er/petroleum ether) to give two fractions: (1) dimer **6b** (210 mg, 34%) and (2) a mixture (3:2) of **6b-A** and **6b-B** (350 mg, 36%). The mixture was heated in benzene to 80 °C for 2 h. [2.2.1]Bicyclic **6b-A** was converted quantitatively into **6b-B**. **6b-A**: 90-MHz ^{1}H NMR ($CDCl_3$) δ 6.22, 5.98 (dd, J = 6 Hz, J = 3 Hz, 2 H, ring CH=CH), 5.34 (m, 2 H, C*H*=C*H*), 3.72 (s, 3 H, OCH_3), 3.27, 2.81 (m, 2 H, 2 bridgehead H), 1.20-3.00 (m, 4 H, 2 CH_2), 1.60 (m, 3 H, CH_3). ***cis*-Hydrindane 6b-B**: IR ($CHCl_3$) 3060 (w), 2980 (m), 2940 (m), 2880 (m), 2835 (m), 1710 (vs), 1655 (w), 1635 (w), 1400 (s), 1380 (w), 1375 (w), 1345 (w), 1310 (m), 1260 (vs), 1120 (m), 1085 (m), 1035 (w), 995 (w); 90-MHz ^{1}H NMR ($CDCl_3$) δ 6.86 (m, 1 H, C*H*=C), 5.44 (m, 2 H, CH=CH), 3.73 (s, 3 H, OCH_3), 1.8-2.2 (m, 7 H, 2 CH_2, 3 CH), 1.20 (d, J = 7.5 Hz, 3 H, CH_3); 20-MHz ^{13}C NMR ($CDCl_3$) δ 167.4 (s, C=O), 147.3 (d, =CH), 132.3, 130.6 (d, CH=CH), 131.0 (s, *C*=CH), 51.5 (q, OCH_3), 50.1, 35.3, 33.1 (d, 3 CH), 41.1, 27.9 (t, 2 CH_2), 17.7 (q, CH_3); mass spectrum (70 eV, room temperature), m/z (relative intensity) 192 (30, M^+), 160 (13), 133 (8), 127 (33), 126 (11), 121 (10), 115 (18), 91 (13), 67 (23), 66 (100); exact mass calcd for $C_{12}H_{16}O_2$ 192.1150304, found 192.1149426.

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Synthesis and Spectroscopic Analysis of Branched RNA Fragments: Messenger RNA Splicing Intermediates

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RNA splicing is now established as a major RNA processing reaction in eukaryotic cells. Splicing of messenger RNA precursors generates a lariat RNA structure containing a branched RNA core (Wallace J. C.; Edmons M. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 950-954) of the type A(2′p5′G)3′p5′X (A^G_X), where X is a pyrimidine residue. Understanding the mechanism which generates these molecules as well as the role they play in the splicing reaction are central issues in cell biology. Chemically synthesized branched RNA fragments of defined sequence and structure are likely to play a key role in understanding the full biological role of these molecules. Herein we describe the chemical synthesis of a series of trinucleotides (A^U_U, A^G_G, A^C_C, A^G_U, A^U_G, A^G_C, A^C_G, A^T_T, G^U_U, aA^U_U) and a tetranucleotide (UpA^U_U) with branched linkages. These molecules were fully characterized by UV, NMR (^{1}H, ^{13}C, ^{31}P), HPLC analyses of enzymatic digests, and gel electrophoresis.

Introduction[1]

In vivo and in vitro studies of the biosynthesis of messenger RNA precursors (pre-mRNA) have established that splicing of these primary transcripts proceeds via a novel RNA form, a lariat RNA.[2] This intermediate is an RNA branch with RNA chains linked to an adenosine residue by both 2′-5′ and 3′-5′ vicinal phosphodiester linkages. Analysis of branch structures in yeast and higher eukaryotes has indicated that the adenosine-branched nucleoside is usually linked to guanosine and a pyrimidine through the vicinal 2′-5′ and 3′-5′ phosphodiester linkages, respectively. The structure of the branch is A(2′p5′G)-3′p5′U (A^G_U) in the case of adenovirus 2 transcripts[2b] and A(2′p5′G)3′p5′C (A^G_C) in both β-globin[2c] and yeast[2d] actin RNA precursors. In spite of the remarkable advances made in the elucidation of the splicing process, many aspects of this reaction are still poorly understood. The exact mechanism of splice site and branch point selection is unknown as are the role and conformational properties of the lariat molecules. Some authors have speculated that the branch point sequences of the lariat molecules may serve as a recognition signal for achieving accurate splicing.[2c,3] Alternatively, the primary sequence and/or the three-dimensional structure of the branch may play a key role (e.g. may possess catalytic activity) in directing and regulating the splicing process. In order to gain a better understanding of the role of lariat RNA, the development of chemical synthesis and the study (e.g. conformational analysis) of branched nucleotide fragments is a prerequisite. In this report we describe the chemical synthesis and structural characterization of a series of trinucleotides and a tetranucleotide with branched linkages. A preliminary report of some of these results has appeared.[4]

Results and Discussion

Synthesis of Branched RNA. Branched RNA fragments were rapidly prepared by introducing both 2′-5′ and 3′-5′ vicinal phosphate linkages simultaneously. The basis of this strategy, as presently applied to the phosphite triester synthesis of oligonucleotides using nucleoside phosphoramidites, is summarized in Scheme II. The key

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(1) Abbreviations: the branched nucleotide sequence X(2′-p-5′Y)3′-p-5′Z where X is the branched nucleoside is abbreviated as X^Y_Z. The linear sequences X3′-p-5′Y3′-p-5′Z and X2′-p-5′Y2′-p-5′Z are written as XpYpZ and XpYpZ, respectively. The branched tetramer U3′-p-5′A-(2′-p-5′U)3′-p-5′U is abbreviated as UpA^U_U.

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(3) Hornig, H.; Aebi, A.; Weissmann, C. *Nature (London)* **1987**, *324*, 589-590.

(4) Damha, M. J.; Pon, R. T.; Ogilvie, K. K. *Tetrahedron Lett.* **1985**, *26*, 4839-4842.

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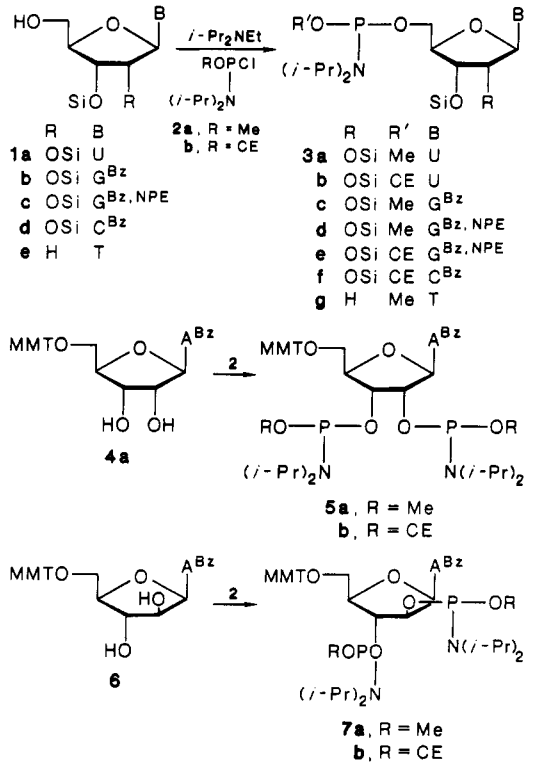
Scheme I[a]



[a] OSi = (*tert*-butyldimethylsilyl)oxy.

reagents used were the nucleoside 5′-phosphoramidites **3** and the 2′,3′-diphosphoramidite derivatives **5** and **7**. These derivatives were prepared by the reaction of nucleosides **1** with either methyl (**2a**) or β-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (**2b**) in 60–95% yields by using established procedures (Scheme I).[5-7]

The branched trinucleotides A^{U}_{U}, A^{G}_{G}, A^{C}_{C}, A^{G}_{U}, A^{U}_{G}, A^{G}_{C}, A^{C}_{G}, A^{T}_{T}, and G^{U}_{U} (**8a–l**) were prepared via one-flask procedures using either route A or B (Scheme II). Thus, 5′-*O*-(monomethoxytrityl)-N^6-benzoyladenosine (**4a**), the corresponding 5′-methyl or cyanoethyl phosphoramidites **3a–g** (3.0 equiv) and tetrazole (12.0 equiv) were stirred in anhydrous tetrahydrofuran (THF) for 1 to 2 h (route A). Similarly, the adenosine 2′,3′-diamidites **5a** and **5b**, uridine **1a** (3.0 equiv), and tetrazole (8.0 equiv) were reacted under identical conditions (route B). After completion of reactions, collidine and an excess of an aqueous iodine solution were added to oxidize the phosphite triester intermediates. The organic layer was washed with an aqueous solution of sodium bisulfite and evaporated, and the crude products were detritylated with a 0.1 M benzenesulfonic acid (BSA)/acetonitrile solution (0 °C, 10 min). After chromatography on silica gel, the detritylated methyl- and cyanoethyl-protected nucleotides **9** were isolated in 15–30% and 50–75% yields, respectively.

The use of an excess of 5′-phosphoramidite is essential for obtaining good yields of the trinucleotides (route A). When the ratio of uridine amidite **3a** to adenosine **4a** was changed from 3:1 to 2:1 the yield of trinucleotide A^{U}_{U} (**9a**) decreased by 20% (Table I). We have found the ratio of 3:1:12 of reactants 5′-methyl phosphoramidite **3**:nucleoside

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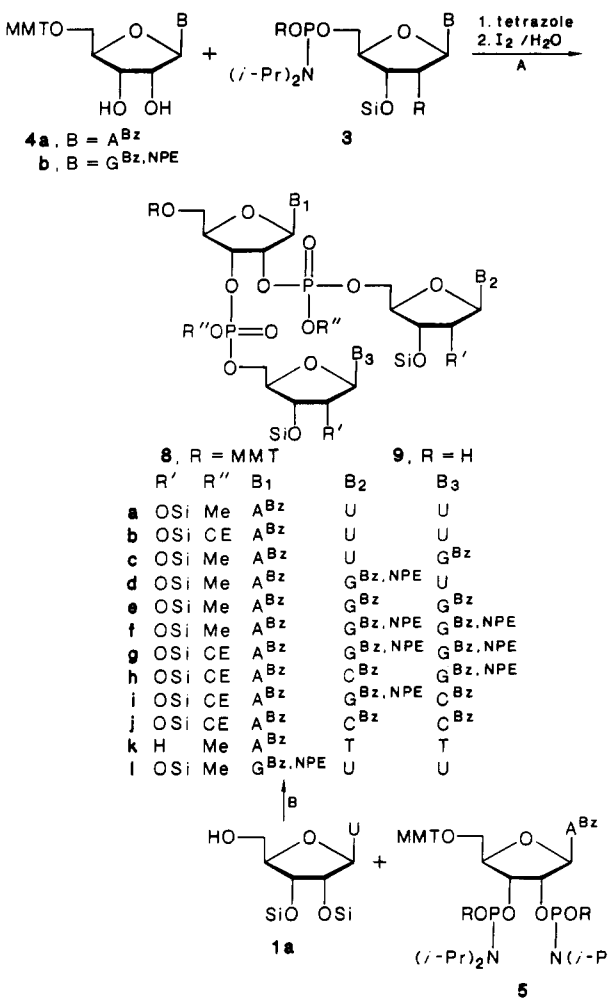
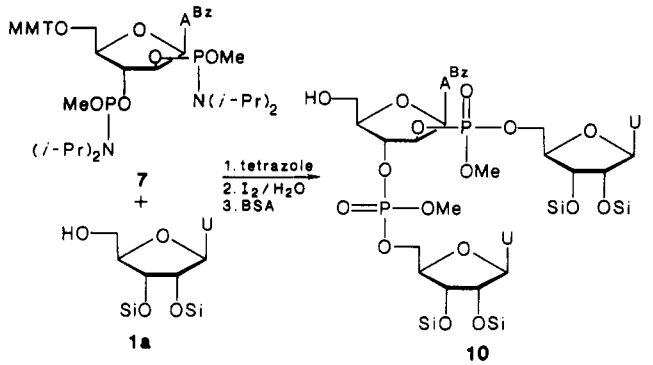
Scheme II[a]



[a] OSi = (*tert*-butyldimethylsilyl)oxy.

Scheme III[a]



[a] OSi = (*tert*-butyldimethylsilyl)oxy.

4a:tetrazole gives the best results for branched RNA synthesis using route A. The experimental conditions employed in these condensations are summarized in Table I.

