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A new approach to the synthesis of branched and branched cyclic oligoribonucleotides

Colin B. Reese* and Quanlai Song

Department of Chemistry, King's College London, Strand, London WC2R 2LS, UK

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ABSTRACT

The six-step synthesis of the di-triethylammonium salt of 5'-*O*-trityl-6-*N*-pivaloyladenosine-2'-(*H*-phosphonate)-3'-[(2-chlorophenyl) phosphate] 9 from 3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxan-1,3-diyl)-6-*N*-pivaloyladenosine 1 in 68% overall yield is described. Compound 9 is converted into a branched pentaribonucleoside tetraphosphate 24 and a branched cyclic pentaribonucleotide ('lariat') 25 by solution phase triester chemistry involving both *H*-phosphonate and conventional phosphotriester coupling reactions. The monomeric building block 9 is proposed as a universal synthon for the preparation of branched and branched cyclic oligoribonucleotides derived from adenosine.

INTRODUCTION

It has been established (1–3) that, in the course of eukaryotic messenger RNA splicing, introns are excised in the form of branched circular RNA sequences, so-called 'lariats'. Branching occurs when the 2'-hydroxy function of a non-terminal adenosine residue is phosphorylated by a phosphomonoester function attached to the guanosine residue that is situated at the 5'-terminus of the intron. As well of being of interest as possible substrates for enzymatic and biological studies, relatively low molecular weight branched oligoribonucleotides and particularly 'lariats' have presented a synthetic challenge to nucleotide chemists. Soon after the original reports (1,2) in the literature relating to the splicing of messenger RNA precursors, several research groups successfully undertook the chemical synthesis of branched oligoribonucleotides (4–20) and, more recently, Chattopadhyaya and his co-workers (21,22) have accomplished the chemical synthesis of 'lariats'.

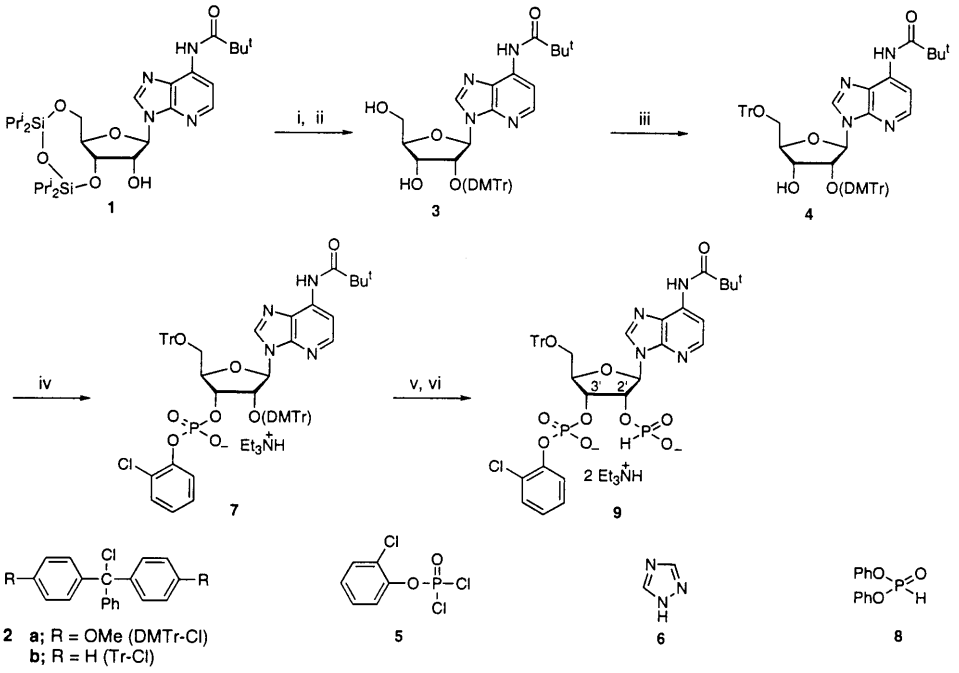
A number of successful strategies have been developed for the synthesis of branched oligoribonucleotides. A particularly widely used solution phase strategy (7–11,13,14) involves the reaction between a monomeric 5'-phosphoramidite and a partially protected oligoribonucleotide, containing an unprotected phosphodiester internucleotide linkage vicinal to the hydroxy function at which branching is to occur. If a branched oligoribonucleotide containing identical 2'- and 3'-sequences is required, it may be prepared by reacting a 5'-protected ribonucleoside derivative with free 2'- and 3'-hydroxy functions with an excess of a 5'-phosphoramidite. This approach has been used successfully both in solution (6) and on a solid support (19). Chattopadhyaya's approach (21,22) to the solution phase synthesis of 'lariats' involves the 2'-phosphorylation of a partially protected oligoribonucleotide vicinal to its 3'-terminal 2-chlorophenyl phosphate (i.e. 3'-phosphodiester) function, followed by appropriate elaboration, cyclization and deprotection.

RESULTS AND DISCUSSION

We recently described (23) a new approach to the synthesis of linear and cyclic oligoribonucleotides, based on *H*-phosphonate coupling. It occurred to us that, if a monomeric building block such as the di-triethylammonium salt of 5'-*O*-trityl-6-*N*-pivaloyladenosine-2'-(*H*-phosphonate)-3'-[(2-chlorophenyl) phosphate] **9** were available, it should be possible to extend our original work (23) and develop a completely general synthesis both of branched oligoribonucleotides and branched cyclic oligoribonucleotides ('lariats'). The synthesis of the required building block (the 'lariat' synthon) **9**, which is illustrated in outline in Scheme 1, proved to be relatively straightforward. The previously reported (24) partially-protected adenosine derivative **1** reacted slowly with 4,4'-dimethoxytrityl chloride **2a** in pyridine solution at 60°C. Treatment of the product with tetraethylammonium fluoride in acetonitrile solution gave the 2'-*O*-(4,4'-dimethoxytrityl) derivative **3**. When the latter compound was allowed to react with trityl chloride **2b** in pyridine solution, compound **4** was obtained and isolated in 92% overall yield for the three steps (i–iii). Phosphorylation of this compound with 2-chlorophenyl phosphorodichloridate **5**, 1,2,4-1*H*-triazole **6** and triethylamine (25) in pyridine solution, followed by treatment with triethylamine and water, gave its 3'-(2-chlorophenyl) phosphate ester **7**. This material was treated with dichloroacetic acid and methanol in dichloromethane solution at 0°C for 10 min. The product was dissolved in diphenyl phosphite **8** (26) at room temperature and triethylamine was added. Following hydrolysis with aqueous triethylamine, the products were worked up and chromatographed on silica gel to give the 'lariat' synthon **9**, which was isolated in 74% overall yield for the three steps (iv-vi) or in 68% overall yield for the six steps (i–vi) starting from compound **1**.

