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A novel solid support for synthesis of 2',3'-cyclic phosphate terminated oligonucleotides

Joseph S. Vyle*[1], Nicholas H. Williams, Jane A. Grasby

Department of Chemistry, Krebs Institute, University of Sheffield, Sheffield S3 7HF, UK.

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Abstract

Michaelis-Arbusov chemistry was used to prepare *O,S*-dialkyl 3'-*O*-nucleosidyl phosphorothiolate triesters in solution and attached to CPG. The support-bound nucleoside was utilised in the synthesis of a pentaribonucleotide that was fully deprotected on the support. Subsequent treatment with a buffered solution of iodine cleaved the RNA from the CPG with concomitant formation of a terminal 2',3'-cyclic phosphate.

Keywords: Solid-phase synthesis; Nucleic Acids; Cyclisation; Arbuzov reactions

2',3'-Cyclic phosphate terminated RNAs have been characterised as products from a wide variety of biological processes including small ribozyme autolysis[1], pre-tRNA cleavage[2], small nuclear RNA 3'-uridylation[3] and as initial products in ribonuclease mediated RNA cleavage[4]. Synthetic 2',3'-cyclic phosphates were first utilised in 1952 to help identify the products from incomplete RNase digestion of RNA[5] and have since facilitated mechanistic studies on ribozyme ligation[6] and both chemical[7] and enzymatic[8] cleavage of RNA. Despite the utility of these moieties, all preparative methods reported to-date have, to the authors' knowledge, been based upon solution-phase reactions such as ribozyme cleavage[6, 9], phosphitylation[10] and phosphate coupling[11]. The ability to prepare cyclic phosphate terminated oligonucleotides on solid support would significantly increase the diversity of readily accessible sequences with applications both to the study of biological processes involving these moieties and also in generating nucleic acid libraries with ligating activity. Herein, we describe protocols for the solid-phase synthesis and isolation of a pentanucleotide containing a terminal 2',3'-cyclic phosphate. These protocols should be generally applicable to the synthesis of oligonucleotides containing terminal cyclic phosphates.

Liu and Reese[12] have demonstrated that neutral and mild alkaline transesterification of a 5'-*S*-phosphorothiolate diester (uridylyl-(3'→5')-(5'-thiouridine); UpsU) gives almost exclusively the corresponding 2',3'-cyclic phosphate, U>p, under conditions in which the natural congener, UpU, remains intact. This is consistent with other reports[13,14] of the lability of this linkage although in all cases extended reaction times were required for complete reaction in the absence of catalytic metal ions. More rapid P-S bond cleavage in 3'-*S*-phosphorothiolates has been effected using thiophilic agents such as I_2 and Ag^+ again without apparent damage to phosphodiester linkages[15]. The viability of using iodine catalysed

[1] Corresponding author; e-mail address - j.s.vyle@sheffield.ac.uk

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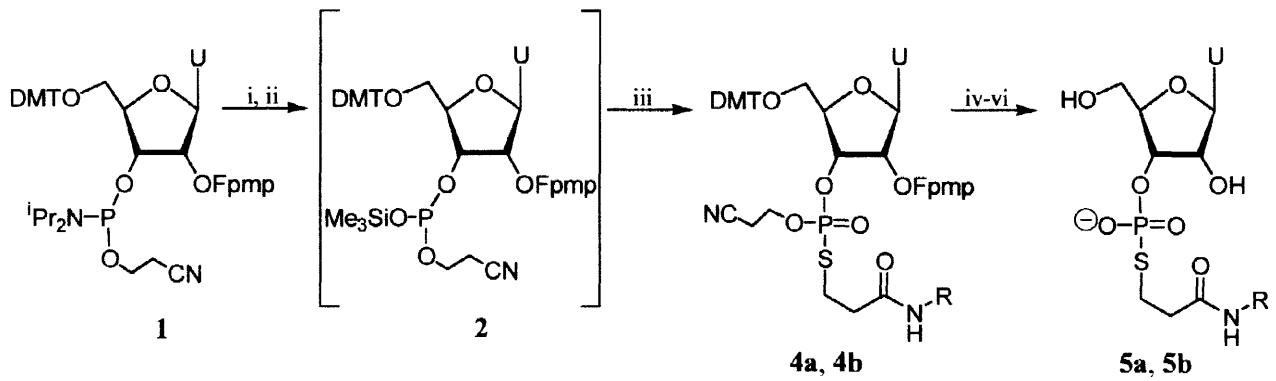
intramolecular transesterification of an *S*-alkyl phosphorothiolate vicinal to a 2'-hydroxyl as a method for the solid-phase preparation of 2',3'-cyclic phosphates was therefore investigated.