Similarly, the arabinoadenosine-diuridine trimer **10** (aA^{U}_{U}) was prepared by the reaction of the arabinonucleoside bisamidite **7** with the nucleoside **1a** (route B, Scheme III). A better yield of this nucleotide was obtained when the condensation time was increased from 3 to 16 h (Table I). This result reflects, in part, the marked difference in steric environment of the 2′-position in arabinonucleosides.[8] In addition, we found that activation

Table I. Preparation of Branched RNA Fragments

entry	nucleoside (mmol)	amidite (mmol)	tetrazole (mmol)	THF (mL)	time (h)	BSA (mmol)	product	%[a]
1a	**4a** (0.25)	**3a** (0.55)	1.7	2.0	2.5	38	A^{U}_{U} **9a**	41
1b	**4a** (0.25)	**3a** (0.75)	3.0	1.5	0.8	38	A^{U}_{U} **9a**	65
2	**1a** (0.75)	**5a** (0.25)	2.0	1.5	2.0	38	A^{U}_{U} **9a**	68
3	**4a** (0.25)	**3d** (0.75)	3.0	1.5	2.0	38	A^{G}_{G} **9f**	76
4	**4a** (0.25)	**3c** (0.75)	3.0	1.5	1.0		A^{G}_{G} **8e**	0
5	**4a** (0.25)	**3a** (0.38)	3.0	1.5	2.5		A^{U}_{U} **8a**	16
		3d (0.38)					A^{G}_{G} **8c**	39
							A^{G}_{U} **8d**	
							A^{G}_{G} **8f**	24
6	**4b** (0.25)	**3a** (0.75)	3.0	1.5	2.0	38	G^{U}_{U} **9l**	61
7	**4a** (0.25)	**3g** (0.75)	2.8	1.5	2.0	38	A^{T}_{T} **9k**	42
8a	**1a** (0.83)	**7** (0.20)	1.0	1.2	3.5		aA^{U}_{U} **10**	27
8b	**1a** (2.00)	**7** (0.50)	4.2	3.0	160	76	aA^{U}_{U} **10**	61
9	**4a** (0.25)	**3f** (0.75)	3.0	1.5	1.0	38	A^{C}_{C} **9j**	21
10	**4a** (0.25)	**3b** (0.75)	3.0	1.5	0.8	38	A^{U}_{U} **9b**	13
11	**1a** (0.75)	**5b** (0.25)	2.0	1.5	2.0		A^{U}_{U} **9b**	20
12	**4a** (0.25)	**3e** (0.38)	3.0	1.5	2.0	38	**8g–j**	32
		3f (0.38)						
13	**9a** (0.14)	**15** (0.30)	1.2	0.9	2.0		$U_pA^{U}_{U}$ **16a**	53

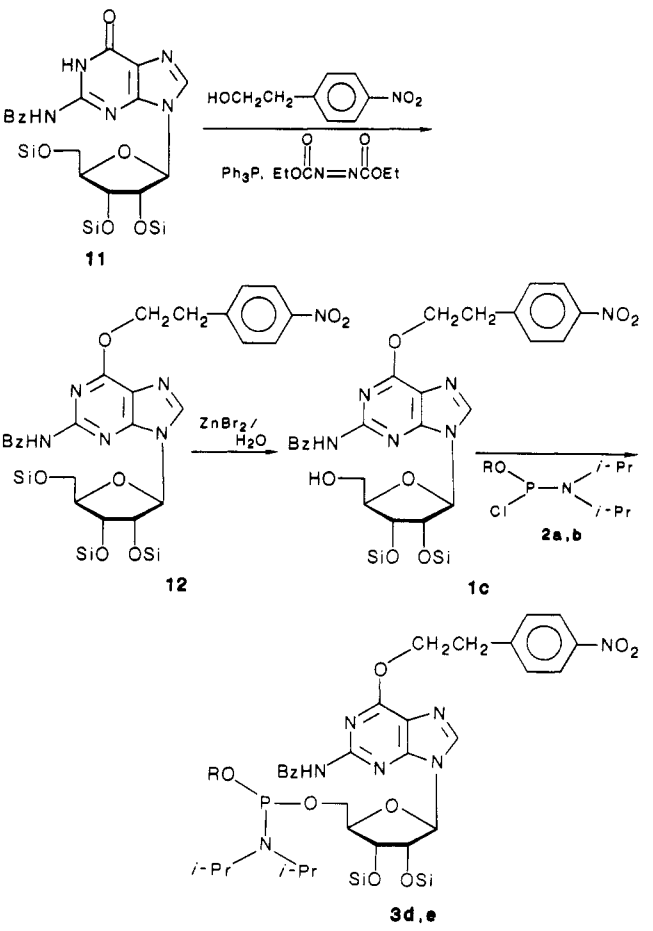
[a] Estimated yield by ^{31}P NMR.

of the arabinoadenosine diamidite **7** with tetrazole was slower than that of its ribo counterparts **5a** and **5b** (^{31}P NMR).[9]

The adenosine-diguanosine trinucleotide MMT-A^{G}_{G} (**8e**) could not be prepared by using guanosine units lacking O^6 protection. TLC of the crude reaction of adenosine **4a**, guanosine amidite **3c**, and tetrazole showed, after the oxidation step, mainly unreacted **4a**, fast-moving non-tritylated products, and base-line material. However, when the guanosine amidite **3d** bearing O^6-*p*-nitrophenylethyl (NPE) protection[10] was used instead, the corresponding trinucleotide **9f** was isolated in 76% yield after the detritylation step. The failure of the former reaction may be ascribed to the phosphitylation of the O^6-position of guanine under the phosphoramidite condensation conditions.[11] The ^{31}P NMR spectrum of a mixture of O^6-unprotected amidite **3c** and tetrazole (1:4) in acetonitrile showed complete conversion of **3c** to products with peaks at 128.6 ppm (tetrazoyl intermediate,[12] ca. 60%) and at 139.0–135.2 ppm (O^6 adducts,[11] ca. 40%), confirming the reaction of the tetrazolyl phosphoramidite moiety with the O^6-position of guanine. As expected, only the tetrazolylamidite product (127.8 ppm) was observed when the O^6-NPE protected amidite **3d** was treated under the same conditions conditions. The necessity of the protection of the O^6-position of guanine residues has also been reported by Imbach et al.[13] and Nielsen et al.[14] in analogous phosphoramidite condensations.

The O^6-*p*-nitrophenylethyl protected guanosine phosphoramidites **3d** and **3e** were prepared by the route shown in Scheme IV. The O^6-protected derivative **12** was prepared from 5′,3′,2′-tris-*O*-(*tert*-butyldimethylsilyl)-N^2-benzoylguanosine[15] (**11**) in 87% yield via a Mitsunobu reaction according to Pfleiderer et al.[10] Selective removal of the 5′-TBDMS group of **12** with an aqueous solution of zinc bromide[16] gave nucleoside **1c** in 58% yield. Guanosine

Scheme IV[a]



[a] OSi = (*tert*-butyldimethylsilyl)oxy.

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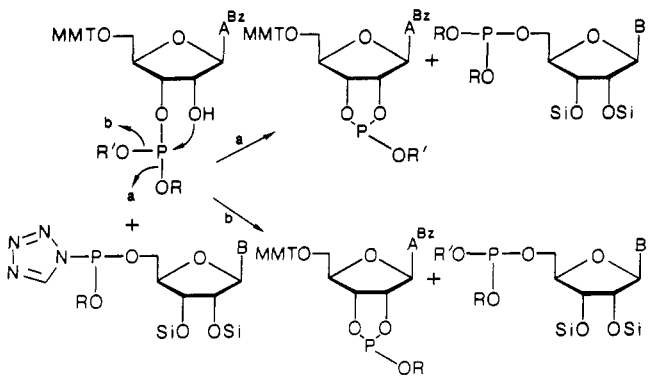
Scheme V[a]



[a] OSi = (*tert*-butyldimethylsilyl)oxy.

amidites **3d** and **3e** were prepared by the reaction of **1c** with either phosphoramidochloridite **2a** or **2b**, in 78% and 70% yields, respectively. Accordingly, the O^6-protected guanosine **4b**[10,11b] was used in the synthesis of the guanosine-diuridine trimer **9l**.

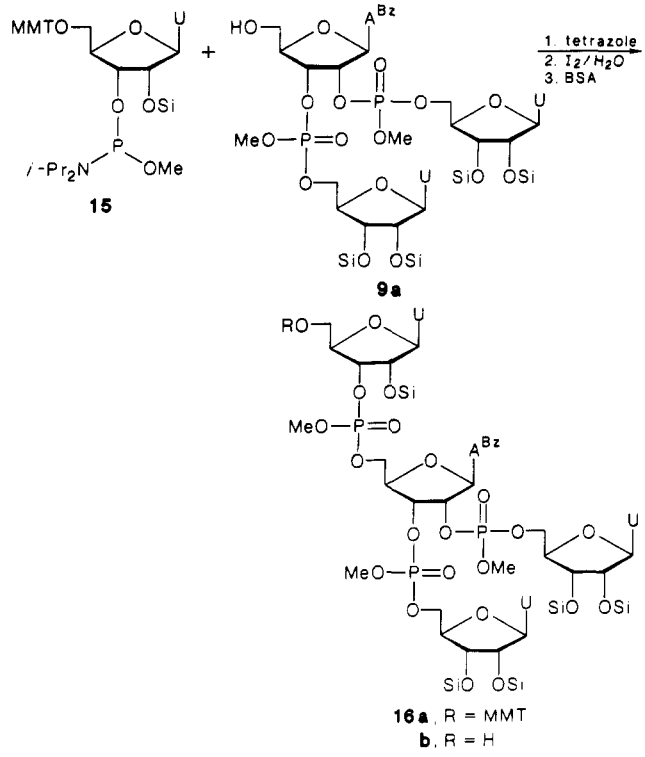
The isomers of MMT-A^U_G (**8c**) and MTT-A^G_U (**8d**) were prepared simultaneously, via route A, by condensing adenosine **4a** with the uridine and guanosine methyl phosphoramidites **3a** and **3d**. The nucleotide isomer mixture of MMT-A^U_G (**8c**) and MMT-A^G_U (**8d**) was first separated (39%, combined yield) from MMT-A^G_G (**8f**, 24%) and MMT-A^U_U (**8a**, 16%) by silica gel column chromatography and subsequently detritylated with BSA to give the isomer mixture of A^U_G (**9c**) and A^G_U (**9d**) in 72% yield. These products could not be separated at this stage. They were, however, fully separated by high pressure liquid chromatography (HPLC) after complete deprotection (vide infra).

The isomers MMT-A^C_G (**8h**) and MMT-A^G_C (**8i**) were obtained from the reaction of adenosine **4a** with the cytidine and guanosine cyanoethyl phosphoramidites **3e** and **3f**. The crude mixture of MMT-A^C_G (**8h**), MMT-A^G_C (**8i**), MMT-A^G_G (**8g**), and MMT-A^C_C (**8j**) was detritylated with BSA to give nucleotides **9g**, **9h**, **9i**, and **9j** in 32% overall yield. These trinucleotides were cleanly separated after complete deprotection (vide infra).

The significant difference obtained in the coupling yields between the methyl and cyanomethyl phosphoramidites (compare entries 1b and 10, Table I) appears to be related, at least in part, to the relative ease of activation of these derivatives with tetrazole. Activation studies of the uridine methyl (**3a**) and cyanoethyl phosphoramidite (**3b**) with tetrazole (4 equiv) in acetonitrile indicated that **3a** was 98% converted to the tetrazolyl intermediate[12] (130.4 ppm) while only 80% conversion of **3b** (128.5 ppm) was observed under these conditions (^{31}P NMR).

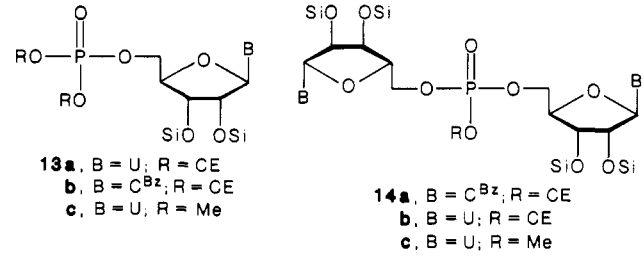
In addition, significant amounts of triesters **13** and **14** were detected in the condensations of adenosine **4a** with the nucleoside 5′-(cyanoethylphosphoramidites). For instance, triesters pU (**13a**) and the 5′–5′ symmetrically linked dimer UpU (**14a**) were obtained (30–40% combined yield) in the condensation of adenosine **4a** and the uridine amidite **3b** (entry 10, Table I). Similarly, the cytidine byproducts pC (**13b**) and CpC (**14b**) were obtained in the condensation of adenosine **4a** and cytidine amidite **3f** (entry 9, Table I). These products are most likely formed via 2′- or 3′-hydroxy-assisted cleavage of the vicinal phosphite linkage of the 2′–5′- or 3′–5′-linked dimer intermediates (Scheme V). The released terminal nucleoside (ROH) and/or 2-cyanoethanol (R′OH) can then react with the activated nucleoside phosphoramidite present in excess

Scheme VI[a]



[a] OSi = (*tert*-butyldimethylsilyl)oxy.

in the condensation reaction to yield, after the oxidation step, compounds **13** and **14**.



These side products were also obtained by using the methyl phosphoramidites [e.g. pU (**13c**) and UpU (**14c**)]. They formed, however, in only ca. 5% combined yield (^{31}P NMR) and were easily separated during the purification step. As expected, side products did not form (^{31}P NMR, TLC) in the preparation of the trinucleotides A^U_U (**9a** and **9b**) from the bis(methyl and cyanoethyl amidites) **5a** and **5b** (route B, Scheme II).

The branched tetranucleotide MMT-UpA^U_U (**16a**) was prepared in 53% yield by the reaction of uridine 3′-phosphoramidite **15**, A^U_U (**9a**), and tetrazole (Table I) and was subsequently detritylated with BSA to yield UpA^U_U (**16b**) in 92% yield (Scheme VI).

Deprotection and Purification of Branched RNA Fragments. The nucleotides were completely deprotected by consecutive treatment with (i) *tert*-butylamine to remove the methyl phosphate protecting groups where present, (ii) aqueous ammonia/ethanol to remove the benzoyl protecting groups, and (iii) 1 M tetra-*n*-butylammonium fluoride/THF to simultaneously remove silyl and (*p*-nitrophenyl)ethyl groups. Nucleotides containing guanosine residues had to be treated with ammonium hydroxide for about 24 h to insure complete removal of the N^2-benzoyl groups. When the debenzoylation time of A^G_G was decreased to 6 h, considerable amounts of di- and monobenzoylated products were isolated (^1H NMR). After complete deprotection the crude material obtained was

Table II. Physical Properties of Free Branched and Linear RNA Fragments

nucleotide	UV[a] max	UV[a] min	^{31}P NMR[b] P-3′	^{31}P NMR[b] P-2′	R_f A	R_f F	R_f G	EM[d] R_m	HPLC t_R,[e]
A^{U}_{U}	261	234	−0.74	−1.68	0.07	0.37	0.81	0.78	12.7
A^{G}_{U}	256	228	−0.53	−1.27	0.05	0.31	0.72	0.71	12.4
A^{U}_{G}	257	234	−0.67	−1.53	0.05	0.32	0.72	0.71	13.2
A^{G}_{G}	254	227	−0.69	−1.37	0.04	0.29	0.74	0.61	12.8
A^{C}_{C}	264	238	−0.66	−1.63	0.11	0.43	0.76	0.77	12.1
A^{G}_{C}	257	228	−0.66	−1.38	0.06	0.35	0.73	0.71	11.8
A^{C}_{G}	258	228	−0.69	−1.73	0.06	0.37	0.73	0.71	13.3
G^{U}_{U}	257	230	−0.63	−1.70	0.06	0.34	0.78	0.71	10.4
A^{T}_{T}	264	234	−0.94	−1.50	0.14	0.49	0.81	0.48	15.1
aA^{U}_{U}	261	232	−1.21	−1.56	0.08	0.40	0.78	0.85	12.8
UpA^{U}_{U}	260	232	−0.79 −0.85	−1.22	0.05	0.34	0.79	0.72	11.0
UpApU	260	232	−0.66 −0.66		0.09	0.41	0.79	0.67	13.3
$U^{p}A^{p}G$	258	230		−0.75 −0.79	0.07	0.34	0.76	0.66	10.2
$UpA^{p}G$	258	229	−0.72	−0.79	0.07	0.37	0.77	0.66	10.2

[a] H_2O. [b] Sample conditions described in the Experimental Section. [c] Cellulose TLC, solvents, A, F, G. [d] Electrophoretic mobility (24% polyacrylamide/8 M urea gel) relative to xylene cyanol (XC) EM R_m = 0.00 and bromophenol blue (BPB) EM R_m = 1.00. [e] HPLC condition A.