The 'lariat' synthon **9** was characterised on the basis of its ^{1}H (Materials and Methods) and ^{31}P (Fig. 1b and Materials and Methods) NMR spectra. Two distinct phosphorus resonance signals can be observed in Figure 1b: an upfield singlet (δ –5.87), which is assigned to the resonance of the phosphodiester phosphorus, and a double-doublet (δ 3.79, $J_{P,H}$ 10.1 and 629.1), which is assigned to the resonance of the *H*-phosphonate

*To whom correspondence should be addressed. Tel: +44 207 848 2260; Fax: +44 207 848 2899



Scheme 1. Reagents and conditions: (i) DMTr-Cl **2a**, 2,4,6-collidine, C_5H_5N, 60°C, 24 h; (ii) Et_4NF, MeCN, room temperature, 30 min; (iii) Tr-Cl **2b**, 2,4,6-collidine, 60°C, 15 h; (iv) (a) **5**, **6**, Et_3N, C_5H_5N, 0°C to room temperature, (b) 0.5 M aq. triethylammonium phosphate buffer (pH 7.0); (v) Cl_2CHCO_2H, MeOH, CH_2Cl_2, 0°C, 10 min; (vi) (a) diphenyl phosphite **8**, Et_3N, room temperature, 1 h, (b) Et_3N, H_2O, 0°C, 20 min.

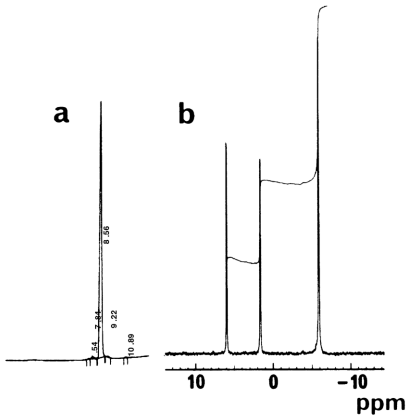
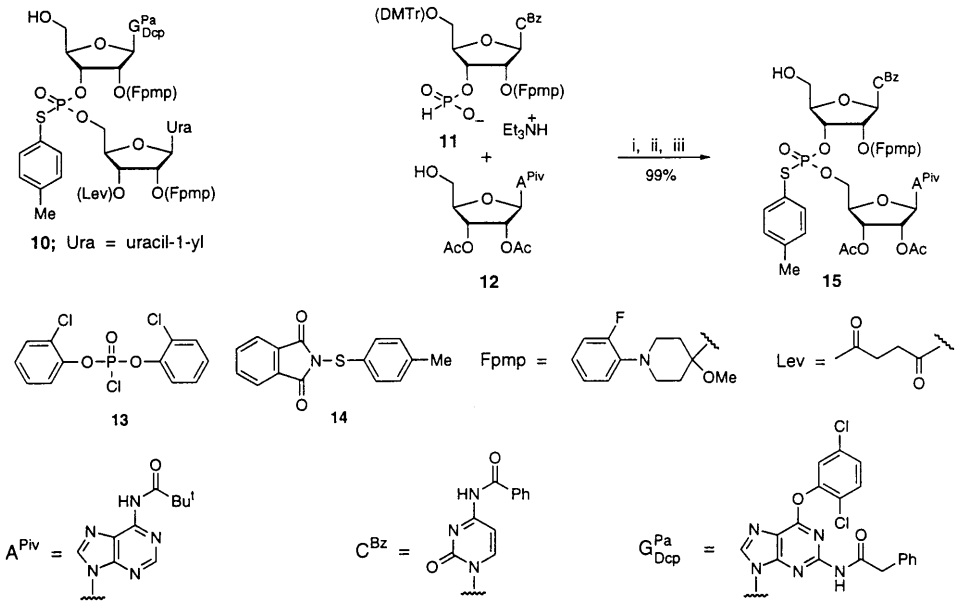


Figure 1. (**a**) Reverse phase HPLC profile (programme A) of the 'lariat' synthon **9** and (**b**) the ^{31}P NMR spectrum [$CDCl_3$] of **9**.

phosphorus. It is clear from its reverse phase HPLC profile (Fig. 1a) and from its NMR spectra (Fig. 1b and Materials and Methods) that the isolated material was of relatively high purity (~97–98% by HPLC). It is particularly noteworthy that the *H*-phosphonylation step (Scheme 1, step vi) appeared to proceed without concomitant 2',3'-cyclic phosphate formation. Experience so far suggests that the 'lariat' synthon **9** is a robust, relatively stable building block.

In order to demonstrate the utility of the 'lariat' synthon **9** as an intermediate in the synthesis both of branched oligonucleotides and 'lariats', two partially-protected dinucleoside phosphorothioates **10** and **15** (Scheme 2) were required. The preparation of one of these dimers **10** in 98% isolated yield has already been reported (23). The other dimer **15** was similarly prepared in 99% isolated yield from the *H*-phosphonate **11** (23,27) and 2',3'-di-*O*-acetyl-6-*N*-pivaloyladenosine **12**. The latter compound **12** was prepared (Materials and Methods) in three steps from 6-*N*-pivaloyladenosine and was isolated as a crystalline solid in 74% overall yield.

The procedure followed for conversion of the 'lariat' synthon **9** into the partially protected branched pentamer **18** is illustrated in Scheme 3. The strategy adopted was based on the assumption that it should be possible to carry out an *H*-phosphonate coupling reaction in the presence of a vicinal phosphodiester group. This has indeed proved to be the case. When the 'lariat' synthon **9** (1.2 mol equiv.) was allowed to react with the dimer **10** (1.0 mol equiv.) in the presence of di-2-chlorophenyl phosphorochloridate **13** (23) (3.3 mol equiv.) in pyridine/dichloromethane (9:1 v/v) at –40°C, coupling was complete within 10 min. The products were then treated, *in situ*, with 2-(4-methylphenyl)sulfanyl-1*H*-isoindole-1,3(2*H*)-dione **14** (2.0 mol equiv.) at –40°C for 15 min (23). Following work-up and chromatography, the trimer **16** was isolated as a colourless solid in 94% overall yield for the two steps (i and ii). Using the methodology of the conventional phosphotriester approach (28), a solution of this material **16** (1.3 mol equiv.) and the other dimer **15** (1.0 mol equiv.) in anhydrous pyridine solution was treated with 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-1*H*-triazole (MSNT) **17** (28) (6.0 mol equiv.) over a period of 5.5 h. The trityl protecting group was then removed by treatment with the trifluoroacetic acid and pyrrole (29) in dichloromethane

