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Solid-phase synthesis of terminal oligonucleotide–phosphoramidate conjugates

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Abstract—A novel phosphoramidite, *N*,*N*-diisopropylamino-2-cyanoethyl-9-anthracenemethyl phosphoramidite **1**, was prepared and coupled with the terminal 5′-hydroxyl of support-bound T_{10} and the putative phosphite triester intermediate was subsequently reacted with iodine in the presence of either water or a series of primary and secondary amines. The reactivity of **1** compared to a previously reported benzyl phosphoramidite **2** was also investigated: oxidation of the product of coupling **2** with CPG-T_{10}-5′OH under aqueous conditions resulted in greater than 30% of the benzyl moiety being retained. In contrast, essentially complete loss of the 9-anthracenemethyl group was observed using **1** under the same conditions. Oligonucleotides modified with a terminal phosphate monoester, lipophilic, fluorescent or cationic groups were thus prepared.

Oligonucleotide conjugates incorporating cell targeting/delivery agents, reporter groups, capture tags or nuclease resistant moieties at the 3′- or 5′-termini are currently widely prepared.[1] The utility of such conjugates can often be optimised using 'programmable linkers', the properties of which respond to environmental changes. This technology is well established for chemical- or light-cleavable linkers applied to affinity purification.[2] More recently, acid-cleavable linkers such as phosphoramidates have been utilised for the in vivo delivery of oligonucleotide–PEG conjugates.[3] Within 5 h at the endosomal pH (4.7), complete cleavage of a phosphoramidate linking an antisense oligonucleotide and a PEG group was observed.

*Inter*nucleotide phosphoramidate linkages and also mononucleoside phosphoramidate prodrugs have been installed via a diverse range of solid-phase chemistries.[4] In contrast, few developments in the solid-phase preparation of oligonucleotides bearing *terminal* phosphoramidates have been reported following descriptions of efficient solution-phase methods in the 1980s.[5] Solid-phase methods, which have been reported typically involve reactions of phosphate monoesters, phosphoimidazolides or *H*-phosphonates rather than phosphites.[6] Vasseur and co-workers have recently described[7] a simple route to *H*-phosphonates using a 4-methoxybenzyl phosphoramidite first reported by Li et al.[8] Using a related *ortho*-methylbenzyl phosphoramidite, we sought to exploit the reactivity of the corresponding phosphite triester derivatives[9] for the preparation of

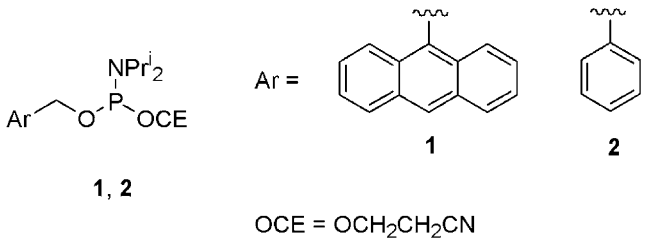

Figure 1. Phosphoramidites utilised in this study.