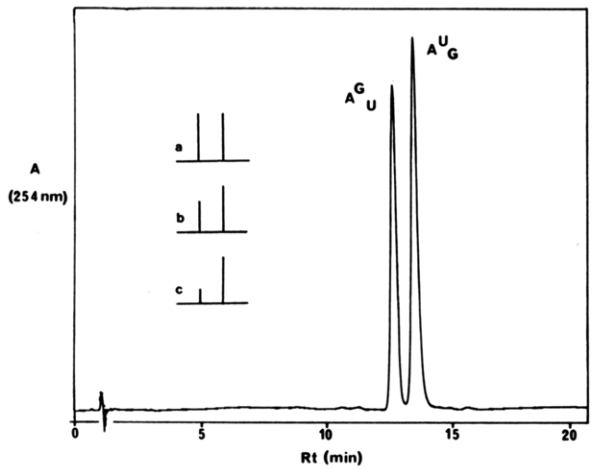


Figure 1. HPLC analysis of the crude mixture of A^{U}_{G} and A^{G}_{U} after complete deprotection and desalting on a Sephadex G-25 column. Insert: HPLC analysis of fractions a, b, and c of a mixture of A^{U}_{G} and A^{G}_{U} eluting, in this order, from a Sephadex G-25 column (Experimental Section). HPLC condition A (Experimental Section).

passed through a column of Dowex Na^+ ion exchange resin and subsequently desalted by size exclusion column chromatography (Sephadex G-25) and cellulose TLC. Milligram quantities (5–35 mg) of the fully deprotected nucleotides were obtained. The isomeric mixture A^{C}_{G} and A^{G}_{C} was separated from A^{G}_{G} and A^{C}_{C} by chromatography on cellulose TLC. Each of the isomer mixtures A^{U}_{G}, A^{G}_{U} and A^{C}_{G}, A^{G}_{C} was separated into their respective components by HPLC using a reverse-phase column (Figure 1).

The purity of all fully deprotected nucleotides was checked by analytical polyacrylamide gel electrophoresis, cellulose TLC, HPLC, and NMR (^{1}H, ^{13}C, ^{31}P) spectroscopy. All these methods indicated that the nucleotides isolated were homogeneous. The ^{31}P NMR, HPLC, UV, TLC, and gel electrophoresis data of the branched and some linear nucleotides are collected in Table III. It is worth noting the following.

(i) In the series A^{U}_{U}, A^{U}_{G}, A^{G}_{U}, and A^{G}_{G} the HPLC t_R values follow the order $A^{G}_{U} < A^{U}_{U} < A^{G}_{G} < A^{U}_{G}$. Similarly, in the series A^{C}_{C}, A^{C}_{G}, A^{G}_{C}, and A^{G}_{G} the HPLC t_R values follow the order $A^{G}_{C} < A^{C}_{C} < A^{G}_{G} < A^{C}_{G}$. Therefore, under our HPLC conditions, adenosine-branched nucleotides containing 3′–5′-linked guanosine residues elute more slowly than those containing 3′–5′-linked pyrimidines.

(ii) The branched trinucleotides exhibit base-(e.g. A^{T}_{T}, A^{U}_{U}) and structure-(e.g. aA^{U}_{U}, A^{U}_{U}) specific mobility under

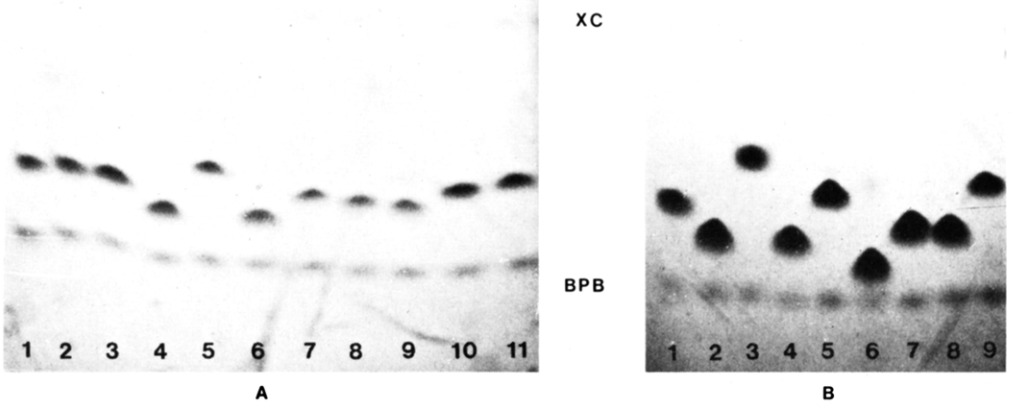


Figure 2. Electrophoresis of branched RNA fragments on a 24% polyacrylamide/8M urea gel. A: lane 1, A^{G}_{C}; lane 2, A^{C}_{G} + A^{G}_{C}; lane 3, A^{C}_{G}; lane 4, A^{C}_{C}; lane 5, A^{G}_{G}; lane 6, A^{U}_{U}; lane 7, A^{G}_{U}, lane 8, A^{U}_{G} + A^{G}_{U}; lane 9, A^{U}_{G}; lane 10, $U^{p}A^{p}G$; lane 11, $UpA^{p}G$. B: lane 1, UpApU; lane 2, A^{U}_{U}; lane 3, A^{T}_{T}; lane 4, A^{C}_{G}; lane 5, A^{G}_{G}; lane 6, aA^{U}_{U}; lane 7, G^{U}_{U}; lane 8, UpA^{U}_{U}; lane 9, ApGpApUpC. All samples were loaded with xylene cyanol (XC) and bromophenol blue (BPB).

Table III. ^{1}H NMR (400 MHz) Spectral Data of Free Branched RNA Fragments[a]

	A			3'p5'B			2'p5'C		
	H1'	H2	H8	H1'	H5(2)	H6(8)	H1'	H5(2)	H6(8)
A^U_U	6.21	8.00	8.31	5.96	5.88	7.88	5.60	5.65	7.36
	7.3			5.5			2.8		
A^U_G	6.07	8.03	8.30	5.90		8.02	5.60	5.66	7.37
	7.5			5.4			2.8		
A^G_U	6.19	7.86	8.23	5.92	5.84	7.87	5.63		7.72
	5.7			4.4			4.9		
A^G_G	6.10	7.79	8.21	5.88		8.01	5.60		7.65
	6.4			5.5			4.8		
A^C_G	6.15	7.95	8.30	5.90		8.02	5.55	5.74	7.34
	7.1			6.2			1	5.74	7.34
A^G_C	6.16	7.79	8.22	5.91	5.96	7.84	5.61		7.61
	6.1			4.4			4.4		
A^C_C	6.16	7.94	8.28	5.94	6.01	7.88	5.54	5.74	5.34
	7.3			4.5			0.0		
A^T_T	6.19	8.00	8.33	6.30	1.85	7.69	6.00	1.78	7.23
	7.4			6.9			6.5		
				6.9			5.3		
G^U_U	6.06		7.99	5.97	5.92	7.93	5.71	5.79	7.69
	7.0			4.4			2.4		
aA^U_U	6.50	8.16	8.30	5.92	5.88	7.89	4.69	5.71	7.44
	3.9			4.2			4.2		
UpA^U_U -pA^U_U	6.25	8.16	8.44	5.97	5.92	7.92	5.63	5.57	7.41
	5.9			4.6			3.0		
5'U-				5.72	5.81	7.70			
				5.4					

[a] Sample conditions described in the Experimental Section; $^3J_{H1'-H2'}$ (and $^3J_{H1-H2''}$, in the case of A^T_T) values are shown below the H1' chemical shifts.

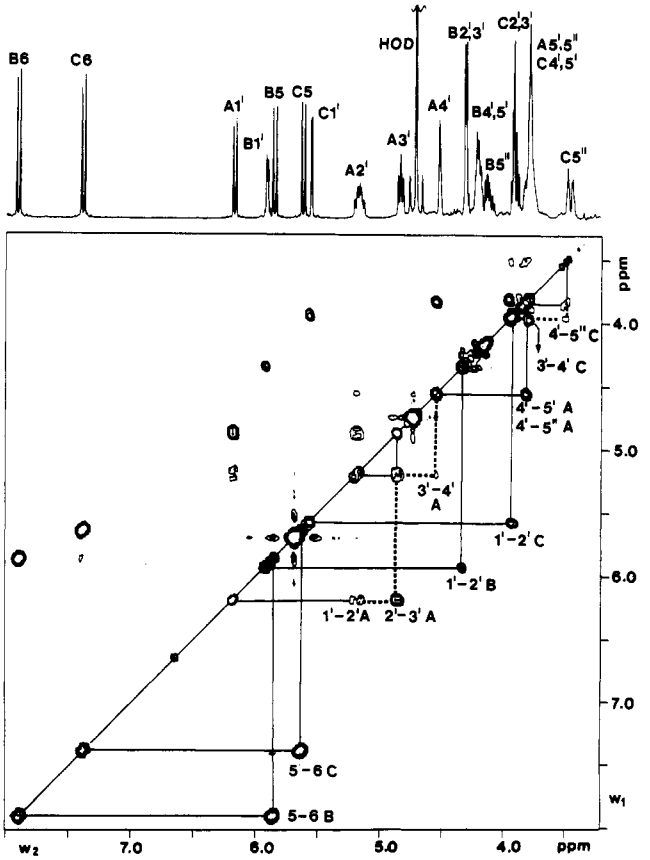


Figure 3. 2D ^{1}H-RELAYED-COSY (300 MHz) spectrum of A^U_U, with the ^{1}H NMR spectrum shown along the top axis. Solid and broken lines indicate the COSY and RELAYED-COSY off-diagonal resonances, respectively.

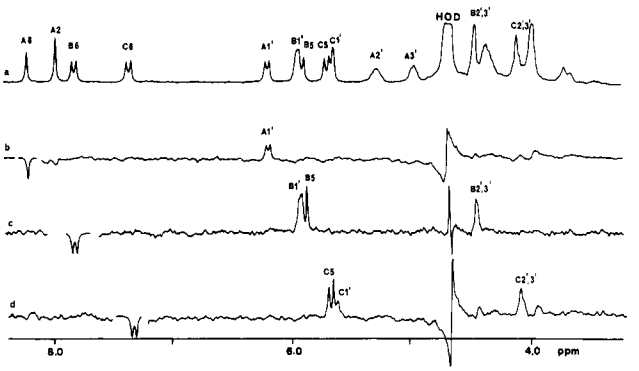


Figure 4. Pre-steady-state NOE measurements on A^U_U at 37 °C: (a) ^{1}H NMR (200 MHz, with line broadening) spectrum between 8.5 and 3.0 ppm; (b–d) difference spectra (on-resonance minus off-resonance preirradiation) after presaturation for 5.0 s of (b) the proton resonance at 8.31 ppm (peak A_{H8}), (c) the proton resonance at 7.88 ppm (doublet B_{H6}), (d) the proton resonance at 7.36 ppm (doublet C_{H6}). The assignments of the proton resonances are given in Table V. The experimental conditions are described in the Experimental Section.

our electrophoretic conditions as shown in Figure 2. The trinucleotides A^U_G, A^G_U, A^C_G, and A^G_C had, however, the same mobility. The linear trimers UpApU, U^pA^pG, and UpA^pG had slower electrophoretic mobility than the corresponding A^U_U, A^U_G, and A^G_U branched trimers. A^T_T exhibited lower electrophoretic mobility than the pentanucleotide ApGpApUpC.

(iii) A^U_G and A^G_U have slightly different elution behavior on a Sephadex G-25 column; on this column A^U_G was eluted more slowly than A^G_U (see insert, Figure 1).

Structure Determination of Branched RNA Fragments. Nuclear Magnetic Resonance. The general procedure can be illustrated by the structure determination of A^U_U. The first step consisted of the identification of the three ribose spin systems A, B, and C with 2D-*J* correlated spectroscopy (^{1}H-RELAYED-COSY,[17] Figure 3). Since the chemical shift dispersion of some proton reso-

(17) (a) Bolton, P. H.; Bodenhausen, G. *Chem. Phys. Lett.* **1982**, *89*, 139–144. (b) Eich, G.; Bodenhausen, G.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 3731–3732.