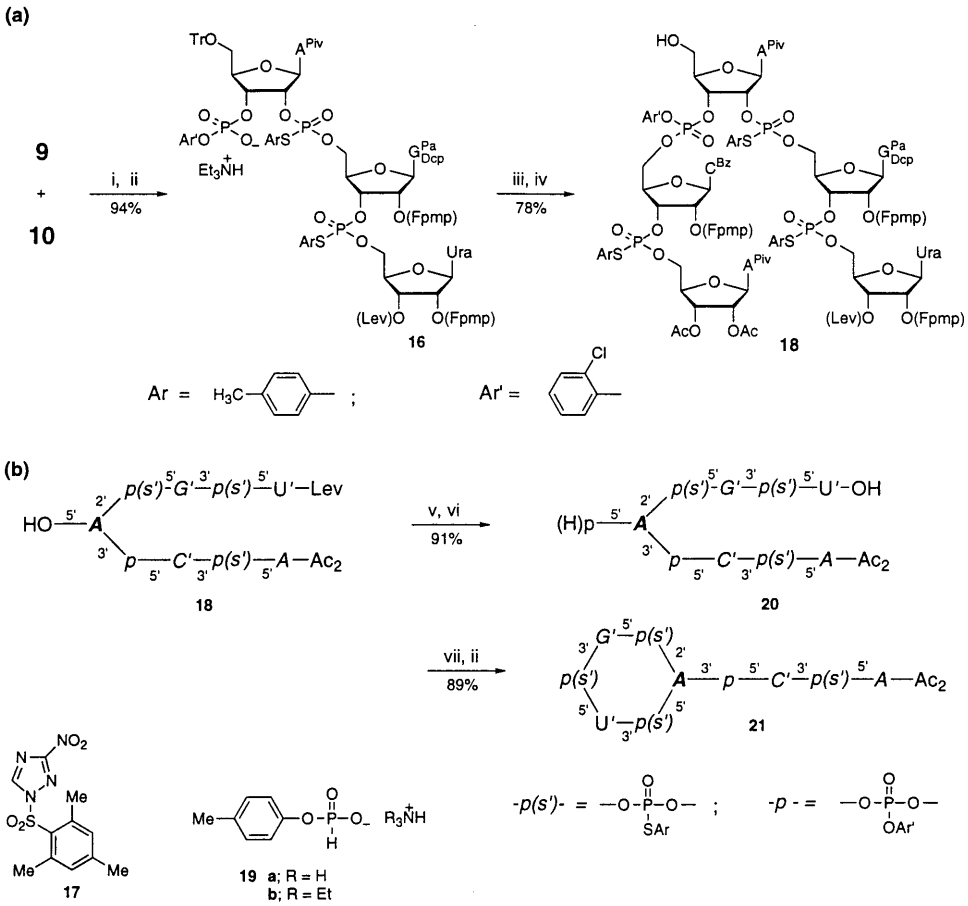


Scheme 2. Reagents and conditions: (i) **13**, CH_2Cl_2/C_5H_5N (1:9, v/v), –40°C, 10 min; (ii) **14**, CH_2Cl_2, C_5H_5N, –40°C, 15 min; (iii) CF_3CO_2H, CH_2Cl_2, room temperature, 1 min.

solution to give the partially protected branched pentamer **18** in 78% isolated yield for the two steps (iii and iv). No loss of the 2'-*O*-Fpmp protecting groups (30) was detected under these detritylation conditions. It should be emphasized that, in principle, this strategy permits the attachment of oligonucleotide chains of different lengths and different sequences to the 2'- and 3'-hydroxy functions of the branched nucleoside residue. Not unexpectedly (23), *H*-phosphonate coupling followed by the transfer of an arenesulfanyl group leads to higher yields than those obtained in conventional phosphotriester coupling reactions (31).

We have introduced (23,32) a system of abbreviations for protected and partially protected oligoribonucleotides in which ribonucleoside residues are italicized (as in *A*, *C* and *G*) if their base residues are protected and a prime is added (as in *A*', *C*', *G*' and U') if their 2'-hydroxy functions are protected in a defined way (i.e. with the Fpmp group in the present study). The base protecting groups used in this study are indicated in Scheme 2. If the internucleotide linkages are protected, they are also italicized. Thus *-p(s')-* and *-p-* (Scheme 3b) represent an *S*-(*p*-tolyl)-protected phosphorothioate (as in **10**) and a (2-chlorophenyl)-protected natural phosphodiester internucleotide linkage, respectively. For example, the dimers **10** and **15** are abbreviated to HO-*G'p(s')*U'-Lev and HO-*C'p(s')A*-Ac_2, respectively. These abbreviations can be extended to include fully- and partially-protected branched and branched cyclic oligoribonucleotides by clearly indicating the sites of attachment of the internucleotide linkages to the nucleoside residues. Thus, the partially protected branched pentaribonucleotide **18** (Scheme 3a) is abbreviated in Scheme 3b. This intermediate **18** was converted into the fully protected 'lariat' pentaribonucleotide **21** in four steps (Scheme 3b). First, it was allowed to react with large excesses each of the putative triethylammonium salt of *p*-tolyl *H*-phosphonate **19b** (27) and pivaloyl chloride in dry pyridine at –30°C. The product was then treated with hydrazine hydrate in pyridine/acetic acid solution (33) at room temperature to give the 'lariat' precursor **20** in 91% overall yield for the two steps (v and vi). Cyclization (step vii) was effected by adding a solution of **20** in dichloromethane to a large excess of di-(2-chlorophenyl) phosphorochloridate **13** in pyridine solution at –40°C, under conditions of high dilution (23). Following *in situ* treatment with 2-(4-methylphenyl)sulfanyl-1*H*-isoindole-1,3(2*H*)-dione **14** at –40°C, the fully protected 'lariat' pentamer **21** was isolated as a colourless solid in 89% overall yield for the two steps (vii and ii). It was clear from TLC [in solvent system A: dichloromethane/methanol (9:1 v/v)] that an uncharged species had been obtained. The R_f (0.40) of the product **21** was very close to that of **18** (R_f 0.41). Charged species, such as the 'lariat' precursor **20**, generally run close to the baseline (i.e. $R_f < 0.1$) in solvent system A (23). Under the present reaction conditions (Scheme 3b, steps vii and ii), an uncharged product can only be formed by cyclization. Firm evidence in favour of the assigned structure **21** comes also from its ^{31}P NMR spectrum {δ_P [$CDCl_3$] –7.41 to –4.92 (1P, phosphotriester group) and 23.51 to 30.34 (4P, *S*-(*p*-tolyl) phosphorothioate triester groups}. The phosphorus atom of an *S*-(*p*-tolyl) phosphorothioate diester, which would have been obtained if cyclization (step vii) had not occurred, would be expected to resonate at approximately δ 10 (C.B.Reese and C.Visintin, unpublished observations).

The protocols followed for the unblocking of the partially protected branched pentamer **18** and the fully-protected 'lariat' pentamer **21** are indicated in outline in Scheme 4. Following acetylation of the 5'-terminal hydroxy function (Scheme 4a, step i), the branched pentamer was unblocked by the previously described (23) three-step procedure. First, the internucleotide linkages were unblocked and the 6-*O*-(2,5-dichlorophenyl) protecting group was removed from the guanine residue by oximate treatment (34) (step ii). Secondly, all of the acyl protect-

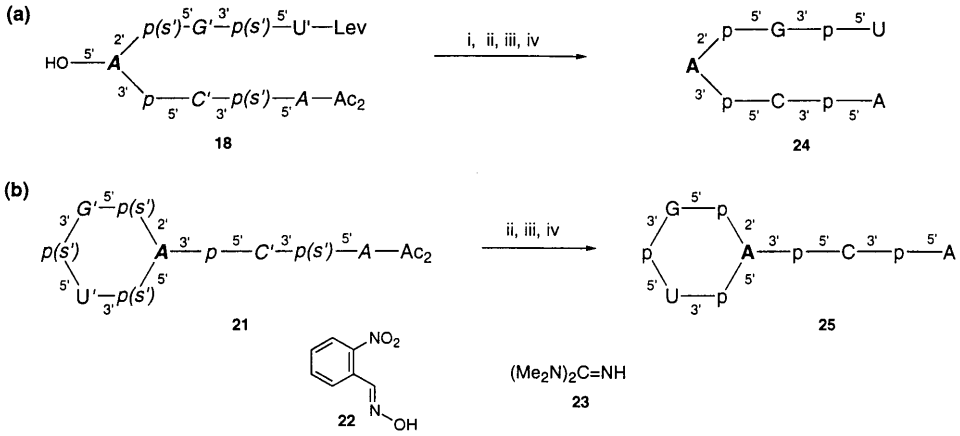


Scheme 3. Reagents and conditions: (i) **13**, CH_2Cl_2/C_5H_5N (1:9 v/v), –40°C, 10 min; (ii) **14**, CH_2Cl_2, C_5H_5N, –40°C, 15 min; (iii) **15**, **17**, C_5H_5N, room temperature, 5.5 h; (iv) CF_3CO_2H, pyrrole, CH_2Cl_2, room temperature, 3 min; (v) **19b**, $Me_3C{\cdot}COCl$, C_5H_5N, –30°C, 30 min; (vi) $N_2H_4{\cdot}H_2O$, $AcOH/C_5H_5N$ (1:4 v/v), room temperature, 10 min; (vii) **13**, CH_2Cl_2/C_5H_5N (1:5 v/v), –40°C, 20 min.

