediates from HeLa and yeast cells, and of RNA lariats formed *in vitro*. Since nuclease P1 hydrolyzes (3′-5′)-, and not (2′-5′)-, phosphodiesters of linear oligonucleotides specifically, it appears that the 2′-5′-linked nucleotidyl unit of bRNA fragments 'protects' the vicinal 3′-5′-phosphodiester from endonucleotidic cleavage.

Finally, analysis of enzymatic digestion by a mixture of SVPDE (which can split either 3′-5′- or 2′-5′-linkages of linear oligonucleotides) and AP gave the expected nucleoside composition in each case (Figure 8).

CONCLUSIONS

The solid-phase silyl-phosphoramidite chemistry in conjunction with nucleoside 2′,3′-O-bisphosphoramidites provides a convenient means for introducing the vicinal (2′-5′) and (3′-5′)-phophodiester linkages which occur in RNA splicing intermediates. Good yields and the relative simplicity of automated operation makes this approach an attractive alternative to other synthetic procedures. The chemistry is applicable to syntheses of symmetric 'V-like' as well as 'Y-like' branched oligonucleotides with base sequences related to natural bRNA. In view of these excellent results, studies are being extended to the regiospecific solid-phase synthesis of natural sequences (*i.e.*, 5′-$(Xp)_nA2'(pY)_n3'(pZ)_n$) and the preparative (mg-quantity) synthesis and NMR studies of long bRNA chains. Furthermore, several synthetic bRNA related to the sequence of pre-mRNA intermediates from HeLa and yeast cells are currently being used to probe the mechanism of bRNA recognition during RNA splicing.

MATERIALS AND METHODS

Reagents

Ribonucleosides phosphoramidites, i.e., 5′-O-DMT-2′-O-TBDMS-uridine-3′-O-(2-cyanoethyl)-N,N′-diisopropylphosphoramidite; N^4-benzoyl-5′-O-DMT-2′-O-TBDMS-cytidine-3′-O-(2-cyanoethyl N,N′-diisopropylphosphoramidite); N^6-benzoyl-5′-O-DMT-2′-O-TBDMS-adenosine-3′-O-(2-cyanoethyl N,N′-diisopropylphosphoramidite); N^2-isobutyryl-5′-O-DMT-2′-O-TBDMS-guanosine-3′-O-(2-cyanoethyl N,N′-diisopropylphosphoramidite) were purchased from Dalton Chemical Laboratories, Inc. (Toronto). N^2-((dimethylamino)methylene)-5′-O-DMT-2′-O-TBDMS-guanosine-3′-O-(2-cyanoethyl N,N′-diisopropylphosphoramidite) was generously provided by Dr Ravi Vinayak (Applied Biosystems, CA). N^6-benzoyl-5′-O-MMT-2′,3′-bis-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) 3 was prepared as previously described (19).

Reagents for solid-phase synthesis were obtained from Applied Biosystems or can be prepared as follows: (a) anhydrous acetonitrile: we recommend its use for both amidite dissolution and intermediate washing steps. (b) Detritylation: 3% trichloroacetic acid/dichloroethane for deblocking DMT groups, and 5% trichloroacetic acid/dichloroethane for deblocking MMT groups. (c) Activation: 0.5 M tetrazole in acetonitrile. (d) Capping: Cap A, 10% acetic anhydride/10% 2,6-lutidine/THF