Table IV. ^{13}C NMR (75.4 MHz) Spectral Data[a] of Free Branched RNA Fragments

nucleotide		1′	2′	3′	4′	5′	5	6	2	8
A^{U}_{U}	A	90.2d	78.3q	78.0[q]	88.8[d]	64.4			155.1	143.9
	-2′pU	91.7	77.0	70.8	84.1[d]	66.0[d]	104.8	143.2		
	-3pU	91.4	76.4	72.6	86.0[d]	68.0[d]	105.4	144.3		
A^{G}_{G}	A	90.0[d]	78.2[q]	77.4[q]	87.8[d]	64.0			154.8	143.5
	-2′pG	89.9	76.6	72.2	85.1[d]	66.8[d]				139.3
	-3′pG	89.7	76.3	73.1	86.3[d]	68.2[d]				139.7
A^{C}_{C}	A	90.1[d]	78.1[q]	77.8[q]	88.6[d]	64.3			155.0	143.8
	-2′pC	92.4	77.3	70.3	83.5[d]	65.6[d]	98.4	143.0		
	-3′pC	92.1	76.8	72.2	85.5[d]	67.7[d]	99.2	144.2		
A^{T}_{T}	A	90.1[d]	78.3[q]	77.9[q]	88.5[d]	64.3			155.0	*b*
	-2′pT	87.0	14.3	71.7	87.1[d]	66.5[d]	41.1	139.3		
	-3′pT	87.9	14.5	73.6	88.2[d]	68.4[d]	42.0	140.1		
G^{U}_{U}	G	89.7[d]	77.6[q]	77.5[q]	88.2[d]	64.0				141.1
	-2′pU	91.7	76.9	71.0	84.3[d]	65.9[d]	104.8	143.1		
	-3′pU	91.2	76.3	72.4	86.0[d]	67.7[d]	105.3	144.1		
aA^{U}_{U}	aA	86.4[d]	80.0[q]	81.1[q]	86.9[d]	64.0			155.6	144.2
	-2′pU	91.4	76.6	71.9	85.2[d]	67.1[d]	105.0	143.6		
	-3′pU	91.6	76.4	72.3	85.7[d]	67.8[d]	105.2	144.3		
UpA^{U}_{U}	5′pU	91.5	75.5[d]	76.2[d]	86.1[d]	63.2	105.0	144.1		
	A	88.1[d]	78.7[q]	76.7[q]	85.5[q]	67.7[d]			155.8	142.6
	-2′pU	91.6	76.8	71.0	84.6[d]	66.2[d]	104.6	143.1		
	-3′pU	91.1	76.4	72.4	86.0[d]	67.7[d]	105.3	144.1		

[a] Sample conditions described in the Experimental Section. [b] Signal not observed due to exchange of H8 signal with deuterium. [d] Doublet. *q* Quartet; C2′ and C3′ of central branched residue consisted of two sets of doublets due to geminal and vicinal coupling to $^{31}P2'$ and $^{31}P3'$.

nances was limited (300 MHz) some proton–proton connectivities could not be established from the ^1H-RELAYED-COSY spectra alone and it was therefore necessary to make use of ^1H–^{13}C heteronuclear 2D NMR[18] (spectrum not shown). Secondly, the bases of each sugar residue were assigned via one-dimensional ^1H–^1H nuclear Overhauser enhancement (NOE) measurements (Figure 4). Therefore, the ^1H spin system A belongs to adenosine, and B and C to the uridine residues. In the third step, the scalar coupling connectivities between the ^1H spin systems and the ^{31}P NMR resonances were identified using ^1H–^{31}P heterodecoupling[19] (Figure 5). The ^{31}P NMR spectrum of A^{U}_{U} showed two well-resolved proton-decoupled ^{31}P signals (I and II, see insert Figure 5). Figure 5a shows the proton NMR spectrum (400 MHz, sugar proton region) of A^{U}_{U}. Figure 5b shows that the spectral pattern of A(H3′,H2′) and B(H4′,H5′,H5″) has been perturbed by irradiation at peak I of the ^{31}P resonance. The perturbation of the AH2′ signal is due to a four-bond phosphorus–proton coupling with $^4J_{H2'-P3'}$ = 2.2 Hz (vide infra). Therefore, the ^{31}P resonance I and ^1H spin system B are assigned to the 3′-p-5′-uridine residue. When peak II was irradiated AH2′ and C(H4′,H5′,H5″) was perturbed (Figure 5c). Therefore, the ^{31}P resonance II and ^1H spin system C are assigned to the 2′-p-5′-uridine residue. Clearly, the combination of these NMR techniques allowed for the unambiguous characterization of this nucleotide, i.e., presence of vicinal 2′–5′ and 3′–5′ phosphodiester linkages and assignments of ^1H,^{13}C, and ^{31}P NMR resonances (Tables II–IV).

All branched trinucleotides were characterized in this fashion and the following general characteristics were observed.

(i) Without exception, the nucleotide residue attached to the central nucleoside via the 2′–5′ linkage (2′-residue) exhibits ^1H, ^{31}P, and ^{13}C resonances at higher field than that attached through the 3′–5′ linkage (3′-residue).

(ii) The base and anomeric proton chemcical shift (δ) values of the 2′ residues are primarily influenced by the nature of the central nucleoside (i.e. adenosine or guanosine), and vice versa. For instance, the δ values of both the adenine and 2′-guanine in A^{G}_{C}, A^{G}_{U}, and A^{G}_{G} are very similar. In contrast, the δ values of the 2′-uracil protons in A^{U}_{U} and G^{U}_{U} differ substantially.

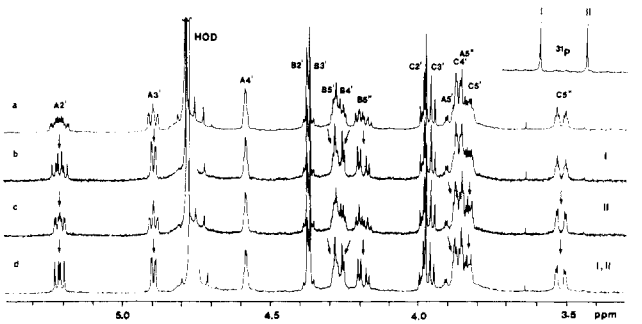


Figure 5. Insert: proton-decoupled ^{31}P resonances of A^{U}_{U} (I = −0.74 ppm, II = −1.68 ppm) obtained at 121.5 MHz: (a) ^1H-NMR (400 MHz) spectrum of A^{U}_{U} between 5.4 and 3.3 ppm ($H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$, $H_{5''}$, proton region); (b) same as (a) except an irradiation power was applied at peak I; (c) same as (a) except an irradiation power was applied at peak II; (d) same as (a) except that an irradiation power was applied at both peaks I and II (broad band decoupling). The arrows indicate the region of changes in the spectral pattern due to decoupling. I, ^{31}P resonance of 3′p5′-uridine residue; II, ^{31}P resonance of 2′p5′-uridine residue. The sugar spin systems A, B, and C correspond to the adenosine, 3′p5′-uridine, and 2′p5′-uridine residues, respectively.

(iii) The base and anomeric proton δ values of a given 3′ residue are nearly independent of the nature of both the central nucleoside and 2′ residue. For example, the 3′-guanosines in A^{C}_{G}, A^{U}_{G}, and A^{G}_{G} have nearly the same δ values. Similarly, the δ values of the 3′-uridines in G^{U}_{U}, A^{U}_{U}, aA^{U}_{U}, and A^{G}_{U} are virtually the same.

(iv) The $^3J_{H1'-H2'}$ values of adenosine residues containing 2′–5′-linked guanosines are 1–1.5 Hz smaller than those containing 2′–5′-linked pyrimidines (e.g. $^3J_{H1'-H2'}$ of A^{G}_{U}, A^{G}_{C}, A^{G}_{G}, < A^{U}_{G}, A^{C}_{G}, A^{U}_{U}, A^{C}_{C}, A^{T}_{T}).

(v) The residue attached to adenosine via the 2′–5′ linkage has smaller $^3J_{H1'-H2'}$ values than that linked via the 3′–5′ bridge. In A^{G}_{U}, A^{G}_{C}, and A^{G}_{G}, however, these residues have nearly the same $^3J_{H1'-H2'}$ values.

(vi) A four-bond $^4J_{AH2'-P3'}$ (or $^4J_{GH2'-P3'}$ in th e case of G^{U}_{U}) coupling was observed. This coupling indicates that

(18) (a) Maudsley, A. A.; Muller, L.; Ernst, R. R. *J. Magn. Reson.* **1977**, *28*, 463. (b) Bodenhausen, G.; Freeman, R. J. *Ibid.* **1977**, *28*, 471.

(19) Cheng, D. M.; Kan, L. S.; Iuorno, V. L.; Ts'o, P. O. P. *Biopolymers* **1984**, *23*, 575–592.

a substantial fraction of the total conformer populations along the flexible P–O3′–C3′–C2′–H2′ bonding system exists in the planar "W" form.[20,21] As expected, this long-range coupling was not observed for the arabinoadenosine-diuridine trimer since no favourable coupling path is possible between AH2′ and P3′.

These observations strongly suggest that an intramolecular base–base stacking interaction occurs between the adenine base (guanine in the case of G^{U}_{U}) of the central nucleotide and the base of the 2′-p-5′ residue.[21,22] For example, the upfield shifts of the base and anomeric protons of the 2′-p-5′ residue relative to those of the 3′-p-5′ nucleotide (observation i) may be due to the influence of the ring current magnetic anisotropy of the adenine base or of guanine in the case of G^{U}_{U}.

Enzyme Degradations. All branched trinucleotides were degraded by snake venom phosphodiesterase (SVPD) while no degradation was observed with spleen phosphodiesterase (HPLC analysis, Experimental Section). It should be noted that spleen phosphodiesterase specifically cleave 3′–5′ (and not 2′–5′) linkages of linear nucleotides producing nucleoside 3′-phosphates. Therefore, the above results indicate that the 2′–5′ phosphodiester linkage of the branched RNA fragments renders the 3′–5′ phosphodiester linkage resistant to spleen phosphodiesterase. The tetramer UpA^{U}_{U} was completely degraded by SVPD. Analysis of the spleen phosphodiesterase digest of UpA^{U}_{U} by reverse-phase HPLC indicated that this tetramer was split into a mixture of uridine 3′-monophosphate and A^{U}_{U} (1:1 ratio), establishing unambiguously its structure. The ^{1}H, ^{13}C, and ^{31}P NMR data of this tetranucleotide also support its assigned structure (Tables II–IV).

Conclusions

A procedure that permits the rapid synthesis, is milligram quantities, of branched RNA fragments by the phosphite triester methodology was developed. This method can be used to simultaneously prepare, via a one-flask procedure, a mixture of A^{X}_{X}, A^{X}_{Y}, A^{Y}_{X}, and A^{Y}_{Y} (X = U or C and Y = G) which can be separated by TLC and HPLC as described. The ease of their preparation and purification allowed us to obtain and compare their physical properties (UV, NMR, HPLC, and electrophoretic mobility). Although the β-cyanoethyl phosphate protecting group offered some advantages over methyl protection (e.g. ease of removal during the deprotection of nucleotides), the yields of the nucleotides prepared from the methylated amidites were significantly higher. This difference was ascribed to differences in the activation of the methyl and cyanoethyl amidites with tetrazole to afford the corresponding tetrazolyl amidite intermediates. Dahl et al.[23] have recently demonstrated that the β-cyanoethyl group causes a fourfold rate reduction, compared to methyl, in the solution synthesis of dinucleotide units using methyl and cyanoethyl phosphoramidites as building blocks. This is in complete agreement with our results.

Since the appearance of our early report,[4] three manuscripts describing the synthesis of A^{G}_{C}[13,24,25] and the tetranucleotide ApA^{G}_{C}[25] have appeared. All three methods allow for the stepwise introduction of the vicinal phosphodiester linkages, via 3′–5′-linked phosphodiester intermediates, to afford A^{G}_{C} as the sole product. The preparation of these nucleotides, however, required four[13,25] or nine[24] steps.

Branched triribonucleotides of defined base composition (e.g. A^{U}_{U}, G^{U}_{U}) and structure (e.g. A^{U}_{U}, aA^{U}_{U}) may be used to examine the requirements of branch recognition during the early steps of eukaryotic mRNA processing. In addition, quantitative structural and conformational data of these molecules[21,22] are vital as starting points for conformation determination of higher chain-length branched RNA fragments and lariat RNA.

Experimental Section

General Methods. Thin layer chromatographic (TLC) data (R_f values) was obtained by using Merck Kieselgel 60 F 254 analytical silica gel (#5735) sheets (0.2 mm × 20 cm × 20 cm). Thick layer chromatography was carried out on glass plates (20 cm × 20 cm, 50–150 mg of crude material/plate) coated with a 1–2 mm thick layer of Kieselgel 60 GF 254 silica gel (#7730). Preparative chromatography was performed on Merck Kieselgel 60 (230–400 mesh, #9385) using 20 g of silica gel per g of crude compound. Thin layer chromatography on cellulose (desalting) was performed on Merck cellulose F (#5565) plates (0.1 mm × 20 cm × 20 cm) in all cases or Eastman cellulose (Eastman Kodak Co., #13254) plates (0.16 mm × 20 cm × 20 cm) for the separation of A^{C}_{G} and A^{G}_{C} from A^{C}_{C} and A^{G}_{G}. The solvents employed were solvent A (2-propanol:29% NH_4OH:water 7:1:2), solvent F (1-propanol:29% NH_4OH:water 55:10:35), and solvent G (1-propanol:29% NH_4OH:water 35:10:55). Nucleosides and derivatives were detected on thin- and thick-layer sheets using a UV light source (Mineralite, output ca. 254 nm.). Compounds containing trityl groups were detected on chromatography by spraying with 10–20% perchloric acid solution and drying in a stream of warm air.

Cation exchange chromatography was performed on a column (0.8 cm × 40 cm) packed with autoclaved Dowex 50W-X8 (Na^+ form, 20–50 mesh) resin (J.T. Baker Chemical Co., Phillipsburg, NJ) in sterilized aqueous soltions (see Reagents and Chemicals). Size exclusion chromatography was performed on a column (1.7 cm × 100 cm) packed with preswollen autoclaved Sephadex G-25F resin (Pharmacia Fine Chemicals Canada Ltd., Dorval, Quebec). Sephadex columns, packed with sterile water, were monitored with a Pharmacia UV-1 UV monitor at 254 nm. Aqueous solutions of the deprotected nucleotides were lyophilized in a Speed-Vac concentrator (Savant Instruments). Gel electrophoresis was performed by using 24% acrylamide/8 M urea and were run at 400–500 V. The gels were visualized and photographed by UV shadowing over a fluorescent TLC plate.

HPLC analysis and purifications were carried out on a Spectra-Physics SP8000 HPLC equipped with a 254-nm UV detector and a chromatography data system under the following conditions. Condition A: reverse phase 4.6 × 250 mm Whatman Partisil ODS-2 column (10 μm particles, Chromatographic Specialities); mobile phase, solvent A, 0.02 M KH_2PO_4 (pH 5.5, adjusted with KOH), solvent B, methanol, gradient 0–50% solvent B in 30 min; flow rate 2 mL/min; temperature 30 °C. Condition B: reversed phase 4.6 × 250 mm Whatman Partisil C-8 column (5 μm particles, Chromatographic Specialities); mobile phase, solvent A, 0.05 M KH_2PO_4, 2 mM tetrabutylammonium phosphate (PIC-A, Water Scientific, Milford, MA), pH 4.9, solvent B, metnanol, solvent A:solvent B 97:3; flow rate 1 mL/min; temperature 22 °C.

Glassware used in the deprotection of nucleotides (pipettes, etc.) was silanized prior to sterilization using Sigmacote (Sigma). All glass- and plasticware used in the deprotection of nucleotides was sterilized by autoclaving (120 °C, 1 h) except for the Sephadex and cation exchange columns which were treated with a 1% diethyl pyrocarbonate (DEP) solution for 2 h prior to use. The water used in the deprotection of all nucleotides (e.g. Sephadex

(20) (a) Hall, L. D.; Malcom, R. B. *Can. J. Chem.* **1972**, *50*, 2092–2101. (b) Hall, L. D.; Malcom, R. B. *Ibid.* **1972**, *50*, 2102–2110. (c) Donaldson, B.; Hall, L. D. *Ibid.* **1972**, *50*, 2111. (d) Sarma, R. H.; Mynott, R. J.; Wood, D. J.; Hruska, F. E. *J. Am. Chem. Soc.* **1973**, *95*, 6457–6459.