ing groups were removed by treatment with concentrated aqueous ammonia at 50°C (step iii) and, finally, the 2'-*O*-Fpmp protecting groups were removed under very mild conditions of acidic hydrolysis (step iv) (23). The fully protected 'lariat' pentamer **21** was unblocked by the same three-step procedure (Scheme 4b, steps ii–iv). In each case, 0.025 g of material was deprotected and, following fractionation of the fully unblocked products by chromatography on DEAE-Sephadex A25, 230 and 240 A_{260} units, respectively, of the triethylammonium salts of the branched pentaribonucleoside tetraphosphate **24** and the 'lariat' pentaribonucleotide **25** were obtained.

The reverse phase HPLC profiles and the ^{31}P NMR spectra of the fully unblocked oligonucleotides **24** and **25** are illustrated in Figure 2. Integration of the HPLC peaks (Fig. 2a and c) reveals that **24** and **25** are almost 99 and 96% pure, respectively. Four and five phosphodiester phosphorus resonance signals (in the region of approximately δ –1 to +1) can be seen in the ^{31}P NMR spectra of the branched pentaribonucleoside tetraphosphate **24** (Fig. 2b) and the 'lariat' pentaribonucleotide **25** (Fig. 2d), respectively. Integration of the ^1H NMR spectra of both **24** and **25** (Materials and Methods) confirms that there are seven protons in the region of δ 7.5–8.5 (five well-resolved singlets, each integrating for one proton, that may be assigned to the resonance of one guanine and four adenine protons and two doublets, each integrating for one proton, that may be assigned to the *H*-6 resonances of the uracil and cytosine residues). In the ^1H NMR spectra both of **24** and **25**, the resonance signals in the region of δ 5.5–6.25 integrate for seven protons (five anomeric protons and *H*-5 of the uracil and cytosine residues). The latter signals are less well resolved.

Further confirmation of the constitutions of the branched pentaribonucleoside tetraphosphate **24** and the 'lariat' pentaribonucleotide **25** was provided by the HPLC analysis of their enzymatic digests. Both pentamers were quantitatively converted to their constituent nucleosides by digestion first with *Crotalus adamanteus* snake venom phosphodiesterase and then with *Escherichia coli* alkaline phosphatase. The adenosine:cytidine:guanosine:uridine ratios for the digests obtained from the branched pentaribonucleoside tetraphosphate **24** and the 'lariat' pentanucleotide **25** were estimated to be 2.05:1.03:1.06:1.00 and 2.01:0.97:1.04:1.00, respectively. HPLC analysis of the products obtained by digesting the branched pentaribonucleoside tetraphosphate **24** with a mixture of ribonucleases A and T_1 (Scheme 5a) revealed three components: adenosine (A), uridine (U) and what is believed to be the branched trinucleoside tetraphosphate **26**. Digestion of the



Scheme 4. Reagents and conditions: (i) Ac_2O, C_5H_5N, room temperature, 5 h; (ii) **22**, **23**, MeCN, room temperature, 12 h; (iii) conc. aq. NH_3 (*d* 0.88), 50°C, 15 h; (iv) 0.5 M aq. NaOAc buffer (pH 4.0), 40°C, 5 h.

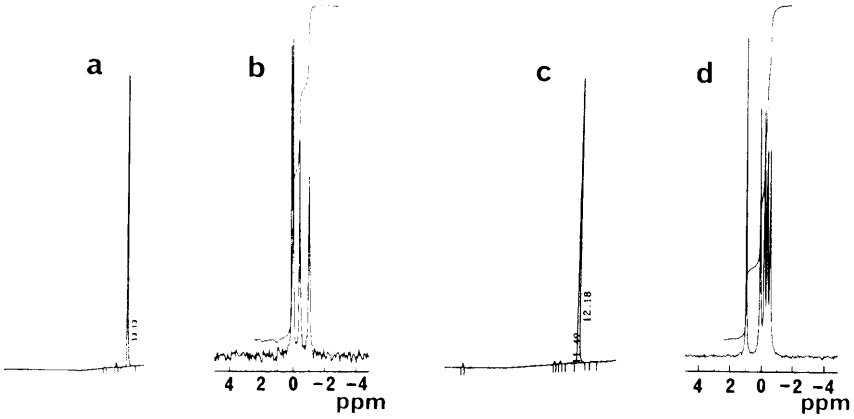


Figure 2. (**a**) Reverse phase HPLC profile (programme C) of the branched pentaribonucleoside tetraphosphate **24**; (**b**) ^{31}P NMR spectrum (D_2O) of **24**; (**c**) reverse phase HPLC profile of the 'lariat' pentanucleotide **25** and (**d**) ^{31}P NMR spectrum (D_2O) of **25**.

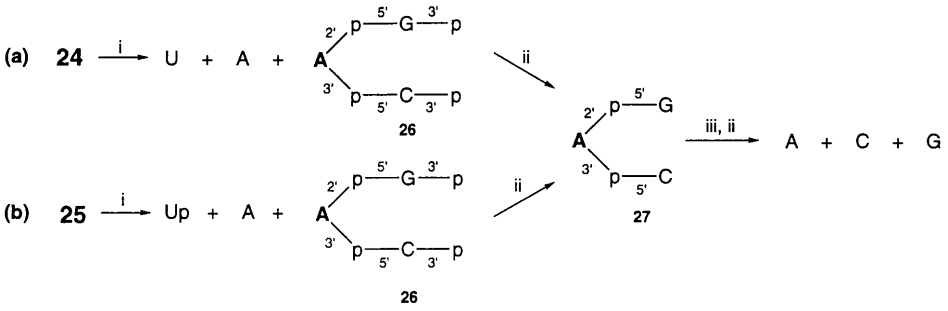
'lariat' pentanucleotide **25** with the same enzyme mixture gave (Scheme 5b) adenosine, uridine 3'-phosphate (Up) and again oligonucleotide **26**. When each of these digests was further incubated with *E.coli* alkaline phosphatase (Scheme 5), the same three products [uridine (R_t ~5.6 min), the putative trinucleoside diphosphate **27** (R_t ~11 min) and adenosine (R_t > 12 min)] were obtained in virtually identical proportions (Fig. 3). The trinucleoside diphosphate **27**, which was isolated by preparative HPLC, was completely unchanged after it had been heated in concentrated aqueous ammonia solution at 50°C for 24 h, thereby confirming the absence of a secondary (2'- or 3'-) hydroxy function vicinal to a (3'→5')- or (2'→5')-internucleotide linkage; when this material **27** was digested with snake venom phosphodiesterase followed by *E.coli* alkaline phosphatase (Scheme 5), it was quantitatively converted into a 1.03:1.00:1.01 mixture of adenosine (A), cytidine (C) and guanosine (G).