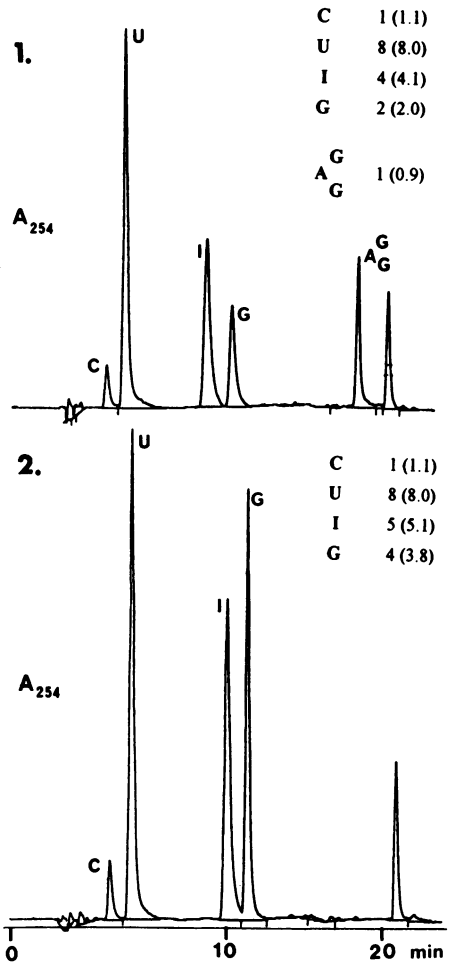


Figure 8. Reversed-phase HPLC analysis of products resulting from (1) nuclease P1/alkaline phosphatase treatments of pure UACUAA(2'pGUAUGU)3'pGUAUGU I. Inosine results from the deamination of adenosine by adenosine deaminase, which is a contaminant in the enzyme preparations. Experimentally found (in parenthesis) and expected molar ratio of products are provided. The last eluting peak is a non-nucleotidic material which leached from polyacrylamide gels.

and Cap B, 16% N-methylimidazole/THF. (e) Oxidation: 0.1M iodine in THF/pyridine/water (75:20:2, v/v/v). Tetra-*n*-butylammonium fluoride (1 M) in THF and diethylpyrocarbonate were purchased from Aldrich. Sephadex G-25 F was purchased from Pharmacia. Polyacrylamide gel electrophoresis (PAGE) reagents were purchased from Bio-Rad. HPLC grade solvents, pyridine, dichloromethane, and acetonitrile were purchased from BDH and Caledon (Toronto).

Derivatization of controlled pore glass supports

Long chain alkylamine controlled-pore glass (LCAA-CPG, 500 Å, Pierce Rockford, IL, or CPG, Inc., N.J.) was derivatized with 5'-O-MMT-uridine, 5'-O-DMT-deoxythymidine, 5'-O-MMT-N^6-Bz-adenosine, and 5'-O-MMT-N^4-Bz-cytidine following minor modifications of the procedures described by Damha *et al.* (30) (for ribonucleoside bound CPG) and Pon *et al.* (31) (for deoxythymidine bound CPG). Final nucleosides loadings were in the range of 28–47 μmol/g and are given in Table 2. A typical preparation of 5'-MMT-uridine bound LCAA-CPG follows: LCAA-CPG (5 g) was slowly stirred or shaken in a solution of 3% trichloroacetic acid in dichloroethane at room