Michaelis-Arbusov chemistry has previously been employed in the synthesis of internucleoside phosphorothiolates[12,16-18] and initial solution-phase studies were performed in order to determine the potential of this strategy to the preparation of such linkages from the commercially available phosphoramidite **1** (Scheme).



Scheme. Reagents and conditions: i) 4 eq. 1-*H*-tetrazole, 10 eq. H_2O, MeCN, r.t., 15 min; ii) 8 eq. *N,O*-bis(trimethylsilyl)acetamide, $CDCl_3$, r.t., 2 h.; iii) *in situ* **6a** (r.t., 2.5 h) or **6b** (r.t., 15.5 h); iv) 5 eq. Cl_3CCO_2H, 15 eq. pyrrole, DCM, r.t., 10 min; v) methanolic ammonia (saturated at -5°C), r.t., 5 h; vi) 97:3 H_2O:AcOH, 22°C, 4.25 h. Key: **4a**, **5a** R = Bu; **4b**, **5b** R = CPG- (long chain alkylamine controlled pore glass); DMT 4,4'-dimethoxytrityl; Fpmp *N*-(2-fluorophenyl)-4-methoxypiperidinyl.

Thus, **1** was hydrolysed to the corresponding H-phosphonate[2] and silylated under neutral conditions to give the activated phosphite triester **2**.[3] *In situ* addition of **2** to the symmetrical disulfide 3,3'-dithiopropionic acid di(*N*-succinimidyl ester) (Lomant's reagent) gave only *ca.* 65% reaction over 2 days (by ^{31}P nmr) and predominantly led to sulfurisation. Previously, such non-Arbusov products have been suppressed by employing more highly activated leaving groups in the disulfide[18] and this was also found using the *S*-alkyl *S*-5-nitropyridyl disulfide (**6a**[4] - Figure 1); complete reaction with **2** was observed within 2.5 hours, only minimal non-Arbusov products were detected and the phosphorothiolate (**4a**) was isolated in 77% yield.[5]

As anticipated from previous studies[12,13], the integrity of the P-S bond in **4a** remained intact during its complete deprotection under conditions appropriate for short 2'-*O*-Fpmp protected oligonucleotides.[6] Iodine mediated transesterification of the resultant *S*-alkyl phosphorothiolate

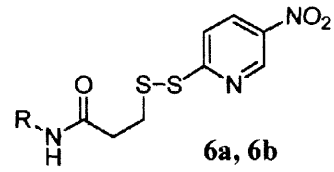


Figure 1. **6a**; R=Bu. **6b**; R=CPG-

[2] Quantitative by tlc; isolated from Na_2CO_3/EtOAc extraction: ^{31}P NMR δ ($CDCl_3$), 7.97, 6.54; FAB+ ms e/z 871 $(M+H)^+$.

[3] ^{31}P NMR δ (10/3 $CDCl_3$/*N,O*-bis(trimethylsilyl)acetamide), 129.52, 128.70

[4] Prepared as follows: Lamont's reagent (0.28 mmol), DCM (2 mL), $BuNH_2$ (4 eq.), rt., 40 min, product used unpurified; DTT (2 eq.), Et_3N (0.5 eq.) MeOH (2 mL), r.t., 105 min, scrubber column (Al_2O_3); DTNP (2,2'-dithiobis(5-nitropyridine) - 1.05 eq.), 4/5 MeOH/DCM (Ar purged - 4.5mL), r.t., 50 min addition of DTNP plus 10 min, isolated by silica chromatography; 43% over 3 steps; **3a**: ^1H NMR δ ($CDCl_3$) 9.26 (dd, PyH6), 8.41 (dd, PyH4), 7.89 (dd, PyH3), 5.70 (s, NH), 3.27 (m, C<u>H</u>$_2$-N), 3.12 (t, C<u>H</u>$_2$-S), 2.58 (t, C<u>H</u>$_2$-CO), 1.48 (m, C<u>H</u>$_2$-CH$_2$N), 1.34 (m, C<u>H</u>$_2$-CH$_3$), 0.92 (t, C<u>H</u>$_3$); EI+ ms e/z 315 (M^+), 160 (100%, $(M\text{-}S5NO_2Py)^+$).

[5] Following silica gel chromatography. **4a**: ^{31}P NMR δ ($CDCl_3$), 31.58, 30.13; FAB+ ms e/z 1031 ($(M+H)^+$).