In conclusion, we believe that what we have termed the 'lariat' synthon **9** will prove to be a universal building block for the preparation of any adenosine-derived branched oligoribonucleotide or 'lariat'. In principle, the partially protected dimers **10** and **15** (Scheme 2) can be replaced by similarly protected oligonucleotides of any sequence and length. In this way, the sequences and sizes of both the circular and the stem regions of a 'lariat' can be varied. There is as yet no evidence to suggest that the yields of individual steps will decrease with increasing molecular weight. However, it is not yet known whether or not the efficiency of the cyclization step (Scheme 3, step vii) depends on ring-size. Clearly, 'lariat' synthons derived from ribonucleosides other than adenosine could also be prepared by the route indicated in Scheme 1.

MATERIALS AND METHODS

General procedures

^1H NMR spectra were measured at 360 MHz with a Bruker AM 360 spectrometer; tetramethylsilane was used as an internal standard. ^{31}P NMR spectra were measured at 145.8 MHz



Scheme 5. Reagents and conditions: (i) ribonuclease A, ribonuclease T_1, 37°C, 20 min; (ii) *E.coli* alkaline phosphatase, 37°C, 14 h; (iii) *C.adamanteus* snake venom phosphodiesterase, 37°C, 20 h.

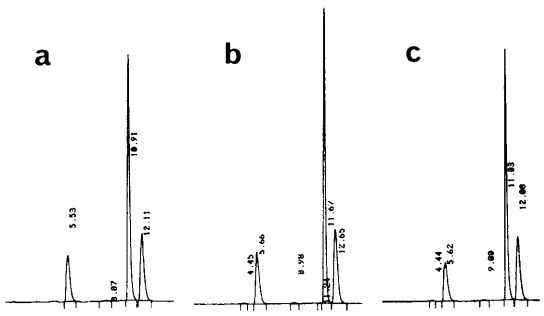


Figure 3. Reverse phase HPLC profiles (programme D) of enzymatic digests (ribonucleases A and T_1, followed by *E.coli* alkaline phosphatase; see Scheme 5) of (**a**) the branched pentaribonucleoside tetraphosphate **24**; (**b**) the 'lariat' pentaribonucleotide **25** and (**c**) **24** and **25** (prepared by mixing digests of approximately equal quantities of pure **24** and **25**). The digestion products have been identified as uridine, the branched trinucleoside diphosphate **27** and adenosine in order of increasing retention time.

with the same spectrometer; 85% orthophosphoric acid was used as an external standard. Merck silica gel 60 F_{254} precoated plates (Art 5715 and 5642), which were developed in solvent system A (CH_2Cl_2/MeOH 9:1 v/v), were used for TLC. High performance liquid chromatography (HPLC) was carried out on a 250 × 4.6 mm Hypersil ODS 5µ column, which was eluted with 0.1 M triethylammonium acetate buffer (pH 7.0)/acetonitrile mixtures [programme A: linear gradient of buffer/acetonitrile (70:30–20:80 v/v) over 10 min; programme B: linear gradient of buffer/acetonitrile (85:15–40:60 v/v) over 15 min and then isocratic; programme C: linear gradient of buffer/acetonitrile (97:3–85:15 v/v); programme D: linear gradient of buffer/acetonitrile (97:3–93:7 v/v) over 10 min and then isocratic]. Peaks were monitored and integrated at 270 nm. Merck Kieselgel H (Art 7729 and 9385) was used for short column chromatography. The general procedure followed for the chromatography of fully- or partially-protected oligoribonucleotide phosphorothioates and oligoribonucleotide/oligoribonucleotide phosphorothioates was as previously described (23). Ion-exchange chromatography was carried out on a column of DEAE-Sephadex A25, which was eluted with triethylammonium bicarbonate buffer (pH 7.5, linear gradient from 0.01 to 1.00 M over 1000 ml). Triethylamine, pyridine and 4-methylmorpholine were dried by heating with calcium hydride, under reflux, and were then distilled; N^1, N^1, N^3, N^3-tetramethylguanidine (TMG) and 2,4,6-collidine were dried by distillation over calcium hydride under reduced pressure; dichloromethane was dried over phosphorus pentaoxide and was then distilled. All solvents were stored over 4 Å molecular sieves in sealed containers. Protected {i.e. *N*-acyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl]- and *N*-acyl-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxan-1,3-diyl)-} ribonucleoside derivatives were supplied by Cruachem Ltd (Glasgow). Phosphorolytic enzymes were purchased from Sigma-Aldrich Co. Ltd. Stock solutions of enzymes were prepared as previously reported (24).

2'-*O*-(4,4'-Dimethoxytrityl)-5'-*O*-trityl-6-*N*-pivaloyladenosine 4

A stirred solution of 3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxan-1,3-diyl)-6-*N*-pivaloyladenosine **1** (24) (2.97 g, 5.0 mmol), 4,4'-dimethoxytrityl chloride (3.39 g, 10.0 mmol) and 2,4,6-collidine (5.29 ml, 40 mmol) in dry pyridine (25 ml) was heated at 60°C. After 24 h, the products were poured into saturated aqueous sodium hydrogen carbonate (100 ml), and the resulting mixture was extracted with dichloromethane (3 × 50 ml). The combined organic extracts were dried ($MgSO_4$) and evaporated under reduced pressure. A solution of tetraethylammonium fluoride in acetonitrile (1 M, 35 ml) was added to the residue and the resulting solution was stirred at room temperature. After 30 min, the products were concentrated under reduced pressure and the residue was partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). The layers were separated and the aqueous layer was extracted with dichloromethane (2 × 50 ml). The combined organic layers were concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: the appropriate fractions which were eluted with dichloromethane/methanol (99:1–98:2 v/v), were evaporated to give 2'-*O*-(4,4'-dimethoxytrityl)-6-*N*-pivaloyladenosine **3** as a colourless solid (3.15 g); R_f 0.65 (system A); δ_H [$(CD_3)_2SO$] 1.30 (9 H, s), 3.38 (1 H, m), 3.53 (2 H, m), 3.63 (3 H, s), 3.68 (3 H, s), 3.98 (1 H, m), 4.87 (1 H, dd, *J* 5.1 and 6.3), 5.10 (1 H, d, *J* 5.2), 5.13 (1 H, m), 6.06 (1 H, d, *J* 6.6), 6.57 (2 H, d, *J* 8.9), 6.69 (2 H, d, *J* 8.9), 7.04 (2 H, d, *J* 8.8), 7.08–7.21 (5 H, m), 7.36 (2 H, d, *J* 6.8), 8.41 (1 H, s), 8.56 (1 H, s) and 10.18 (1 H, br s).