(21) (a) Damha, M. J.; Ogilvie, K. K. 69th Canadian Chemical Conference, June 1986, Saskatoon, Saskatchewan. (b) Damha, M. J.; Ogilvie, K. K. *Biochemistry*, in press.

(22) Remaud, G.; Vial, J.-M.; Nylas, A.; Balgobin, N.; Chattopadhyaya, J. *Tetrahedron* **1987**, *43*, 947.

(23) Dahl, B. H.; Nielsen, J.; Dahl, O. *Nucl. Acids Res.* **1987**, *15*, 1729–1743.

(24) Sekine, M.; Hata, T. *J. Am. Chem. Soc.* **1985**, *107*, 5813–5815.

(25) Kierzek, R.; Kopp, D. W.; Edmonds, M.; Caruthers, M. H. *Nucl. Acids Res.* **1986**, *14*, 4751–4764.

and cation exchange columns) and HPLC separation (including buffers) of A^{U}_{G}, A^{G}_{U} and A^{C}_{G}, A^{G}_{C} was doubl-distilled, sterilized, filtered, and contained sodium azide (0.001%) to inhibit microbial growth. The water was sterilized by treatment with diethyl pyrocarbonate (DEP) as a 1% solution followed by autoclaving at 120 °C for 1 h.

Reactions for the preparation of the nucleoside amidites and branched nucleotides were carried out in Ar-purged, oven-dried hypovials (Pierce chemical Co.) closed with rubber septa. Reagents in solution were injected via syringe. A syringe filled with magnesium perchlorate was used to equalize the pressure in reaction vessels.

Reagents and Chemicals. Tetrahydrofuran (THF) was continuously refluxed over sodium and benzophenone under nitrogen until a purple color persisted. The THF was distilled and removed from the collection bulb by syringe via a septum port. $CHCl_3$ was distilled from P_2O_5 and stored over activated (450 °C) 4A molecular sieves. The ethanol used was 95%. Acetonitrile used for carrying out reactions in NMR tubes was HPLC grade and was first dried over activated (450 °C) 4A molecular sieves and then continuously refluxed over, and distilled from, CaH_2. Chromatographic solvents were fractionally distilled prior to use with the exception of triethylamine. Methanol, used for HPLC, was HPLC grade and was filtered through 0.45-μm filters (Millipore Corp., Bedford, MA) before use. Deuteriated solvents were obtained from Merck, Sharp and Dome (Montreal, Quebec) (99.8%, 99.96% D_2O, CD_3CN) or Aldrich Chemical Co. ($CDCl_3$).

N,N-Dimethylformamide (DMF), diisopropylethylamine, and 2,4,6-trimethylpyridine (2,4,6-collidine) were dried by mild heating and stirring over calcium hydride (50–60 °C, 16 h) followed by distillation under vacuum. The distilled material was stored over 4A molecular sieves. Pyridine was refluxed over phthalic anhydride (16 h) before fractional distillation and stored over 4A molecular sieves. *tert*-Butylamine used for methyl phosphate deprotection of nucleotides was reagent grade and was used as such. 4-(Dimethylamino)pyridine (DMAP) was recrystallized from THF and dried under vacuum over P_2O_5 prior to use. Tetrazole (Aldrich) was stored under vacuum over P_2O_5 or sublimed prior to use. Tetra-*n*-butylammonium fluoride was purchased as a 1 M solution in THF (Aldrich). $MeOPCl_2$,[26] $CEOPCl_2$,[26] and methyl phosphoramidochloridite (**2a**)[6b,27] were prepared according to the literature. Phosphoramidochloridite **2b** was generously donated by American BioNuclear (Emeryville, CA). Nucleosides were obtained from Boehringer Mannheim Canada (Montreal, Quebec). The benzoyl and monomethoxytrityl protected derivatives were prepared by literature procedures.[15,28]

Spectra. Ultraviolet spectra were recorded on a Cary 17 or Hewlett-Packard 8451A spectrophotometer using 95% ethanol as solvent for protected nucleoside and nucleotide derivatives. Water was used for the spectra of completely deprotected compounds. Nuclear magnetic resonance (^1H, ^{13}C, ^{31}P) spectra were recorded on a Varian XL-200 and XL-300 spectrometers. All spectra were obtained at 20–22 °C. ^1H and ^{13}C NMR chemical shifts (organic deuteriated solvents) are quoted in ppm and are downfield from tetramethylsilane (TMS). ^{13}C NMR spectra were referenced to the internal D–^{13}C of the corresponding deuteriated solvent from TMS. ^1H and ^{13}C NMR spectra of the deprotected nucleotides were referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, $[CH_3)_3Si$, external reference, Aldrich Chemical Co.). ^{31}P NMR chemical shifts of all compounds are quoted in ppm and are downfield (positive value) from 85% H_3PO_4 (external capillary standard).

The NMR samples of the deprotected nucleotides were prepared as follows: The sodium salts of the nucleotides were dissolved in 0.50 mL of phosphate buffer (5 mM Na_2HPO_4, 100 mM NaCl, 0.1 mM EDTA, pD of buffer adjusted to 7.4 by addition of concentrated HCl) and lyophilized. The samples were then lyophilized two times from 99.8% D_2O (0.25 mL) and once from 99.96% D_2O (0.25 mL) and finally dissolved in 99.96% D_2O (0.50 mL). These solutions were transferred via syringe to 5-mm NMR tubes (Wilmad Glass Co., #528PP). The pD (pH + 0.4) of these solutions were in the range 7–8. The sample concentrations were in the range of 5–10 mM. The samples for the acquisition of ^1H NMR spectra were the same as used to obtain ^{13}C NMR spectra.

The ^1H NMR spectra with specific ^{31}P resonance decoupling were obtained with a Brucker WH-400 spectrometer (Université de Montrêal, Quebec) using standard Brucker accessories. This method has previously been described.[19] Nuclear Overhauser enhancement (NOE) difference experiments were performed with a Varian XL-200 spectrometer. A delay of 5 s between scans was used to permit relaxation of the system. The power of the selective irradiation pulse used was sufficient to achieve effective instantaneous saturation while at the same time maintaining selectivity. ^1H RELAYED-COSY[17] and ^1H–^{13}C heteronuclear 2D-NMR[18] spectra were recorded on a Varian XL-300 spectrometer.

Enzyme Assays. Spleen Phosphodiesterase. Spleen phosphodiesterase was obtained as a suspension (2 mg/mL) in ammonium sulfate (Boehringer Mannheim). Typically, 0.2 A_{260} units of lyophilized branched nucleotide were dissolved in 0.5 M ammonium acetate (adjusted to pH 6.5 with acetic acid, 75 μL) in a 1.5-mL Eppendorf tube and the enzyme solution was added (10 μL). After incubation (37 °C, 1–16 h) the sample was centrifuged and aliquots were analyzed by HPLC under condition A (General Methods).

Snake Venom Phosphodiesterase (SVPD). The enzyme (Boehringer Mannheim) was obtained as a solution in 50% glycerol containing 1 mg/mL. This enzyme contained a phosphatase impurity and prolonged incubations of branched nucleotides with SVPD converted 5′-mononucleotides into nucleosides. As a result, alkaline phosphatase (Boehringer Mannheim, diluted with water 1:9) was used in conjunction with SVPD to insure complete conversion of all nucleotides into nucleosides. Typically, 0.2 A_{260} units of lyophilized branched nucleotide were dissolved in 50 mM Tris·HCl and 10 mM $MgCl_2$ (75 μL, pH 8) in a 1.5-mL Eppendorf tube and SVPD (10 μL) and alkaline phosphatase (10 μL) solutions were added. After incubation (37 °C, 0.5 h) the digest was centrifuged and aliquots were analyzed by HPLC under condition B (General Methods).

Synthesis of Nucleoside 5′-*O*-(Methyl *N,N*-diisopropylphosphoramidites). N^2-Benzoyl-O^6-(*p*-nitrophenylethyl)-2′,3′-bis-*O*-(*tert*-butyldimethylsilyl)guanosine 5′-*O*-(Methyl *N,N*-diisopropylphosphoramidite) (3d). (a) N^2-Benzoyl-O^6-(*p*-nitrophenylethyl)-2′,3′,5′-tris-*O*-(*tert*-butyldimethylsilyl)guanosine (12). Derivative **12** was prepared from **11**[15] according to the procedure of Pfleiderer et al.[10] To a solution of **2c** (2.0 mmol, 1.46 g), triphenylphosphine (3.0 mmol, 0.79 g), and 2-(*p*-nitrophenyl)ethanol (3.0 mmol, 0.50 g) in anhydrous dioxane (25 mL) was added diethyl azidodicarboxylate (3.0 mmol, 0.52 g, 0.46 mL) at room temperature. The solution became warm and purple colored and faded to yellow after 10 min. After 45 min the solution was concentrated to an oil, redissolved in $CHCl_3$ (50 mL), washed with water/NaCl (5 × 100 mL), and dried (anhydrous Na_2SO_4). The crude material was purified on a silica gel column by elution with $CHCl_3$. The pure product was obtained as a white foam in 87% yield (1.53 g). In a larger scale reaction (10.0 mmol of **11**), product **12** eluted from the column together with triphenylphosphine oxide and was used without further purification in the next step. TLC: ether/$CHCl_3$ 1:3, R_f 0.83; ether/hexane 3:1, R_f 0.39; ethyl acetate/$CHCl_3$ 1:3, R_f 0.61. UV: max 271 nm; min 244 nm. ^1H NMR ($CDCl_3$, 200 MHz): 8.30 (s, 1, N*H*-benzoyl), 8.14 (s, 1 H_8), 8.1–7.4 (m, 10, aryl), 5.96 (d, $J_{H1'-H2'}$ = 5.7 Hz, 1 $H_{1'}$), 4.83 (t, 2, OCH_2CH_2), 4.67 (dd, 1, $H_{2'}$), 4.28 (dd, 1, $H_{3'}$), 4.10 (m, 1, $H_{4'}$), 4.02 (dd, 1, $H_{5'}$), 3.78 (dd, 1, $H_{5''}$), 3.34 (t, 2, CH_2NO_2), 0.92, 0.91, 0.78 (s, 27, *t*-BuSi), 0.11, 0.10, −0.05, −0.28 (s, 18, CH_3Si).

(b) N^2-Benzoyl-O^6-(*p*-nitrophenylethyl)-2′,3′-bis-*O*-(*tert*-butyldimethylsilyl)guanosine (1c). Derivative **1c** was prepared from **12** by using a slight modification of the procedure of Seela and Potter.[16] To a solution of **2d** (1.5 mmol, 1.35 g) in nitromethane (10 mL) was added $ZnBr_2$/waterr/nitromethane (25 mL, solution prepared by stirring 70 g of $ZnBr_2$, 5 mL of water, and 500 mL of nitromethane) and the reaction mixture stirred

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at 60 °C. After 24 h 1 M NH_4OAc (50 mL) was added followed by $CHCl_3$ (100 mL), and the mixture was transferred to a separatory funnel. After extraction, the organic layer was dried (anhydrous Na_2SO_4) and evaporated to yield an oil. The crude product was purified by silica gel chromatography by elution with 1:4 ether/$CHCl_3$. The pure product was obtained as a white foam in 68% yield (0.74 g). In a larger scale reaction (8.0 mmol of **12**), the crude product crystallized from 3:1 ether/hexane in 50% yield. TLC: ethyl acetate, R_f 0.85; methanol/$CHCl_3$, 0.5:9.5 R_f 0.38. UV: max 227 nm (sh), 270 nm; min 243 nm. ^1H NMR ($CDCl_3$, 200 MHz): 8.42 (s, 1, N*H*-benzoyl), 8.15 (d, 2 aryl), 7.92 (d, 2, aryl), 7.87 (s, 1 H_8), 7.51 (m, 4, aryl), 5.77 (d, 1 $H_{1'}$), 5.75 (bd, 1, 5'OH), 4.98 (dd, 1, $H_{2'}$), 4.85 (m, 2, OCH_2CH_2), 4.34 (d, $J_{H3'-H4'}$ = 0, 1, $H_{3'}$), 4.16 (b, 1, $H_{4'}$), 3.97 (bd, 1, $H_{5'}$), 3.72 (bdd, 1, $H_{5''}$, coupling with 5'OH), 3.30 (t, 2, CH_2NO_2), 0.93, 0.74 (s, 18, *t*-BuSi), 0.10, 0.09, −0.12 (s, 12, CH_3Si).

(c) N^2-Benzoyl-O^6-(*p*-nitrophenylethyl)-2',3'-bis-*O*-(*tert*-butyldimethylsilyl)guanosine 5'-*O*-(Methyl *N*,*N*-diisopropylphosphoramidite) (3d). To a stirred anhydrous $CHCl_3$ (7.5 mL) solution of derivative **1c** (2.5 mmol, 1.92 g) and diisopropylethylamine (11.6 mmol, 2.0 mL) was added, dropwise over 90 s, methyl diisopropylphosphoramidochloridite (**2a**) (2.88 mmol, 552 μL) at room temperature. The solution became orange colored. After stirring for 2 h ethyl acetate (200 mL, prewashed with 5% $NaHCO_3$) was added, and the solution washed with saturated NaCl solution (4 × 200 mL). The organic phase was dried (anhydrous Na_2SO_4) and the solvent was removed under vacuum to yield the crude material as a foam. The crude product was purified by silica gel chromatography by elution with 97:3 hexane/triethylamine, followed by 47:50:3 CH_2Cl_2/hexane/triethylamine. The pure product was obtained in 78% yield (1.8 g). TLC: ether/CH_2Cl_2 1:1, R_f 0.86. UV: max 228 nm (sh), 270 nm; min 243 nm. ^1H NMR ($CDCl_3$, 200 MHz), mixture of diastereoisomers 1:1 ratio: 8.45, 8.43 (s, 2, N*H*-benzoyl), 8.34, 8.28 (s, 2, H_8), 8.2–7.5 (m, 18, aryl), 5.98 (apparent d, 2, $H_{1'}$), 4.84, 4.83 (t, 4, OCH_2CH_2), 4.77, 4.69 (dd, 2, $H_{2'}$), 4.1–3.5 (m, 4, $H_{5'}$, $H_{5''}$), 3.43 (d, $^3J_{H-P}$ = 13.4 Hz, 3, $POCH_3$), 3.37 (d, $^3J_{H-P}$ = 13.4 Hz, 3, $POCH_3$), 3.35 (bt, 4, CH_2NO_2), 0.93, 0.92, 0.78, 0.75 (s, 36, *t*-BuSi), 0.10, 0.09, −0.06, −0.10, −0.28, −0.33 (s, 24, CH_3Si). ^{31}P NMR ($CDCl_3$): 150.0, 149.9.