[6] Only single products were observed by tlc and ^{31}P NMR for each of the deprotection steps. Detritylated **4a** was treated with ammonia both as shown in the scheme and also under more vigorous conditions (3:1 conc. NH_3(aq):EtOH, 55°C, O/N - appropriate for removing the base protecting groups found in oligonucleotides derived from commercially available Fpmp protected phosphoramidites); no P-S bond cleavage was detected in either case. **5a**: ^{31}P NMR δ (D_2O), 21.28; ^1H NMR δ (D_2O) 7.86 (d, H6), 5.94 (d, H1'), 5.87 (d, H5), 4.61 (m, H3'), 4.43 (m, H2'), 4.31 (m, H4'), 3.84 (m, H5', H5''), 3.33 (t, C<u>H</u>$_2$-N) 3.02 (m, C<u>H</u>$_2$-S), 2.58 (t, C<u>H</u>$_2$-CO), 1.43 (m, C<u>H</u>$_2$-CH$_2$N), 1.28 (m, C<u>H</u>$_2$-CH$_3$), 0.84 (t, C<u>H</u>$_3$); FAB- ms e/z 466 (100%, M^-).

diester (**5a**) was initially attempted without buffering. Although complete reaction was observed within 10 minutes at room temperature, the reaction mixture after this time was found to be acidic (pH<1) and the cyclic phosphate had been almost completely hydrolysed to the corresponding 2'- and 3'-monophosphates. In a buffered environment[7] cyclisation of **5a** proceeded smoothly at room temperature with greater than 90% reaction after 2 hours and no other side-products observed.

The solid-supported phosphorothiolate **4b** was synthesised using an analogous strategy to that adopted for **4a**; **2** was added *in situ* to the activated disulfide (**6b** - prepared according to slightly modified literature protocols[19,20][8]) and shaken overnight at room temperature. Applying deprotection conditions used on the model compound **4a** to a sample of **4b** gave **5b**, which was characterised by release of U>p[9] following iodine treatment. Consistent with the solution-phase studies, no nucleoside material was released from the support during ammonolytic decyanoethylation or acid catalysed removal of the acetal. Unreacted amine functions on the support from the preparation of **4b** were capped, the nucleoside loading determined by trityl release (15 μmol g^{-1}) and a further sample (0.6 μmol) used in the synthesis of the Fpmp and cyanoethyl protected pentanucleotide ((Up)$_4$Ups-CPG) using standard RNA chemistry.[10]

Deprotection of the pentamer and iodine-mediated transesterification of the phosphorothiolate-CPG linkage[11] liberated the crude 2',3'-cyclic phosphate terminated pentamer **7** (Figure 2 - (Up)$_4$U>p). This was purified by RP HPLC, desalted[12] and the product characterised by ^{31}P NMR and nucleoside composition analysis.[13] Selective acid catalysed hydrolysis followed by phosphomonoesterase digestion has previously been used[21] to characterise terminal cyclic phosphates in the presence of other phosphodiester linkages and this was applied to **7**; acid hydrolysis putatively giving a mixture of (Up)$_4$U2'p and (Up)$_4$U3'p which coeluted as one peak (**8**) which was further digested enzymatically to (Up)$_4$U (**9**) - Figure 3.[14]

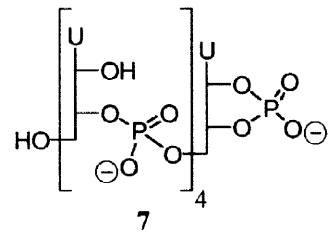


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Figure 2.

In conclusion, the CPG-nucleoside phosphorothiolate linkage reported herein has been

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[7] 8.75 mM I_2, 100 mM triethylammonium acetate (TEAA) pH 6.5, MeCN:H_2O (1:4), r.t., 2 h; extracted with Et_2O, lyophilised: ^{31}P NMR δ (D_2O *ca.* 125 mM TEAA pH 6.5), 20.40 (93% - U>p), 21.05 (7% - UpsR)

[8] CPG (150 mg) was treated with: 0.1M Lomant's reagent, 0.02M Et_3N, DCE, r.t., 7.75 h; 0.2M DTT, 0.2M Tris.HCl pH8.5, 1M NaCl, H_2O, r.t., 1.67h; 0.1M DTNP, DCM, rt., 1.5 h - using the conditions exactly as described by Gupta *et al.*[20] gave a nucleoside loading of less than 7 μmolg^{-1} by trityl assay.

[9] Characterised by comparison with a standard (Sigma) on RP-hplc and normal phase tlc.

[10] Using 1 μmol RNA ABI cycle; synthesised trityl-off; on-line trityl monitoring performed at every step; average step-wise coupling yield 100%.

[11] Deprotection: methanolic ammonia (saturated at -5°C), r.t., O/N; 97:3 H_2O:AcOH (sterile), 22°C, 5 h. Transesterification: 3.9 mM I_2, 75 mM TEAA, 1:4 MeCN:H_2O (sterile), r.t., 7 h.