A stirred solution of the latter material **3** (2.94 g), trityl chloride (1.88 g, 6.7 mmol) and 2,4,6-collidine (4.76 ml, 36 mmol) in anhydrous pyridine (25 ml) was heated at 60°C. After 15 h, the products were poured into saturated aqueous sodium hydrogen carbonate (100 ml) and the resulting mixture was extracted with dichloromethane (3 × 50 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: the appropriate fractions, which were eluted with ethyl acetate/petroleum ether (b.p. 40–60°C) (30:70–60:40 v/v) were evaporated under reduced pressure to give the title compound **4** as a pale yellow solid (3.85 g, 92% overall yield based on **1**); R_f 0.80 (system A); δ_H [$(CD_3)_2SO$] 1.30 (9 H, s), 3.95 (1 H, m), 3.16 (1 H, m), 3.32 (1 H, m), 3.62 (3 H, s), 3.65 (3 H, s), 4.05 (1 H, m), 5.02 (1 H, d, *J* 5.4), 5.08 (1 H, m), 6.00 (1 H, d, *J* 6.1), 6.56 (2 H, d, *J* 8.9), 6.64 (2 H, d, *J* 8.9), 7.05 (2 H, d, *J* 8.8), 7.11–7.37 (22 H, m), 8.37 (1 H, s), 8.40 (1 H, s) and 10.20 (1 H, br s).

Di-triethylammonium salt of 5'-*O*-trityl-6-*N*-pivaloyladenosine-2'-(*H*-phosphonate)-3'-[(2-chlorophenyl) phosphate] 9

2-Chlorophenyl phosphorodichloridate **5** (2.63 ml, 16.0 mmol) was added dropwise over 1 min to a stirred solution of 2'-*O*-(4,4'-dimethoxytrityl)-5'-*O*-trityl-6-*N*-pivaloyladenosine **4** (3.58 g, 4.0 mmol), 1,2,4-triazole **6** (2.21 g, 32 mmol) and triethylamine (4.46 ml, 32 mmol) in dry pyridine (40 ml) at 0°C (ice-water bath). The reactants were allowed to warm to room temperature. After 1 h, the products were poured into a mixture of 0.5 M triethylammonium phosphate buffer (pH 7.0, 100 ml) and dichloromethane (100 ml). After shaking, the organic layer was separated, washed with the same triethylammonium phosphate buffer (2 × 50 ml), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: the appropriate fractions, which were eluted with dichloromethane/methanol/triethylamine (97:3:0.1–95:5:0.1 v/v), were evaporated under reduced pressure to give a colourless solid (4.62 g); δ_P [$(CD_3)_2SO$] –5.46.

The latter material (2.38 g) was dissolved in a stirred solution of dichloroacetic acid (2.0 ml, 24 mmol) in methanol (3 ml) and dichloromethane (95 ml) at 0°C (ice-water bath). After 10 min, the products were poured into 1.0 M triethylammonium phosphate buffer (pH 4.0, 100 ml). The organic layer was separated, washed with the same buffer (2 × 50 ml), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was dissolved in dichloromethane (10 ml), and petroleum ether (b.p. 30–40°C, 100 ml) was added. The resulting precipitate, which was collected by filtration, was added to diphenyl phosphite **8** (20 ml). Triethylamine (1.39 ml, 10 mmol) was then added and the reaction solution was stirred at room temperature. After 1 h, the products were cooled to 0°C (ice-water bath), and triethylamine (15 ml) and water (5 ml) were added. After a further period of 20 min, the products were partitioned between 0.5 M triethylammonium phosphate buffer (pH 7.0, 100 ml) and dichloromethane (100 ml). The organic layer was separated, washed with the same buffer (3 × 20 ml), dried ($MgSO_4$) and then concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: the appropriate fractions, which were eluted with dichloromethane/methanol/triethylamine (95:5:0.1–85:15:0.1 v/v) were evaporated under reduced pressure to give the title compound **9** as a colourless solid (1.60 g, 74% overall yield, based on compound **4**); R_t 8.56 min (programme A); δ_H [$CDCl_3$] 1.22 (18 H, t, *J* 7.3), 1.40 (9 H, s), 3.00 (12 H, quart, *J* 7.3), 3.40 (2 H, m), 4.62 (1 H, m), 5.18 (1 H, m), 5.51 (1 H, m), 5.91 (0.5 H, s), 6.41 (1 H, d, *J* 6.8), 6.90 (1 H, m), 7.08 (1 H, m), 7.22 (10 H, m), 7.39 (6 H, m), 7.66 (1.5 H, m), 8.24 (1 H, s), 8.57 (1 H, s), 8.65 (1 H, s) and 11.99 (2 H, br); δ_p [$CDCl_3$] –5.87 (1 P, s), and 3.79 (1 P, dd, *J* 10.1 and 629.1).

2',3'-Di-*O*-acetyl-6-*N*-pivaloyladenosine 12

A solution of 6-*N*-pivaloyladenosine (24) (3.51 g, 10.0 mmol) in dry pyridine (20 ml) was evaporated to dryness under reduced pressure. The residue was redissolved in dry pyridine (30 ml), and chlorotriphenylmethane (4.18 g, 15.0 mmol) was added. The resulting solution was heated with stirring at 60°C. After 24 h, dichloromethane (100 ml) was added and the resulting solution was extracted with saturated aqueous sodium hydrogen carbonate (3 × 50 ml). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was evaporated with dry pyridine (20 ml) and then redissolved in dry pyridine (30 ml). Acetic anhydride (4.72 ml, 50 mmol) was added to the stirred solution at room temperature. After 5 h, the products were poured into saturated aqueous sodium hydrogen carbonate (100 ml), and the resulting mixture was extracted with dichloromethane (2 × 100 ml). The combined organic extracts were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was evaporated with toluene (3 × 20 ml) and then dissolved in dichloromethane (100 ml). Trifluoroacetic acid (3.85 ml, 50 mmol) and freshly distilled pyrrole (6.94 ml, 0.10 mol) were added to the stirred solution at room temperature. After 5 min, the products were poured into saturated aqueous sodium hydrogen carbonate (100 ml). The layers were separated and the aqueous layer was extracted with dichloromethane (2 × 50 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: the appropriate fractions, which were eluted with dichloromethane/methanol (98:2–96:4 v/v), were evaporated under reduced pressure to give the title compound **12** as a colourless solid (3.25 g, 74%) (Found, in material recrystallized from ethyl acetate: C, 52.35; H, 5.62; N, 15.92. $C_{19}H_{25}N_5O_7$ requires: C, 52.41; H, 5.79; N, 16.08%), m.p. 161–163°C; R_f 0.51 (system A); δ_H [$(CD_3)_2SO$] 1.29 (9 H, s), 2.00 (3 H, s), 2.16 (3 H, s), 3.67 (1 H, m), 3.77 (1 H, m), 4.27 (1 H, m), 5.46 (1 H, t, *J* 5.6), 5.56 (1 H, dd, *J* 3.0 and 5.4), 5.99 (1 H, m), 6.34 (1 H, d, *J* 6.6), 8.72 (1 H, s), 8.74 (1 H, s) and 10.27 (1 H, br s).