Keywords: Solid-phase synthesis; Nucleic acids; Arbuzov reactions; Phosphoramidate.
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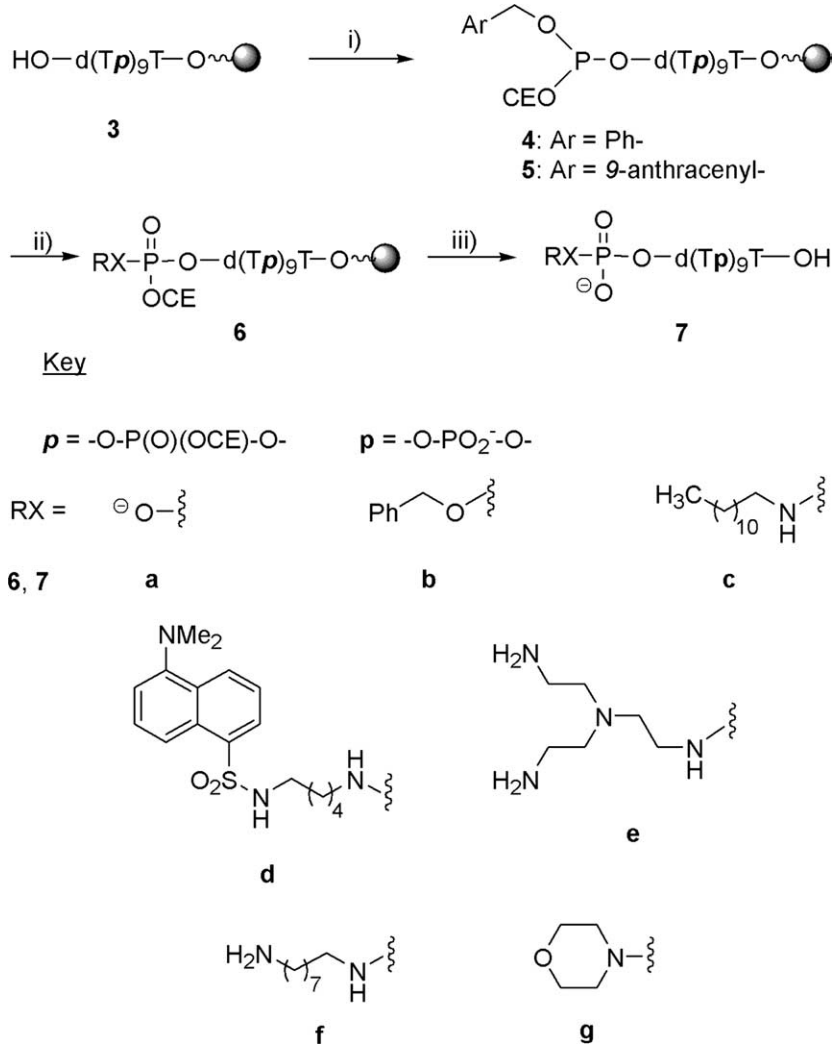

Scheme 1. Reagents and conditions: (i) 0.1 M **1** or **2** 0.2 M 5-benzylthiotetrazole or 0.45 M tetrazole, MeCN; (ii) oxidation—see Table 1 for conditions; (iii) deprotection via A: 40% $MeNH_2$ (aq), 65 °C, 30 min (**7a**–**f**); or B: (a) 10 % (v/v) Et_3N, 1:1 bistrimethylsilylacetamide/anhydrous pyridine, rt, 10 min; (b) 30% NH_3 (aq), rt, 30 min (**7g**).

phosphorothiolate-derived oligonucleotides.[10] However, the instability of the phosphoramidite and the intermediate phosphite triesters led us to seek more stable aryl methyl-derivatives for the preparation of terminal oligonucleotide–phosphoramidate conjugates.

Phosphoramidite derivatives of 9-anthracenemethanol (**1**: Fig. 1) or benzyl alcohol[11] **2** were prepared and isolated without chromatography using the methodology developed in this laboratory and since used by others.[10,12]

Thus, a 0.1 M solution of either 9-anthracenylmethanol or benzyl alcohol and Hünigs base (4.1 equiv) in anhydrous DCM was stirred during addition of *N*,*N*-diisopropylamino-2-cyanoethylchlorophosphoramidite (1.2 equiv) and the reaction stirred at room temperature for 30 min. Excess phosphitylating reagent was quenched following addition of solid-supported benzyl alcohol and agitation for a further 30 min under ambient conditions. Compounds **1** and **2** were isolated in good yield (>80%) and high purity (>90%) following treatment with activated basic alumina.[‡] A solution of **2** in acetonitrile (0.1 M) was found to be stable for over 1 month at room temperature in the presence of Molecular Traps™. The same solution of **1** was used within 1 week of its preparation.