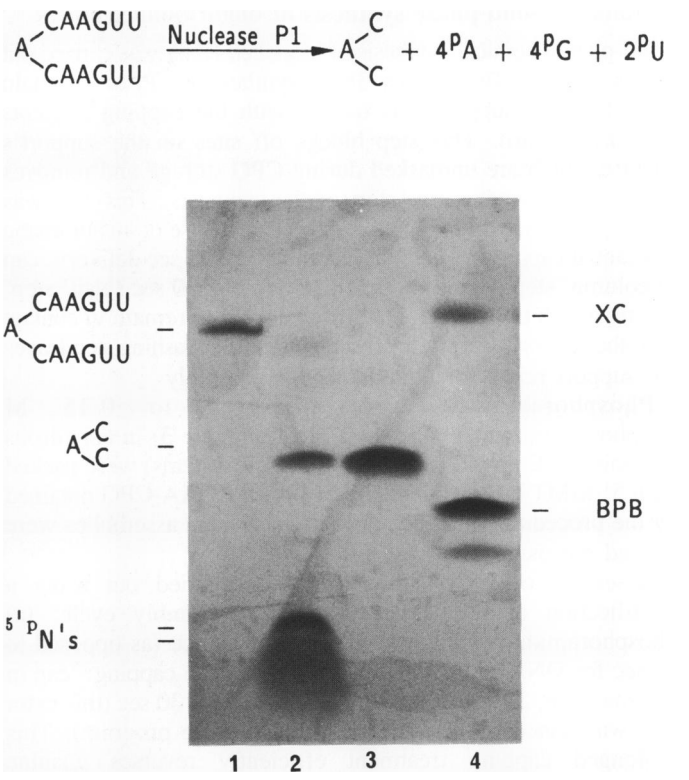


Figure 9. Analysis of a Nuclease P1 digest of bRNA F on a 24% polyacrylamide/7 M urea gel. **Lane 1**, purified oligomer F. **Lane 2**, products resulting from nuclease P1 treatment of F. **Lane 3**, pure trimer A(2'pC)3'pC prepared as in ref. 19. **Lane 4**, Xylene Cyanol (XC) and Bromophenol Blue (BPB) dyes.

temperature for 16 h. The activated LCAA-CPG was then filtered off and washed with 9:1 triethylamine:diisopropylethylamine (50 mL), and then with dichloromethane and ether. The activated support was dried under vacuum over P_2O_5 before use. The activated CPG sample (1 g), succinic anhydride (2 mmol, 0.20 g) and DMAP (0.33 mmol, 40 mg) were placed into a 6 mL glass Hypovial (Pierce). Anhydrous pyridine (6 mL) was added via syringe and the vial was shaken on a wrist action shaker for 16 h (r.t.). The support was then filtered off and washed successively with pyridine, methylene chloride and ether (this sample may be stored indefinitely at r.t. until required). Succinylated LCAA-CPG (0.5 g), 5'-MMT-uridine (52 mg, 0.10 mmol), DMAP (6 mg, 0.05 mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (192 mg, 1.0 mmol) were placed in a 6-mL Hypovial. Triethylamine (40 μL) and anhydrous pyridine (6 mL) were then added, and the mixture shaken at room temperature for 16 h. Pentachlorophenol (67 mg, 0.25 mmol, dissolved in 100 μL pyridine) was then added and shaking was continued for another 16 h. Finally, piperidine (2 mL) was added and after 5 min (*Important*: prolonged treatment with piperidine will cleave the CPG bound nucleoside and will decrease the nucleoside loading on the support) the CPG was filtered off, washed successively with dichloromethane and ether, and dried. The derivatized support was then 'capped' by suspending the beads in acetic anhydride capping reagent (6 mL; prepared by combining equal volumes of Cap A and Cap B reagents supplied for use on the Applied Biosystem DNA synthesizer). The support was shaken for 2 h and then washed successively with pyridine and methylene chloride. The nucleoside loading can then be obtained by trityl analysis.

Automated solid-phase synthesis of oligoribonucleotides

Solid-phase chemical synthesis of branched RNA was carried out on an Applied Biosystems 381A synthesizer. Prior to chain assembly, the support was treated with the capping reagents (Ac_2O/N-MeIm). This step blocks off sites on the support's surface which are unmasked during CPG storage and removes any trace of water at the start of the synthesis. This step was easily performed on the DNA synthesizer by use of an automatic or manual capping cycle. We recommend a 45 sec delivery 'cap to column' step followed immediately by a 300 sec 'wait' step, during which time the reagents are allowed to remain in contact with the support. Finally, a 60 sec acetonitrile washing step leaves the support ready for oligonucleotide assembly.

Phosphoramidites were dissolved to 0.15 M (3'-phosphoramidites) and 0.02 M (bisamidite 3) in anhydrous acetonitrile. Empty columns (Applied Biosystems) were packed with 5'-MMT-ribonucleoside-derivatized LCAA-CPG obtained by the procedure described above (27). Chain assemblies were carried out using the 'trityl off' mode.

Assembly of bRNA sequences was carried out using a modification of the 1.0 mmol DNA assembly cycle: (a) Phosphoramidite coupling: 'wait; step, 600 sec (as opposed to 30 sec for DNA synthesis). (b) Ac_2O/N-MeIm capping: 'cap to column' step, 20 sec followed by a 'wait' step, 30 sec (this extra step was added by editing the standard cycle program). This prolonged capping treatment efficiently reverses guanine modification which takes place during the amidite coupling step (32, 33). (c) Oxidation: 'iodine to column' step, 30 sec, followed by a 'wait' step, 20 sec. (d) Detritylation: 'TCA to column' step, 120 sec for DMT deblocking, and 160 sec for MMT deblocking.