Preparation of HO-*C'p*(*s'*)*A*-Ac_2 15

The triethylammonium salt of 5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl]-4-*N*-benzoylcytidine-3'-*H*-phosphonate [DMTr-*C'*p(H)] **11** (23) (1.228 g, 1.20 mmol) and 2',3'-di-*O*-acetyl-6-*N*-pivaloyladenosine **12** (0.435 g, 1.0 mmol) were co-evaporated with dry pyridine (2 × 10 ml) and the residue was then redissolved in dry pyridine (9 ml). The stirred solution was cooled to –40°C [industrial methylated spirits (IMS)—dry ice bath], and a solution of di-(2-chlorophenyl) phosphorochloridate **13** (23) (1.01 g, 3.0 mmol) in dry dichloromethane (1 ml) was added dropwise over 5 min. After a further period of 5 min, 2-(4-methylphenyl)sulphanyl-1*H*-isoindole-1,3(2*H*)-dione **14** (23) (0.539 g, 2.0 mmol) was added and the reactants were stirred at –40°C

for 15 min. Then pyridine/water (1:1 v/v, 1 ml) was added with continuous stirring. After 5 min, the products were evaporated under reduced pressure. The residue was dissolved in dichloromethane (50 ml) and the solution was washed with saturated aqueous sodium hydrogen carbonate (3 × 25 ml). The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. After it had been co-evaporated with dry toluene (2 × 20 ml), the residue was dissolved in dichloromethane (40 ml) at room temperature and trifluoroacetic acid (0.77 ml, 10 mmol) was added. After 1 min, 4-methylmorpholine (1.65 ml, 15 mmol) was added, and the products were poured into saturated aqueous sodium hydrogen carbonate (50 ml). The layers were separated, and the aqueous layer was extracted with dichloromethane (2 × 50 ml). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by short column chromatography on silica gel, following the previously recommended procedure (23): the appropriate fractions, which were eluted with ethyl acetate/acetone (100:0–70:30 v/v), were combined and evaporated under reduced pressure to give HO-*C'p*(*s'*)*A*-Ac_2 **15** as a colourless solid (1.15 g, 99%); R_f 0.50 (system A); δ_P [$CDCl_3$] 26.10, 26.33.

Preparation of partially protected pentaribonucleoside triphosphorothioate triester mono-phosphotriester 18

Di-triethylammonium 5'-*O*-trityl-6-*N*-pivaloyladenosine-2'-(*H*-phosphonate)-3'-[(2-chlorophenyl) phosphate] **9** (1.135 g, 1.08 mmol) and HO-*Gp*(*s'*)U'-Lev **10** (23) (1.326 g, 0.90 mmol) were co-evaporated with dry pyridine (2 × 10 ml), and the residue was then redissolved in dry pyridine (9 ml). The stirred solution was cooled to –40°C (IMS—dry ice bath), and a solution of di-(2-chlorophenyl) phosphorochloridate **13** (1.01 g, 3.0 mmol) in dry dichloromethane (1 ml) was added dropwise over 5 min. After a further period of 5 min, 2-(4-methylphenyl)sulphanyl-1*H*-isoindole-1,3(2*H*)-dione **14** (0.485 g, 1.8 mmol) was added and the reactants were stirred at –40°C for 15 min. Then pyridine/water (1:1 v/v, 1 ml) was added with continuing stirring. After 5 min, the products were evaporated under reduced pressure. The residue was dissolved in dichloromethane (50 ml) and the solution was washed with saturated aqueous sodium hydrogen carbonate (3 × 25 ml). The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by short column chromatography on silica gel: the appropriate fractions, which were eluted with dichloromethane/methanol (97:3–93:7 v/v) were evaporated under reduced pressure to give the intermediate **16** as a colourless solid (2.150 g, 94%); δ_P [$CDCl_3$] –6.66 to –6.01 (1 P), 24.19 to 26.99 (2 P).

The latter intermediate **16** (1.642 g, 0.65 mmol) and HO-*C'p*(*s'*)*A*-Ac_2 **15** (0.580 g, 0.50 mmol) were co-evaporated with dry pyridine (2 × 5 ml), and then dissolved in dry pyridine (5 ml). 1-(Mesitylene-2-sulphonyl)-3-nitro-1,2,4-1*H*-triazole **17** (28) (0.881 g, 3.0 mmol) was added in six equal portions at intervals of 30 min to the stirred solution at room temperature. After a further period of 3 h, the products were poured into saturated aqueous sodium hydrogen carbonate (50 ml), and the resulting mixture was extracted with dichloromethane (2 × 50 ml). The combined organic extracts were dried ($MgSO_4$), evaporated and then co-evaporated with toluene (3 × 10 ml). The residue was dissolved in dichloromethane (20 ml) and pyrrole (0.69 ml, 10.0 mmol) and then trifluoroacetic acid (0.39 ml, 5.0 mmol) were added to the stirred solution at room temperature. After 3 min, 4-methylmorpholine (0.83 ml, 7.5 mmol) was added, and the reaction solution was poured into saturated aqueous sodium hydrogen carbonate (50 ml). The organic layer was separated, and the aqueous layer was extracted with dichloromethane (2 × 25 ml). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: the appropriate fractions, which were eluted with ethyl acetate/acetone (100:0–70:30 v/v), were evaporated under reduced pressure to give the title product **18** as a colourless solid (1.301 g, 78%); R_f 0.41 (system A); R_t 12.84 min (programme B); δ_P [$CDCl_3$] –7.70 to –5.66 (1 P), 24.46 to 27.88 (3 P).

Preparation of fully-protected branched cyclic pentaribonucleoside tetraphosphorothioate triester mono-phosphotriester (fully-protected 'lariat') 21

A solution of ammonium *p*-tolyl *H*-phosphonate **19a** (27) (0.302 g, 1.6 mmol) and triethylamine (0.45 ml, 3.2 mmol) were dissolved in methanol (5 ml). The solution was concentrated under reduced pressure and co-evaporated with dry pyridine (5 ml). The partially protected branched pentaribonucleoside triphosphorothioate triester mono-phosphotriester **18** (0.665 g, 0.20 mmol) was added. The mixture was dissolved in dry pyridine (5 ml) and the solution was evaporated under reduced pressure. The residue was redissolved in dry pyridine (5 ml) and the solution was cooled to –30°C (IMS—dry ice bath). Pivaloyl chloride (0.25 ml, 2.0 mmol) was added dropwise over a period of 1 min to the stirred solution. The reactants were maintained at –30°C for a further period of 30 min. Water (1 ml) was then added, and the stirred products were allowed to warm to room temperature. After 1 h, the products were partitioned between dichloromethane (50 ml) and water (50 ml). The organic layer was separated, washed with 0.5 M triethylammonium phosphate buffer (pH 7.0, 3 × 25 ml), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was dissolved in a solution of hydrazine monohydrate (0.50 ml, 10 mmol) in pyridine (16 ml) and acetic acid (4 ml) at room temperature. The reaction solution was stirred for 10 min, and the products were then partitioned between dichloromethane (50 ml) and water (50 ml). The organic layer was separated, washed with water (2 × 25 ml) and then with 0.5 M triethylammonium phosphate buffer (pH 7.0, 25 ml), dried ($MgSO_4$) and applied to a short column of silica gel. Appropriate fractions, eluted from the column with dichloromethane/methanol (95:5–90:10 v/v) were evaporated under reduced pressure to give the intermediate 5'-*H*-phosphonate **20** as a colourless solid (0.618 g, 91%); δ_P [$CDCl_3$] –7.17 to –6.47 (1 P), 4.63 (d, *J* 626.1) and 4.42 (d, *J* 624.3) (1P combined), and 25.24–26.49 (3 P).