2',3'-Bis-*O*-(*tert*-Butyldimethylsilyl)uridine 5'-*O*-(Methyl *N*,*N*-diisopropylphosphoramidite) (3a). (a) 2',3'-Bis-*O*-(*tert*-butyldimethylsilyl)uridine (1a). Derivative **1a** was prepared from 5',3',2'-tris-*O*-(*tert*-butyldimethylsilyl)uridine[28b] by using a slight modification of the procedure of Seela et al.[16] To a solution of 5',3',2'-tris-*O*-(*tert*-butyldimethylsilyl)uridine (20.0 mmol, 11.7 g), in nitromethane (200 mL) was added a solution of $ZnBr_2$/water/nitromethane (200 mL, solution prepared by stirring 70 g of $ZnBr_2$, 5 mL of water, and 500 mL of nitromethane), and the solution was stirred at room temperature. After 48 h 1 M NH_4OAc (500 mL) was added followed by $CHCl_3$ (200 mL) and the mixture transferred to a separatory funnel. After extraction, the organic layer was dried (anhydrous Na_2SO_4) and evaporated under vacuum to yield an oil. The crude product was purified by silica gel chromatography by elution with 1:3 ethyl acetate/$CHCl_3$. The pure product was obtained as a white powder in 80% yield (7.5 g). Alternatively, the crude product could be purified in high yield by crystallization from nitromethane. The pure product had identical UV and ^1H NMR spectra with that reported by Ogilvie et al.[28b] mp 216–217 °C (lit.[28b] mp 224–226 °C).

(b) 2',3'-Bis-*O*-(*tert*-butyldimethylsilyl)uridine 5'-*O*-(Methyl *N*,*N*-diisopropylphosphoramidite) (3a). To a stirred THF (22 mL) solution of derivative **1a** (7.4 mmol, 3.5 g) and diisopropylethylamine (30.0 mmol, 5.2 mL) was added dropwise, over 90 s, **2a** (8.5 mmol, 1.65 mL) at room temperature. A white precipitate appeared after 1 min, indicative of the desired reaction. After stirring for 15 min, ethyl acetate (200 mL, prewashed with 5% $NaHCO_3$) was added and the solution washed with saturated brine solution (5 × 200 mL). The organic phase was dried (anhydrous Na_2SO_4) and the solvent removed under vacuum to yield the crude material as a foam. The crude product was purified by silica gel chromatography by elution with 50:47:3 CH_2Cl_2/hexane/triethylamine. After evaporation of the pooled fractions the product was coevaporated, first with 95% EtOH to remove traces of triethylamine and then with ether, providing the pure product as a foam. Derivative **3a** was obtained in 85% yield (4.0 g). TLC: ether/CH_2Cl_2 1:1, R_f 0.66. UV (EtOH, nm): max 264; min 223. ^1H NMR ($CDCl_3$, 200 MHz): 8.01 (d, 1, H_6), 8.20 (d, 1 H_6), 5.91 (d, $J_{H1'-H2'}$ = 4.7 Hz, 1, $H_{1'}$), 5.85 (d, $J_{H1'-H2'}$ = 3.1 Hz, 1 $H_{1'}$), 5.67 (d, 1, H_5), 3.41 (d, $^3J_{H-P}$ = 13.6 Hz, 3, $POCH_3$), 3.40 (d, $^3J_{H-P}$ = 13.5 Hz, 3, $POCH_3$), 0.88 (×2), 0.87, 0.86 (s, 36, *t*-BuSi), 0.06, 0.05, 0.03 (s, 24, CH_3Si). ^{31}P NMR ($CDCl_3$): 150.24, 150.17.

N^2-Benzoyl-2',3'-bis-*O*-(*tert*-butyldimethylsilyl)guanosine 5'-*O*-(Methyl *N*,*N*-diisopropylphosphoramidite) (3c). To a stirred THF (4.5 mL) solution of **1b** (1.50 mmol, 0.93 g) and diisopropylethylamine (6.9 mmol, 1.2 mL) was added dropwise **2a** (1.95 mmol, 375 μL) at room temperature. A precipitate appeared after 5 min. After stirring for 1 h, ethyl acetate (100 mL, prewashed with 5% $NaHCO_3$) was added and the reaction mixture was worked up and purified as for **3a**. Chromatography solvent: a, CH_2Cl_2/hexane/triethylamine 50:45:5; b, CH_2Cl_2/triethylamine 97:3. Derivative **3c** was obtained in 65% yield (752 mg). TLC: ether/CH_2Cl_2 1:1, R_f 0.47–0.17. UV (EtOH, nm): max 240, 260, 266; min 224, 254, 275. ^1H NMR ($CDCl_3$, 200 MHz): 8.16 (s, 1, H_8), 8.15 (s, 1, H_8), 5.87 (d, $J_{H1'-H2'}$ apparent = 6.4 Hz), 2, $H_{1'}$), 3.49 (d, $^3J_{H-P}$ = 13.8 Hz, 3, $POCH_3$), 3.37 (d, $^3J_{H-P}$ = 13.3 Hz, 3, $POCH_3$), 0.92 (×2), 0.77, 0.76 (s, 36, *t*-BuSi), 0.09, −0.06, −0.08, −0.27, −0.28 (s, 24, CH_3Si). ^{31}P NMR ($CDCl_3$): 150.0, 149.4.

3'-*O*-(*tert*-Butyldimethylsilyl)thymidine 5'-*O*-(Methyl *N*,*N*-diisopropylphosphoramidite) (3g). To a stirred THF (5.0 mL) solution of 3'-*O*-(*tert*-butyldimethylsilyl)thymidine[29] (1.00 mmol, 357 mg), diisopropylethylamine (4.0 mmol, 0.7 mL), and 4-(dimethylamino)pyridine (0.2 mmol, 25 mg) was added dropwise **2** (1.30 mmol, 250 μL) at room temperature. A precipitate appeared after 1 min. After being stirred for 2 h, the reaction mixture was worked up and purified as for **3a**. Chromatography solvent: CH_2Cl_2/hexane/triethylamine 50:46:4. Derivative **3g** was obtained as a gum in 93% yield (480 mg). ^1H NMR ($CDCl_3$, 200 MHz): 8.10 (b, 2, NH), 7.76 (d, 1, H_6), 7.61 (d, 1, H_6), 6.34 (t, 1, $H_{1'}$), 6.33 (t, 1, $H_{1'}$), 3.42 (d, $^3J_{H-P}$ = 13.8 Hz, 3, $POCH_3$), 3.41 (d, $^3J_{H-P}$ = 13.7 Hz, 3, $POCH_3$), 1.94 (d, 3, CH_3-base), 1.92 (d, 3, CH_3-base), 0.883, 0.877 (s, 18, *t*-BuSi), 0.07 (×2), 0.064, 0.057 (s, 12, CH_3Si). ^{31}P NMR ($CDCl_3$): 150.0, 149.9.

N^6-Benzoyl-5'-*O*-(monomethoxytrityl)adenosine 2',3'-Bis-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (5a). To a THF (5.6 mL) solution of **4a** (1.85 mmol, 1.20 g) and diisopropylethylamine (19.4 mmol, 3.4 mL) was added dropwise **2a** (5.6 mmol, 1.1 mL) at room temperature. A precipitate appeared after 1 min. After being stirred for 30 min, the reaction mixture was worked up and purified as for **3a**. Chromatography solvent: CH_2Cl_2/hexane/triethylamine 50:47:3. The bisamidite derivative **5a** was obtained as a white foam in 62% yield (1.10 g). TLC: ether/CH_2Cl_2 1:1, R_f 0.63, 0.52. UV (EtOH, nm): max 234, 282; min 255. ^{31}P NMR ($CDCl_3$): isomer 1, 152.0, 150.9 ($^5J_{P-P}$ = 8.5 Hz); isomer 2, 151.7, 151.2 ($^5J_{P-P}$ = 6.5 Hz); isomer 3, 151.6, 150.4 ($^5J_{P-P}$ = 8.8 Hz); isomer 4, 151.6, 149.9 ($^5J_{P-P}$ = 9.0 Hz).

N^6-Benzoyl-5'-*O*-(monomethoxytrityl)arabinoadenosine 2',3'-Bis-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (7). (a) N^6-Benzoylarabinoadenosine was synthesized by the "transient" protection procedure developed by Jones et al. as described for N^6-benzoyl-2'-deoxyadenosine.[30] When using this procedure more trimethylsilyl chloride was employed to account for the additional 2'-hydroxyl group. Scale of reaction: arabinoadenosine (20.0 mmol, 5.4 g). Recovered product: 94% yield (7.0 g). TLC: ethanol/$CHCl_3$, 2:8 R_f 0.48. UV (EtOH, nm): max 282; min 250. mp: 138–140 °C.

(b) N^6-Benzoyl-5'-*O*-(monomethoxytrityl)arabinoadenosine (6). To a stirred pyridine/dimethylformamide (15 mL/15 mL) solution of N^6-benzoylarabinoadenosine (3.9 mmol, 1.45 g) was added, at once, monomethoxytrityl chloride (4.7 mmol, 1.45 g) at room temperature. The reaction was monitored by TLC (24 h) and more tritylating reagent was added (1.6 mmol, 0.5 g) until the starting material was completely consumed. After a total reaction time of 48 h the solution was poured into vigorously stirred ice water. The resulting solution was transferred to a separatory funnel and the product extracted with $CHCl_3$ (200 mL).

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The organic phase was dried (anhydrous Na_2SO_4) and evaporated under reduced pressure to yield an oil. The crude product was coevaporated with toluene (2 × 200 mL) and purified by silica gel chromatography by elution with methanol/$CHCl_3$, gradient: 0–5% methanol. The pure product was isolated as a foam in 90% yield (2.25 g). TLC: ethyl acetate, R_f 0.14; THF, R_f 0.81. UV (EtOH, nm): max 230, 280; min 264.

(c) N^6-Benzoyl-5′-*O*-(monomethoxytrityl)arabinoadenosine 2′,3′-Bis-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (7). To a stirred THF (5.6 mL) solution of **6** (2.0 mmol, 1.3 g) and diisopropylethylamine (28.2 mmol, 5.0 mL) was added dropwise **2a** (8.0 mmol, 1.6 mL) at room temperature. A precipitate appeared after 1 min. After being stirred for 4 h, the reaction mixture was worked up and purified as for **3a**. Chromatography solvent: CH_2Cl_2/hexane/triethylamine 46:46:8. Two purifications were required. The arabino bisamidite **7** product was obtained in 55% yield (1.1 g). TLC: ether/CH_2Cl_2 1:1, R_f 0.84; ethanol/ethyl acetate/$CHCl_3$ 5:50:45, R_f 0.96 (isomers 1, 2), 0.89 (isomers 3, 4). UV (EtOH, nm): max 234, 282; min 255. A slower moving material identified as N^6-benzoyl-5′-(monomethoxytrityl)arabinoadenosine 3′-*O*-(methyl *N*,*N*-diisopropylphosphoramidite)[8] was isolated in 19% yield (0.30 g).

Synthesis of Nucleoside (β-Cyanoethyl *N*,*N*-diisopropylphosphoramidites). 2′,3′-Bis-*O*-(*tert*-butyldimethylsilyl)uridine 5′-*O*-(β-Cyanoethyl *N*,*N*-diisopropylphosphoramidite) (3b). To a stirred THF (15 mL) solution of **1a** (5.0 mmol, 2.36 g), 4-(dimethylamino)pyridine (0.5 mmol, 62 mg), and diisopropylethylamine (19.3 mmol, 3.3 mL) was added dropwise *N*,*N*-diisopropyl [(β-cyanoethyl)oxy]phosphonamidic chloride (**2b**) (5.5 mmol, 1.2 mL) at room temperature. A precipitate appeared after 5 min. After being stirred for 2 h, the reaction mixture was worked up and purified as for **3a**. Chromatography solvent: CH_2Cl_2/hexane/triethylamine 17:80:3. Derivative **3b** was isolated as a white foam in 81% yield (2.72 g). TLC: ether/CH_2Cl_2 1:1, R_f 0.55, 0.53. UV (EtOH, nm): max 264; min 223. ^{1}H NMR ($CDCl_3$, 300 MHz): 8.07 (d, 1, H_6), 7.83 (d, 1, H_6), 5.89 (d, $J_{H1'-H2'}$ = 5.0 Hz, 1, $H_{1'}$), 5.80 (d, $J_{H1'-H2'}$ = 2.2 Hz, 1, $H_{1'}$), 5.69 (d, 1, H_5), 5.68 (d, 1, H_5), 4.2–3.6 (m), 2.64 (t, 2, CH_2CN), 2.63 (t, 2, CH_2CN), 0.91 (×2), 0.90, 0.88 (s, 36, *t*-BuSi), 0.12, 0.10, 0.09, 0.08, 0.06 (s, 24, CH_3Si). ^{31}P NMR ($CDCl_3$): 149.3, 149.1.

***N*⁴-Benzoyl-2′,3′-bis-*O*-(*tert*-butyldimethylsilyl)cytidine 5′-*O*-(β-Cyanoethyl *N*,*N*-diisopropylphosphoramidite) (3f).** To a stirred THF (9 mL) solution of N^4-benzoyl-2′,3′-bis-*O*-(*tert*-butyldimethylsilyl)cytidine (3.0 mmol, 1.73 g), 4-(dimethylamino)pyridine (0.3 mmol, 37 mg), and diisopropylethylamine (11.6 mmol, 2.0 mL) was added dropwise **2b** (3.3 mmol, 0.70 mL) at room temperature. A precipitate appeared after 1 min. After being stirred for 1 h, the reaction mixture was worked up and purified as for **3a**. Chromatography solvent: CH_2Cl_2/hexane/triethylamine 30:67:3 changed gradually to 47:50:3. Derivative **3f** was isolated as a white foam in 82% yield (1.90 g) (92% yield based on recovered starting material). TLC: ether/CH_2Cl_2 1:1, R_f 0.52. UV (EtOH, nm): max 256, 310; min 250, 290. ^{1}H NMR ($CDCl_3$, 300 MHz): 5.80 (d, $J_{H1'-H2'}$ = 1.5 Hz, 1, $H_{1'}$), 5.77 (bs, $J_{H1'-H2'}$ = 0 Hz, 1, $H_{1'}$), 4.3–3.6 (m), 2.71 (t, 2, CH_2CN), 2.70 (t, 2, CH_2CN), 0.92, 0.91, 0.89, 0.88 (s, 36, *t*-BuSi), 0.28, 0.23, 0.14, 0.12, 0.08, 0.06, 0.05 (s, 24, CH_3Si). ^{31}P NMR ($CDCl_3$): 149.4, 149.2.