Quantitation of the trityl cation released each cycle from the 5'-terminus of the growing oligonucleotide chain is used as a preliminary monitor of synthesis performance. Comparison of each synthesis cycle to the previous one usually indicates 95–97% coupling yield per cycle for 3'-phosphoramidites and 40–60% for the bisphosphoramidite (3) (Note: 100% branch formation would theoretically yield, in the TCA step, half the amount of trityl cations released in the previous coupling step, *i.e.*, an apparent '50% coupling yield'). Full details of the methods of RNA synthesis are given elsewhere (34).

Deblocking of synthetic branched oligoribonucleotide sequences

Oligomer bound-LCAA-CPG's were treated with NH_4OH/ethanol (3:1, v/v) at room temperature for 2–3 days to cleave the oligomer from the support, and to remove 2-cyanoethyl (phosphates), N-isobutyryl (G residues) and N-benzoyl protecting groups (A and C residues). Under these conditions, deprotection of oligomers assembled with rG^{Dmf}, rA^{Bz}, and rC^{Bz} amidites required only a 16 h treatment. (Note: Heating, or the use of totally aqueous ammonia, is not recommended since this leads to cleavage of the assembled ribonucleotide chains. Concentrated NH_4OH should be stored tightly sealed in a refrigerator and opened only briefly before use). Upon completion, the sample was frozen (dry ice/ethanol bath) and lyophilized to dryness using a Speed-Vac concentrator (Savant Instruments).

TBDMS protecting groups were then removed by treatment of the residue obtained from the deacylation step with 1.0 M tetra-$_n$-butylammonium fluoride (TBAF)/THF solution (50 equivalents of TBAF per TBDMS group, r.t., 24 h). The reaction was quenched with sterile water (5.0 mL) and the amount of recovered crude product quantitated in A_{260} units (the amount of material which will produce an absorbance of 1.0 at 260 nm, when dissolved in 1.0 mL of water, in a 1-cm cell). Following quantitation, the solution can be safely stored frozen at −20°C. Lyophilize to a white powder immediately prior to purification.

Purification of branched oligonucleotides

It is critical that sterile equipment, reagents, and handling techniques be used in handling free oligoribonucleotides. Water, Sephadex, silanized glassware and plasticware must be autoclaved in the presence of diethylpyrocarbonate. During all steps of the deprotection, gloves must be worn to prevent enzymatic degradation by ribonucleases present on one's hands.

Polyacrylamide gel electrophoresis (PAGE). The crude oligoribonucleotides obtained after the TBAF treatment were purified on preparative 24% polyacrylamide/7 M urea gels (up to 30 A_{260} crude oligomer per gel) at constant current for 4–5 h (10 mA for the first 15 min followed by 20 mA to completion) using 0.09 M Tris–borate–EDTA buffer (pH 8.3). Following electrophoresis, the gels were wrapped in plastic wrap (Saran Wrap®), placed over a fluorescent TLC plate illuminated with a UV lamp (254 nm), and analyzed (*Caution*: Eye protection required). The illuminated gels can be photographed using Polaroid PolaPan® 4×5 cm Instant Sheet Film (#52, medium contrast, ISO 400/27°) through a Kodak Wratten gelatin filter (#58 green, cat. no. 1495860). The least mobile band was excised, and the gel piece incubated in a 16 mL polypropylene tube in sterile water (5 mL) at 37°C (16 h). The tube was vortexed and centrifuged and the supernatant transferred to a polypropylene tube. The gel pieces were washed with more water (3 mL) and the combined washings were lyophilized and quantitated in A_{260} units.

Following purification by PAGE, the sequences were desalted by either size exclusion chromatography on Sephadex G-25F (Parmacia), or reversed-phase chromatography on C_{18} SEP-PAK® cartridges (C_{18} SEP-PAK® generally yielding better results).