A solution of the 5'-*H*-phosphonate intermediate **20** (0.339 g, 0.10 mmol) in dichloromethane (4 ml) was added dropwise over a period of 15 min to a stirred solution of di-(2-chlorophenyl) phosphorochloridate **13** (0.68 g, 2.0 mmol) in dry pyridine (20 ml) at –40°C (IMS—dry ice bath). After a further period of 5 min, 2-(4-methylphenyl)sulphanyl-1*H*-isoindole-1,3(2*H*)-dione **14** (0.135 g, 0.50 mmol) was added while the stirred reaction solution was maintained at –40°C. After 15 min, pyridine/water (1:1 v/v) was added, and the cooled products were stirred for a further period of 5 min. The

products were then concentrated under reduced pressure, and redissolved in dichloromethane (50 ml). The resulting mixture was washed with saturated aqueous sodium hydrogen carbonate (3 × 25 ml), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: the appropriate fractions, which were eluted with dichloromethane/methanol (98:2–96:4 v/v), were evaporated under reduced pressure to give the fully-protected 'lariat' **21** as a colourless solid (0.302 g, 89%); R_f 0.40 (system A); R_t 11.40 min (programme B); δ_P [$CDCl_3$] –7.41 to –4.92 (1 P), 23.51 to 30.34 (4P).

Complete unblocking of protected oligonucleotides

(a) A solution of the partially-protected pentaribonucleoside triphosphorothioate triester monophosphotriester **18** (0.025 g, 7.5 μmol) in dry pyridine (2 ml) was evaporated under reduced pressure. Acetic anhydride (0.028 ml, 0.30 mmol) was added to a stirred solution of the residue in dry pyridine (1 ml) at room temperature. After 5 h, the products were poured into saturated aqueous sodium hydrogen carbonate (10 ml), and the resulting mixture was extracted with dichloromethane (3 × 10 ml). The combined organic extracts were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was dissolved in a stirred solution of *E*-2-nitrobenzaldoxime **22** (34) (0.083 g, 0.50 mmol) and TMG **23** (0.056 ml, 0.45 mmol) in acetonitrile (0.5 ml) at room temperature. After 12 h, the products were evaporated under reduced pressure, and concentrated aqueous ammonia (*d* 0.88, 5 ml) was added. The resulting mixture was heated at 50°C for 15 h, and was then evaporated under reduced pressure. The residue was dissolved in methanol (2 ml) and ethyl acetate (4 ml) was added. The precipitate was collected by centrifugation, washed with ethyl acetate (2 × 20 ml) and dried to give an off-white solid (0.017 g). This material was dissolved in 0.5 M sodium acetate buffer (pH 4.0, 5 ml). The resulting solution was maintained at 40°C for 5 h, cooled to room temperature and extracted with dichloromethane (3 × 5 ml). 1.0 M Triethylammonium bicarbonate buffer (pH 7.5, 2.5 ml) was added and the solution was applied to a column (20 cm × 2 cm diameter) of DEAE-Sephadex A 25, which was eluted with triethylammonium bicarbonate buffer (pH 7.5, 0.01–1.00 M over 1000 ml). Appropriate fractions (eluted with ~0.8 M buffer) were combined and evaporated under reduced pressure to give the triethylammonium salt of the branched pentaribonucleoside tetraphosphate **24** as a colourless solid (230 A_{260} units); R_t 13.13 min (programme C); δ_H [D_2O] includes the following signals: 5.50 (1 H, d, *J* 4.2), 5.63 (1 H, d, *J* 8.2), 5.83 (1 H, d, *J* 4.4), 5.88–5.94 (3 H, m), 5.99 (1 H, m), 7.60 (2 H, m), 7.74 (1 H, d, *J* 8.3), 7.78 (1 H, d, *J* 7.7), 7.96 (1 H, s), 8.06 (1 H, s), 8.30 (1 H, s); δ_P [D_2O] –1.06, –0.46, –0.04 and 0.04.

(b) The fully protected 'lariat' **21** (0.025 g, 7.4 μmol) was unblocked following the procedure described in (a) above except that the initial acetylation step was omitted. The same quantities of reagents and solvents were used and, following the ammonolysis step, an off-white solid precipitate (0.016 g) was obtained. After treatment with 0.5 M sodium acetate buffer (pH 4.0) at 40°C for 5 h and purification of the products on DEAE-Sephadex A 25, the triethylammonium salt of the fully-deprotected 'lariat' pentaribonucleotide **25** (240 A_{260} units) was obtained; R_t 12.18 min (programme C); δ_H [D_2O] includes the following signals: 5.73–5.81 (3 H, m), 5.89 (1 H, d, *J* 7.0), 6.02 (1 H, d, *J* 5.7), 6.10 (1 H, d, *J* 7.5), 6.17 (1 H, d, *J* 8.2), 7.72 (1 H, d, *J* 7.5), 7.75 (1 H, s), 7.91 (1 H, d, *J* 8.2), 7.98 (1 H, s), 8.01 (1 H, s), 8.35 (1 H, s) and 8.36 (1 H, s); δ_P [D_2O] –0.55, –0.36, –0.18, 0.09 and 0.98.

Enzymatic digestion of the branched pentaribonucleoside tetraphosphate 24 and the fully-deprotected 'lariat' pentaribonucleotide 25

(a) *Crotalus adamanteus* snake venom phosphodieterase stock solution (24) (30 μl) was added to a solution of substrate (~2 A_{260} units) in sterile water (20 μl), and the resulting solution was incubated at 37°C for 20 h. *Escherichia coli* alkaline phosphatase stock solution (24) (30 μl) was then added and, after 14 h, the products were analysed by HPLC (programme C). Both substrates **24** and **25** were fully digested to their constituent nucleosides. The adenosine (R_t 12.3 min): cytidine (R_t 4.4 min): guanosine (R_t 8.7 min): uridine (R_t 5.5 min) ratios were found to be 2.05:1.03:1.06:1.00 and 2.01:0.97:1.04:1.00 for **24** and **25**, respectively.

(b) Ribonuclease A stock solution (24) (70 μl) and ribonuclease T_1 stock solution (24) (70 μl) were added to a solution of each substrate **24** and **25** (~5 A_{260} units) in sterile water (50 μl), and the resulting solutions were incubated at 37°C for 20 min. HPLC analysis (programme D) of the branched pentaribonucleoside tetraphosphate **24** digest revealed three components with R_ts 5.51 min (identified as uridine), 11.95 min (believed to be the trimer **26**) and 12.19 min (identified as adenosine); HPLC analysis of the digested 'lariat' pentaribonucleotide **25** revealed three components with R_ts 8.72 min (identified as uridine 3'-phosphate), 11.72 min (believed to be the trimer **26**) and 12.29 min (identified as adenosine). When both digests were further incubated with *E.coli* alkaline phosphatase stock solution (70 μl) at 37°C for 14 h, the same three components [R_ts ~5.6 min (identified as uridine), ~11.0 min (the putative trinucleoside diphosphate **27**) and >12 min (identified as adenosine)] were obtained in the same proportions (Fig. 3). When the major component **27** (R_t ~11.0 min; ~1.0 A_{260} unit), which had been purified by preparative HPLC, was heated in concentrated aqueous ammonia solution (*d* 0.88, 0.2 ml) at 50°C for 24 h, it remained completely unchanged. This material **27** (~2 A_{260} units in 20 μl of sterile water) was digested first with *C.adamanteus* snake venom phosphodiesterase (30 μl of stock solution) and then with *E.coli* alkaline phosphatase (30 μl of stock solution), according to the procedure described in (a) above. HPLC analysis revealed that the material had undergone total digestion to give adenosine (1.03 parts), cytidine (1.00 part) and guanosine (1.01 parts).

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