***N*²-Benzoyl-*O*⁶-(*p*-nitrophenylethyl)-2′,3′-bis-*O*-(*tert*-butyldimethylsilyl)guanosine 5′-*O*-(β-Cyanoethyl *N*,*N*-diisopropylphosphoramidite) (7e).** To a stirred THF (3.8 mL) solution of **1c** (1.25 mmol, 956 mg), 4-(dimethylamino)pyridine (0.1 mmol, 12 mg), and diisopropylethylamine (4.8 mmol, 0.8 mL) was added dropwise **2b** (1.38 mmol, 245 μL) at room temperature. The solution turned orange colored and a precipitate appeared after 5 min. After being stirred for 2 h, the reaction mixture was worked up and purified as for **3a**. Chromatography solvent: CH_2Cl_2/hexane/triethylamine 17:80:3. Derivative **3e** was isolated as a white foam in 70% yield (845 mg) (90% yield based on recovered starting material). TLC: ether/CH_2Cl_2 1:1, R_f 0.78. UV (EtOH, nm): max 232 (sh), 276; min 248. ^{1}H NMR ($CDCl_3$, 300 MHz): 8.47 (s, 1, H_8), 8.41 (s, 1, H_8), 5.97 (d, $J_{H1'-H2'}$ = 5.7 Hz, 1, $H_{1'}$), 5.95 (d, $J_{H1'-H2'}$ = 5.1 Hz, 1, $H_{1'}$), 4.84 (m, 4, $OCH_2CH_2NO_2$), 4.3–3.6 (m), 3.36 (t, 2, CH_2NO_2), 3.35 (t, 2, CH_2NO_2), 2.69 (dt, 2, CH_2CN), 2.60 (t, 2, CH_2CN), 0.95, 0.94, 0.83, 0.79 (s, 36, *t*-BuSi), 0.130, 0.127, 0.121, 0.10, 0.00, –0.05, –0.17, –0.28 (s, 24, CH_3Si). ^{31}P NMR ($CDCl_3$): 149.5, 149.2.

N^6-Benzoyl-5′-*O*-(monomethoxytrityl)adenosine 2′,3′-Bis-*O*-(β-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (5b). To a stirred THF (6 mL) solution of **4a** (2.0 mmol, 1.30 g), 4-(dimethylamino)pyridine (0.6 mmol, 74 mg), and diisopropylethylamine (21.0 mmol, 3.6 mL) was added dropwise **2b** (6.0 mmol, 1.3 mL) at room temperature. A precipitate appeared after 5 min. After being stirred for 2 h, the reaction mixture was worked up and purified as for **3a**. Chromatography solvent: CH_2Cl_2/hexane/triethylamine 50:47:3. Derivative **5b** was isolated quantitatively (2.10 g) as a white foam. TLC: ether/CH_2Cl_2 1:1, R_f 0.51, 0.40, 0.25. UV (EtOH, nm): max 282; min 252. ^{31}P NMR (CD_3CN): isomer 1, 152.2, 150.6 ($^5J_{P-P}$ = 10.1 Hz); isomer 2, 151.9, 150.5 ($^5J_{P-P}$ = 7.1 Hz); isomer 3, 151.8, 151.0 ($^5J_{P-P}$ = 4.6 Hz); isomer 4, 151.3, 151.2 ($^5J_{P-P}$ = 8.6 Hz).

Synthesis of Linear RNA Fragments. UpA^{P}G, U^{P}A^{P}G, UpApU, and ApGpApUpC were synthesized by the phosphodichloridite procedure[15,26] using β-cyanoethyl phosphodichloridite[26] as coupling reagent. The nucleotides were deprotected as for the branched nucleotides (vide infra) and were characterized by HPLC analysis of their enzymatic digests. Snake venom completely degraded all nucleotides. UpApU and ApGpApUpC were completely degraded by spleen phosphodiesterase. Analysis of the spleen phosphodiesterase digest of U^{P}A^{P}G and UpA^{P}G indicated that U^{P}A^{P}G was not degraded by this enzyme while UpA^{P}G was split into a mixture of $U_{3'p}$ and A^{P}G dimer in a 1:1 ratio.

Synthesis of Branched RNA Fragments. General Procedure. The general procedure can be illustrated by the synthesis of A^U_U (**9a**, route A). Adenosine **4a** (0.25 mmol, 162 mg), uridine phosphoramidite **3a** (0.75 mmol, 478 mg), and tetrazole (3.0 mmol, 211 mg) were transferred into a vial. The absence of solvent prevented any reaction from occurring. The reaction was started by inecting THF (1.5 mL) into the vial and the solution stirred at room temperature for 45 min. At this point 2,4,6-collidine was added (1.5 mmol, 200 μL) followed by addition of an aqueous iodine solution (0.1 M in THF/water 3:1.5, excess) to oxidize the phosphite triester intermediate. After 5 min the reaction mixture was diluted with $CHCl_3$ (50 mL) and washed with an aqueous sodium bisulfite solution (2–5 mL of 5% sodium bisulfite in 50 mL water). The organic phase was washed with saturated brine (2 × 50 mL), dried (anhydrous Na_2SO_4), and evaporated under reduced pressure to yield a foam. The monomethoxytrityl group was removed by treatment of the crude product, A^U_U (**8a**), with a cold (0–5 °C) solution of benzenesulfonic acid in acetonitrile (0.1M, 3.8 mmol, 38 mL) for 10 min. The reaction mixture was dissolved in $CHCl_3$ (100 mL) and washed with 5% sodium bicarbonate solution (75 mL), followed by water (2 × 75 mL). The aqueous phase was separated and back-extracted twice with $CHCl_3$ (20 mL each). The combined $CHCl_3$ extracts were dried (anhydrous Na_2SO_4) and evaporated under reduced pressure. The crude product was purified on a silica gel column by elution with 71:26:3 ether/$CHCl_3$/ethanol, 50–100 mL followed by methanol/$CHCl_3$, gradient 0–5% methanol. The pure trimer A^U_U **9a** was obtained in 65% yield (240 mg). TLC: THF, R_f 0.66, 0.62. UV (EtOH, nm): max 265; min 235. ^{31}P NMR ($CDCl_3$): 0.18, 0.11, –0.10, –0.16, –0.43, –0.72, –0.91, –0.95.

The nucleotides A^U_U (**9b**), A^G_G (**9f**), A^C_C (**9j**), A^T_T (**9k**), G^U_U (**9l**), and a A^U_U (**10**) were prepared in this fashion (Table I). Nucleotides MMT-A^U_G (**8c**), MMT-A^G_U (**8d**), and MMT-UpA^U_U (**16a**) were purified prior to detritylation (vide infra). Nucleotides MMT-A^C_G (,**h**) and MMT-A^G_C (**8i**) were isolated as a mixture with MMT-A^C_C (**8j**) and MMT-A^G_G (**8g**) and were separated after complete deprotection (vide infra). Yields are based on the limiting nucleoside (adenosine **4a** or adenosine bisamidites **5a**, **5b**, and **7**).

A^U_U (.b). Purification (silica gel column): ether/$CHCl_3$/ethanol 71:26:3, 50–100 mL followed by methanol/$CHCl_3$, gradient 0–5% methanol. TLC: THF, R_f 0.47, 0.33, UV (EtOH, nm): max 268; min 232. ^{31}P NMR ($CDCl_3$): –1.36, –1.55, –2.04, –2.15, –2.60, –2.69, –2.80, –2.83.

A^U_G (9c) and A^G_U (9d). Nucleotides **9c** and **9d** were prepared as for A^U_U (**9a**, Table I) except that the crude condensation products [MMT-A^U_G (**8c**) and MmT-A^G_U (**8d**)] were purified on a silica gel column prior to detritylation. Solvent system: ethanol/$CHCl_3$/ether 1:23:76 changed gradually to 4:20:76. The

tritylated nucleotides eluted from the column in the following order: MMT-A^{G}_{G} (**8f**, 24%, 144 mg), the desired mixture of MMT-A^{U}_{G} (**8c**), and MMT-A^{G}_{U} (**8d**, 39%, 190 mg) and MMT-A^{U}_{U} (**8a**, 16%, 70 mg). TLC: ethanol/$CHCl_3$/ether, R_f (**8f**) 0.51, R_f (**8c** and **8d**) 0.3, R_f (**8a**) 0.11. The isolated mixture **8c** and **8d** (0.08 mmol, 150 mg) was detritylated as for MMT-A^{U}_{U} (**8a**) and purified on two preparative TLC plates by development with 95:5 ethyl acetate/ethanol (R_f (**9c** and **9d**) 0.32, 0.23). The pure products **9c** and **9d** were eluted from the silica gel by using 7:3 ethyl acetate/ethanol and were obtained in 72% yield (90 mg). The isomer mixture was separated by HPLC after complete deprotection (vide infra).

A^{G}_{G} (9f). Purification (silica gel column): ethyl acetate/CH_2Cl_2 1:1, 50–100 mL followed by methanol/$CHCl_3$, gradient 0–5% methanol. TLC: THF, R_f 0.81. UV (EtOH, nm): max 272, 232 sh; min 244. ^{31}P NMR ($CDCl_3$): 0.11, −0.06, −0.68, −0.75, −0.85 (overlapping signals), −0.88, −0.89.

A^{C}_{G} (9h) and A^{G}_{C} (9i). A mixture of nucleotides **8g**, **8h**, **8i**, and **8j** was prepared as described in Table I. The crude products were detritylated as for MMT-A^{U}_{U} (**8a**, Table I) and purified on eight preparative TLC plates by development with 5:1 ethyl acetate/CH_2Cl_2 (R_f 0.4–0.1). The pure product mixture **9g**, **9h**, **9i**, and **9j** were eluted from the silica gel by using 7:3 ethyl acetate/ethanol and were obtained in ca. 32% yield (167 mg). The nucleotide mixture was separated by cellulose TLC and HPLC after complete deprotection (vide infra).

A^{C}_{C} (9j). Purification (silica gel column): ethyl acetate/CH_2Cl_2/ethanol, 1:1:0 to 1:0.90:0.10 gradient. TLC: THF, R_f 0.79. UV (EtOH, nm): max 262, 320 sh; min 235. ^{31}P NMR ($CDCl_3$): −1.45, −1.49, −1.90, −1.96, −2.20, −2.24 (overlapping signals), −2.40.

$^{AT}_{T}$ (9k). Purification (silica gel column): methanol/$CHCl_3$, gradient 0–5% methanol. TLC: THF, R_f 0.65. UV (EtOH, nm): max 274, 265 sh; min 237. ^{31}P NMR ($CDCl_3$): −0.16 (overlapping signals), −0.01, −0.05, −0.55, −0.66, −0.91, −0.99. Anal. Calcd for $C_{51}H_{75}N_9O_{19}P_2Si_2 \cdot H_2O$: C, 48.83; H, 6.19; N, 10.05. Found: C, 48.91; H, 6.07; N, 9.99.

G^{U}_{U} (9l). Purification (silica gel column): ether/$CHCl_3$/ethanol 71:26:3, 50–100 mL followed by methanol/$CHCl_3$, gradient 0–5% methanol. TLC: THF, R_f 0.68, 0.61. UV (EtOH, nm): max 270, 243 sh; min 242. ^{31}P NMR ($CDCl_3$): 0.20, 0.11, −0.02, −0.05, −0.32, −0.36, −0.92 (overlapping signals).

aA^{U}_{U} (10). Purification (silica gel column): ether/$CHCl_3$/ethanol 71:26:3, 50–100 mL followed by methanol/$CHCl_3$, gradient 0–5% methanol. TLC: THF, R_f 0.65, 0.62. UV (EtOH nm): max 268; min 232. ^{31}P NMR ($CDCl_3$): 0.45, 0.25, 0.02, −0.05, −0.38, −0.40, −0.73, −0.86. Anal. Calcd for $C_{61}H_{99}H_9O_{21}P_2Si_4 \cdot {}^1/_2H_2O$: C, 49.58; H, 6.82; N, 8.53. Found: C, 49.13; H, 6.64; N, 8.54.

UpA^{U}_{U} (16b). To 5′-*O*-(monomethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine 3′-*O*-(methyl *N*,*N*-diisopropylphosphoramidite) (0.30 mmol, 237 mg), A^{U}_{U} **11a** (0.14 mmol, 198 mg), and tetrazole (1.2 mmol, 85 mg) was added THF (0.9 mL) at room temperature. After being stirred for 2 h, the reaction mixture was oxidized and worked up as for **10a**. The crude product was purified on a silica gel column by elution with 2:50:48 ethanol/ethyl acetate/$CHCl_3$. Pure MMT–UpA^{U}_{U} (**16a**) was obtained in 53% yield (155 mg). Nucleotide **16a** was detritylated as for **8a** in 95% yield (121 mg) and was purified on a silica gel column by elution with methanol/$CHCl_3$, gradient 0–5% methanol. TLC: THF, R_f 0.87. UV (EtOH, nm): max 264; min 230. ^{31}P NMR ($CDCl_3$): 0.14, −0.02, −0.09, −0.15, −0.19, −0.26, −0.37, −0.41, −0.47, −0.52, −0.61, −0.72. Anal. Calcd for $C_{77}H_{126}N_{11}O_{29}P_3Si_5 \cdot H_2O$: C, 48.14; H, 6.72; N, 8.02. Found: C, 47.66; H, 6.57; N, 7.93.

Characterization of Nucleoside Phosphotriesters 13 and 14. The nucleotide triesters **13** and **14** were characterized by chemical and spectroscopic means. The 5′–5′-linked dimers **14** were prepared by the reaction of their respective 5′-hydroxyl nucleoside components and 0.5 molar equiv of methyl phosphodichloridite ($MeOPCl_2$) or β-cyanoethyl phosphodichloridite ($CEOPCl_2$) followed by I_2/H_2O oxidation, according to Ogilvie et al.[26] Similarly, the reaction of the 5′-hydroxyl nucleosides with 1 equiv of $ROPCl_2$ followed by addition of ROH (R = Me or CE) and iodine oxidation afforded the phosphotriesters **13** in good yields.