[12] HPLC was performed on a Waters Column μ-Bondapak™ C_{18} column (3.9 x 300 mm): buffer A 100 mM TEAA pH 6.5; buffer B 100 mM TEAA pH 6.5, 50% aq. MeCN; 260 nm detection; 38 min gradient - isocratic 10% B to 5 min; 10-23% B to 27 min; 23-100% B to 31 min; isocratic 100% B to 33 min; 100-10% B to 38 min - eluting at 1 mL min^{-1}. This removed putative Fpmp-on nucleotidic material (later running) resulting from the limited acid treatment used for acetal cleavage and also minor quantities of shorter sequences. Desalting performed on Sep-Pak™ -product eluted in sterile H_2O. Yield = 0.06 μmol.

[13] ^{31}P NMR δ (D_2O), 20.09 (18%, U>p), -0.54 and - 0.64 (82%, UpU). Digestion with nuclease P_1 and alkaline phosphatase gave U:U>p 4.4:1 (expected 4:1)

[14] 1M HCl (aq - sterile) 18°C, 15 min, quenched with 1M Tris (aq - sterile), alkaline phosphatase, 30°C, 15 min. HPLC analyses for acid catalysed and enzymatic hydrolysis of 7 performed as for purification except: buffer A 6 mM TBA.HSO_4, 30 mM TEAA, pH 6.3; buffer B 6 mM TBA.HSO_4, 30 mM TEAA, pH 6.3, 50% aq. MeCN; 45 min gradient - isocratic 4% B to 12 min; 4-60% B to 30 min; isocratic 60% B to 35 min; 60-100% B to 40 min; 100-4% B to 45 min.

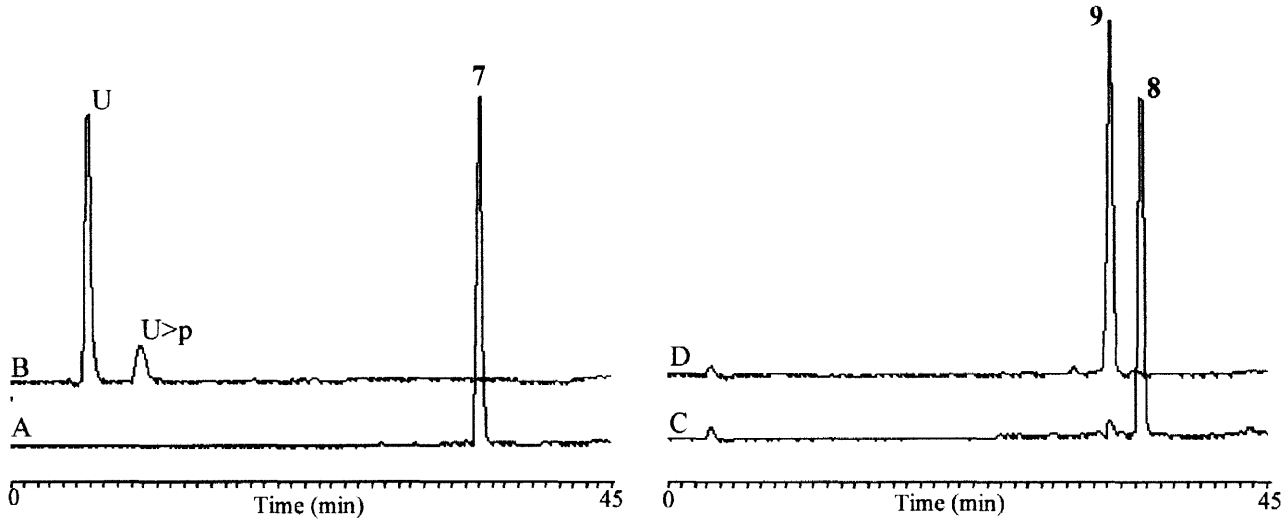

Figure 3. RP HPLC profiles of 7 and its enzymatic and controlled acid hydroylsis - conditions described in footnote 12 (retention times): A - 7 after purification (35.1 min); B - nuclease P_1 and alkaline phosphatase digestion of 7 (to U (5.9 min) and U>p (9.8 min)); C - 1M HCl hydrolysis of 7 (to 8 (35.5 min)); D - 1M HCl and alkaline phosphatase digestion of 7 (to 9 (33.1 min)).

successfully utilised for the preparation of a cyclic phosphate terminated oligonucleotide employing methodology that should be generally applicable to other sequences. In addition, this linkage expands the range of orthogonal linkers for support-bound nucleic acids and demonstrates the viability of solid-phase P-S bond formation by Michaelis-Arbusov chemistry which has previously been restricted to solution-phase.

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