Size exclusion chromatography on Sephadex G-25F. The material was dissolved in water (1 mL), applied to a Sephadex G-25F column (4×2 cm), and eluted with water (*ca.* 15 mL). The eluent was collected in ten 1.5-mL fractions and each of the fractions was lyophilized to dryness. The pure desalted oligomer was usually in the first three fractions (UV quantitation). Fractions containing a mixture of the oligomer and salts were combined and the procedure repeated. In cases where the Sephadex system failed to desalt the oligomer, C_{18} SEP-PAK^R cartridge purification was used following this procedure.

Reversed-phase chromatography on C_{18} SEP-PAK® cartridges. The cartridge was attached to a 10 mL syringe and flushed with methanol (10 mL, HPLC grade) followed by sterilized water (10 mL). The bRNA sample (2–10 A_{260} units) obtained from the TBAF treatment (or after Sephadex purification) was dissolved in 50 mM aqueous triethylammonium acetate (TEAA) and loaded on the cartridge collecting the eluant (A). The cartridge was flushed with 50 mM TEAA (3 mL) collecting again the eluant (B). The oligomer was then eluted from the cartridge with 100 mM TEAA/methanol (1:1, 10 mL) collecting five 1 mL fractions

(C). The oligomer eluted in the first two TEAA/methanol fractions (C) (UV quantitation at A_{260}). When solutions A and B showed absorption maxima at 260 nm, the fractions were combined, lyophilized and repurified.

Enzyme assays

Enzymes were purchased from Boehringer Mannheim (Quebec). Incubation buffers were made-up using autoclaved doubled distilled water and filtered through a 0.45 μ-pore filter. They were stored at −20°C prior to use.

Nuclease P1/alkaline phosphatase. Lyophilized nuclease P1 (from *Pencillium citrinum*) was dissolved in 30 mM NH_4OAc (pH 5.3) (1 mg enzyme/mL or 300 units/mL). Alkaline phosphatase (AP, from calf-intestine) was obtained as a suspension in 3.2 M $(NH_4)_2SO_4$/0.1 mM $ZnCl_2$, pH *ca.* 7.0. Typically, 0.3 A_{260} units of the lyophilized branched oligomer was dissolved in 0.1 M Tris−HCl/1 mM $ZnCl_2$ (pH 7.2, 17 μL) in a 1.5 mL Eppendorf tube, and nuclease P1 (3 μL) and AP solutions (1 μL) were added. After incubation (37°C, 2 h) the sample was centrifuged and analyzed by HPLC as described below.

Snake venom phosphodiesterase (SVPDE)/alkaline phosphatase. The enzyme (from *Crotalus durissus*) (Boehringer Mannheim) was obtained as a solution (2 mg/mL) in 50% (v/v) glycerol, pH *ca.* 6.0. Typically, 0.3 A_{260} units of the lyophilized branched oligomer was dissolved in 50 mM Tris−HCl/10 mM $MgCl_2$ (pH 8.0) in a 1.5 mL Eppendorf tube, and SVPDE (1 mL) and AP solutions (1 mL) were added. After incubation (37°C, 2 h) the sample was centrifuged and analyzed by HPLC as described below.

HPLC analysis of enzymatic digests. HPLC analysis of enzymatic digests of oligomers was used to obtain information concerning purity, and base and branched composition. HPLC grade acetonitrile and mobile phase buffer were filtered through 0.45 μ-pore filter and degassed before use. HPLC analysis were carried out on a Waters Max 480 system (Millipore) equipped with a 254 nm detector under the following conditions: reversed-phase column, Whatman Partisil 5-C_8 (4.6×250 mm, Chromatographic Specialties); mobile phase, solvent A: 50 mM triethylammonium acetate; solvent B, acetonitrile, gradient 0−10% solvent B in 10 min, followed by 10−35% solvent B in 15 min; flow rate 1.2 mL/min; temperature 22°C. Under these conditions the order of elution of nucleosides is C (5.3 min), U (6.3 min), I (11.5 min), G (13.3 min), and A (17.7 min). Peak areas and the previously reported extinction coefficient values (in mL cm^{-1} mmol-1) at 254 nm (35) were used to calculate concentration of monomers: C (6.7), U (9.0), I (10.1), G (13.3) and A (14.0).

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