Nucleoside Triesters 13. pU (13a). TLC: ethyl acetate, R_f 0.17; ether/CH_2Cl_2/EtOH 76:21:3, R_f 0.05. UV (EtOH, nm): max 264; min 233. ^1H NMR ($CDCl_3$, 200 MHz): 7.56 (d, 1, H_6), 5.74 (d, 1, H_5), 5.67 (d, $J_{H1'-H2'}$ = 3.5 Hz, 1, $H_{1'}$), 2.79 (t, 4, 2× CH_2CN), 0.89, 0.87 (s, 18, *t*-BuSi), 0.08, 0.07 (s, 12, CH_3Si). ^{31}P NMR ($CDCl_3$): −1.7.

pC (13b). TLC: ethyl acetate, R_f 0.45; ether/CH_2Cl_2/EtOH 76:21:3, R_f 0.13. UV (EtOH, nm): max 306, 264; min 284, 232. ^1H NMR ($CDCl_3$, 200 MHz): 8.14 (d, 1, H_6), 5.65 (d, $J_{H1'-H2'}$ = 1.1 Hz, 1, $H_{1'}$), 2.84 (t, 2, CH_2CN), 2.83 (t, 2, CH_2CN), 0.91, 0.88 (s, 18, *t*-BuSi), 0.22, 0.14, 0.08, 0.06 (s, 12, CH_3Si). ^{31}P NMR ($CDCl_3$): −1.4.

pU (13c). TLC: ethyl acetate, R_f 0.33; ether/CH_2Cl_2/EtOH 76:21:3, R_f 0.27. UV (EtOH, nm): max 264; min 233. ^1H NMR ($CDCl_3$, 300 MHz): 7.72 (d, 1, H_6), 5.79 (d, 1, H_5), 5.75 (d, $J_{H1'-H2'}$ = 3.5 Hz, 1 $H_{1'}$), 3.81 (d, $^3J_{H-P}$ = 11.3 Hz, 3, $POCH_3$), 3.80 (d, $^3J_{H-P}$ = 11.3 Hz, 3, $POCH_3$), 0.88, 0.87 (s, 18, *t*-BuSi), 0.07, 0.04 (s, 12, CH_3Si). ^{31}P NMR ($CDCl_3$): 2.1.

5′–5′-Linked Dimers 14. UpU (14a). TLC: ethyl acetate, R_f 0.52; ether/CH_2Cl_2/EtOH 76:21:3, R_f 0.16. UV (EtOH, nm): max 264; min 233. ^1H NMR ($CDCl_3$, 200 MHz): 7.54 (d, 1, H_6), 7.52 (d, 1, H_6), 5.61 (d, 1, H_5), 5.60 (d, 1, H_5), 5.76–5.72 (m, 2, $H_{1'}$), 2.75 (t, 2, CH_2CN), 0.89, 0.87 (s, 36, *t*-BuSi), 0.07 (×2), 0.08 (s, 24, CH_3Si). ^{31}P NMR ($CDCl_3$): −1.0.

CpC (14b). TLC: ethyl acetate, R_f 0.85; ether/CH_2Cl_2/EtOH 76:21:3, R_f 0.52. UV (EtOH, nm): max 308, 264; min 283, 234. ^1H NMR ($CDCl_3$, 200 MHz): 8.20 (d, 1, H_6), 8.18 (d, 1, H_6), 5.69 (d, $J_{H1'-H2',apparent}$ = 4.8 Hz, 2, $H_{1'}$), 2.84 (t, 2, CH_2CN), 0.92, 0.91, 0.88, 0.87 (s, 36, *t*-BuSi), 0.27 (×2), 0.17, 0.16, 0.09, 0.08, 0.05, 0.04 (s, 24, CH_3Si). ^{31}P NMR ($CDCl_3$): −0.6.

UpU (14c). TLC: ethyl acetate, R_f 0.67; ether/CH_2Cl_2/EtOH 76:21:3, R_f 0.39. UV (EtOH, nm): max 264; min 233. ^1H NMR ($CDCl_3$, 200 MHz): 7.60 (d, 1, H_6), 7.59 (d, 1, H_6), 5.73 (d, 1, H_5), 5.72 (d, 1, H_5), 5.64 (d, $J_{H1'-H2'}$ = 3.5 Hz, 1 $H_{1'}$), 5.63 (d, $J_{H1'-H2'}$ = 3.5 Hz, 1, $H_{1'}$), 3.81 (d, $^3J_{H-P}$ = 11.4 Hz, 3, $POCH_3$), 0.89, 0.88, 0.87 (×2) (s, 36, *t*-BuSi), 0.101, 0.097, 0.086 (s, 24, CH_3Si). ^{31}P NMR ($CDCl_3$): 1.0.

Deprotection of Branched Nucleotides (See General Methods). A^{G}_{G} (9f). The general procedure can be illustrated by the deprotection of A^{G}_{G} (**9f**). (a) Removal of Methyl Protecting Groups. A^{G}_{G} (**9f**, 72 mg, 35 μmol) was dissolved in *tert*-butylamine (4 mL) in a 5-mL polypropylene test tube. The tube was sealed and heated (50 °C). After 24 h *tert*-butylamine was evaporated under a stream of argon.

(b) Removal of Benzoyl Protecting Groups. The material obtained above was dissolved in ethanol (4 mL) and transferred into two 16-mL polypropylene test tubes (2 mL in each). Concentrated ammonium hydroxide (8 mL) was added in each tube. The tubes were sealed and heated (50 °C) for 24 h. After cooling the samples in an ethanol/dry ice bath, the ammonia solutions were evaporated in a Speed-Vac concentrator and ethanol (1 mL each tube) was added to the residue. The ethanol solutions were combined, transferred to a 5-mL sterile polypropylene tube, and evaporated to leave a foam.

(c) Removal of *tert*-Butyldimethylsilyl and *p*-Nitrophenylethyl Groups. The material obtained above was treated with 1 M TBAF/THF (1.0 mL, 1.0 mmol) for 3 h at room temperature. The solution turned dark, characteristic of desilylations of nucleotides containing O^6-(*p*-nitrophenylethyl)guanosine residues. The solution was diluted with water (2 mL) and passed through a column of Dowex Na^+ ion exchange resin. The eluant (30 mL) was lyophilized to give 900 A_{260} units of crude A^{G}_{G} (UV quantitation) as a yellow solid.

(d) Desalting and Purification. The material recovered from the above reaction was dissolved in water (2 mL) and applied (1 mL at a time) to a column packed with preswollen Sephadex G-25 size exclusion gel. The fractions containing the nucleotide were lyophilized to give 790 A_{260} units of crude A^{G}_{G} as a white powder. The recovered material was dissolved in water (1 mL), applied to cellulose plates, and purified by development in solvent F. The band was removed and transferred to a 16-mL polypropylene tube containing water (4–5 mL). This solution was swirled (Vortex mixer) and centrifuged (20 min) and the supernatant transferred to another tube. The extraction step was repeated two more times and the combined supernatants were lyophilized to yield 380 A_{260} units of material. Finally, the residue was dissolved in water (1 mL) and applied on a Sephadex G-25 column, as above, to remove any residual cellulose and salts. The fractions containing A^{G}_{G}

were lyophilized to yield 280 A_{260} units of pure A^G_G (23% yield, 7 mg).

This general procedure was used in the deprotections of all branched RNA fragments. Each of the isomer mixtures A^U_G, A^G_U and A^C_G, A^G_C were separated into their respective components following deprotection (vide infra). Nucleotides containing cyanoethyl groups did not require *tert*-butylamine treatments since they are removed in the same step that removes *N*-benzoyl groups (step b). All guanosine containing nucleotides were contaminated with a yellow impurity following the TBAF treatment (presumably *p*-$NO_2C_6H_4CH{=}CH_2$). This material was completely removed during size exclusion (Sephadex G-25) and cellulose chromatography. During cellulose TLC purification (solvent F) of the nucleotides there are sometimes traces of material which remain on the base line (due to excess salts). This band is removed from the plate, eluted with water and the material repurified on another plate using a more polar solvent (solvent G). The properties of the deprotected branched trimers are described in Table IV.

A^U_U (9a). Deprotection (35 μmol): (i) *tert*-butylamine (50 °C, 13 h); (ii) 29% aqueous ammonia (50 °C, 6 h); (iii) 0.1 M TBAF/THF (20 °C, 3 h); (iv) Na^+ ion exchange chromatography; isolated crude product: 788 A_{260} units. Purification (394 A_{260} units): (i) Sephadex G-25; (ii) cellulose TLC; (iii) Sephadex G-25; isolated pure product: 130 A_{260} units (4 mg).

A^U_G (9c) and A^G_U (9d). Deprotection (35 μmol): (i) *tert*-butylamine (50 °C, 24 h); (ii) 29% aqueous ammonia (50 °C, 18 h); (iii) 0.1 M TBAF/THF (20 °C, 3 h); (iv) Na^+ ion exchange chromatography; isolated crude product: 730 A_{260} units. Following ion exchange the crude mixture (450 A_{260} units) was applied to three cellulose plates (Merck) and purified by development in solvent F. One diffused band was observed and was divided into two halves referred to "band A" (upper half) and "band B" (lower half). After elution of both bands with water (band A: 171 A_{260} units; band B: 101 A_{260} units, 60%) their composition was determined by ^1H and ^{31}P NMR, and HPLC analysis (see Results and Discussion). Bands A and B were a mixture of the desired nucleotides A^U_G and A^G_U in 1:0.5 and 0.8:1 ratios, respectively. Therefore, the R_f values (cellulose TLC, solvent F) of A^G_G and A^G_U are 0.37 and 0.35, respectively. A mixture of A^U_G and A^G_U (48 A_{260} units, 0.8:1 ratio) was separated by HPLC under condition A. Following separation, the nucleotides were desalted by reverse phase chromatography using C_{18} SEP-PAK cartridges (Waters Associates).[27] After lyophilization of the collected fractions 9 A_{260} and 11 A_{260} units of A^G_G and A^G_U, respectively, were obtained (42% yield, HPLC purification and desalting step).

A^C_G (9h) and A^G_C (9i). Deprotection [(60 mg of a mixture of A^C_C (**9j**), A^G_G (**9g**), A^C_G (**9h**), and A^G_C (**9i**)]: (i) *tert*-butylamine (50 °C, 10 h); (ii) 29% aqueous ammonia (50 °C, 7 h); (iii) 0.1 M TBAF/THF (20 °C, 3 h); (iv) Na^+ ion exchange chromatography; isolated crude product: 706 A_{260} units. Following ion exchange half of the sample (400 A_{260} units) was applied to four cellulose (Eastman Kodak) plates and purified by development in solvent F. Four bands referred to products A, B, C, and D were observed with R_f 0.50 (fluorescent), 0.39, 0.30, and 0.24, respectively. The bands were removed, eluted with water, and each repurified by cellulose TLC (solvent F). The recovered materials were applied to a Sephadex G-25 column and the collected fractions were lyophilized to yield 0 A_{260} units (A), 69 A_{260} units (B), 82 A_{260} units (C), and 17 A_{260} units (D). Products B and D were identical with A^C_C and A^G_G (independently synthesized and deprotected), respectively, with respect to HPLC retention, R_f values (cellulose TLC, solvent A, F, G) and enzymatic (SVPD) digestion products. Product C was a mixture of the desired nucleotides A^C_G and A^G_C (0.5:1 ratio) and were characterized by enzymatic digestion and NMR spectroscopy (see Results and Discussion). A mixture of nucleotides A^C_G and A^G_C (47 A_{260} units, 1:1 ratio) was separated by HPLC under condition A. Following separation, the nucleotides were desalted by reverse phase chromatography using C_{18} SEP-PAK cartridges (Waters Associates).[27] After lyophilization of the collected fractions 16 and 18 A_{260} units of pure A^C_G and A^G_C, respectively, were obtained (73% yield, HPLC purification and desalting step).

A^C_C (9j). Deprotection (25 μmol): (i) 29% aqueous ammonia (50 °C, 16 h); (ii) 0.1 M TBAF/THF (20 °C, 4 h); (iii) Na^+ ion exchange chromatography; isolated crude product: 530 A_{260} units. Purification (250 A_{260} units): (i) cellulose TLC; (ii) Sephadex G-25; isolated pure product: 168 A_{260} units (5 mg).

A^T_G (9k). Deprotection (35 μmol): (i) *tert*-butylamine (50 °C, 16 h); (ii) 29% aqueous ammonia (50 °C, 8 h); (iii) 0.1 M TBAF/THF (20 °C, 3 h); (iv) Na^+ ion exchange chromatography; isolated crude product: 723 A_{260} units. Purification (723 A_{260} units): (i) Sephadex G-25; (ii) cellulose TLC; (iii) Sephadex G-25; isolated pure product: 230 A_{260} units (7 mg).

G^U_U (9l). Deprotection (50 μmol): (i) *tert*-butylamine (50 °C, 16 h); (ii) 29% aqueous ammonia (50 °C, 18 h); (iii) 0.1 M TBAF/THF (20 °C, 3 h); (iv) Na^+ ion exchange chromatography; isolated crude product: 1000 A_{260} units. Purification (700 A_{260} units): (i) Sephadex G-25; (ii) cellulose TLC; (iii) Sephadex G-25; isolated pure product: 612 A_{260} units (20 mg).

aA^U_U (10). Deprotection (50 μmol): (i) *tert*-butylamine (50 °C, 16 h); (ii) 29% aqueous ammonia (50 °C, 8 h); (iii) 0.1 M TBAF/THF (20 °C, 3 h); (iv) Na^+ ion exchange chromatography; isolated crude product, 1255 A_{260} units. Purification (1255 A_{260} units): Sephadex G-25; isolated pure product, 1184 A_{260} units (35 mg).

UpA^U_U (16b). Deprotection (25 μmol): (i) *tert*-butylamine (50 °C, 10 h); (ii) 29% aqueous ammonia (50 °C, 7 h); (iii) 0.1 M TBAF/THF (20 °C, 3 h); (iv) Na^+ ion exchange chromatography; isolated crude product, 706 A_{260} units. Purification (706 A_{260} units): (i) Sephadex G-25; (ii) cellulose TLC; (iii) Sephadex G-25; isolated pure product, 240 A_{260} units (7 mg).

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