Coupling of **1** or **2** to the 5′-hydroxyl of CPG-supported T_{10} **3** was performed under standard conditions (Scheme 1). The putative phosphite triester intermediates (**4** or **5**) were subsequently oxidised under standard conditions using 50 mM I_2 in 8/1/1 THF/pyridine/H_2O. We anticipated conversion to the corresponding cyanoethyl-protected phosphate diester **6a** with complete loss of the benzyl protecting group from **4** as has previously been observed for the *o*-methylbenzyl moiety.[13] However, fol-

[‡] Compound **1a**: δ_H (300.0 MHz, CD_3CN) 1.15–1.25 (12H, m, $2\times(CH_3)_2CH$), 2.45 (2H, $2\times$t $^3J_{HH}$ 6.6 Hz, CH_2CN), 3.65 (2H, m, $2\times CH$), 3.72 (2H, m, OCH_2CH_2), 5.58–5.75 (2H, $2\times$m, CH_2OAr), 7.44–7.59 (4H, m, H2, H7, H3, H6), 8.01 (2H, s, H4, H5), 8.41 (2H, d $^3J_{HH}$ 8.7 Hz, H1, H8), 8.46 (1H, s, H10); $\delta_{P\text{-}31}$ (121.5 MHz, CD_3CN) 147.33; MS-ES, 409.1 (M+H), 431.1 (M+Na); mp 59.7–60.1 °C.

Table 1. Coupling and directed-Arbuzov reactions performed using aryl methyl phosphoramidites **1** and **2**

Phosphoramidite	Oxidation conditions	Deprotection conditions	Product and characterisation MALDI-MS *m/z* *Calculated*		C18-HPLC rt/min (gradient)[a]	Yield/% ($A^{260\,nm}$)
1	I_2 (0.05 M)/THF/H_2O/pyridine (8/1/1)	A	**7a**	3065.38 *3058.47*	20.59 (1)	98
2	I_2 (0.05 M)/THF/H_2O/pyridine (8/1/1)	A	**7a** **7b**	(see above) 3155.84 *3148.52*	22.45 (1)	<70 >30
1/2	$C_{12}H_{25}NH_2$ (0.5 M)/I_2 (0.1 M)/THF (0.5 mL)	A	**7c**	3226.37 *3225.67*	41.17 (1)	86
1/2	DansylNH$(CH_2)_5NH_2$ (0.5 M)/I_2 (0.1 M)/THF (0.5 mL)	A	**7d**	3377.45 *3375.63*	20.03 (2)	76
1	(MMTrHNCH$_2$CH$_2$CH$_2$)$_2$N(CH$_2$)$_2$NH$_2$ (0.5 M)/I_2 (0.1 M)/THF (0.5 mL)	Detritylation then A	**7e**	3185.79[b] *3186.61*	18.59 (1)	93
1	$H_2N(CH_2)_8NH_2$ (0.5 M)/I_2 (0.1 M)/DCM (0.5 mL)	A	**7f**	3187.33 *3184.62*	19.89 (1)	71
1	O$(CH_2)_2$NH (0.5 M)/I_2 (0.1 M)/THF (0.5 mL)	B	**7g**	3136.38 *3127.53*	21.52 (1)	83

[a] HPLC; Monitoring at 260 nm. Column: RP-C18, 5 μm, 250 × 4.6 mm. Flow rate: 1 mL min^{-1}. Buffer A: 0.1 M TEAA, 5% MeCN, pH 6.5; Buffer B: 0.1 M TEAA, 65% MeCN, pH 7. Gradient 1: 0 min, 0% B; 5 min, 0% B; 35 min, 50% B; 38 min, 100% B; 43 min, 100% B; 50 min, 0% B; 55 min, 0% B. Gradient 2: 0 min, 25% B; 5 min, 25% B; 20 min, 45% B, 25 min, 25% B, 35 min, 25% B.

[b] Cyanoethylated material is also observed—observed 3231.46; *calculated 3239.64*.

lowing deprotection in 40% aqueous methylamine, RP-HPLC and MALDI-MS analysis of the products showed only 70% loss of the benzyl group to **7a** with the benzyl-protected phosphate diester **7b** being the only other product (Table 1). In contrast, greater than 98% loss of the anthracene-methyl function from **5** was observed under the same conditions and the 5′-phosphate monoester was formed cleanly following deprotection.

Several phosphoramidate derivatives of primary and secondary amines were also prepared using a standard protocol: the synthesis cycle was interrupted immediately following the coupling step; the support washed with anhydrous acetonitrile, and the phosphite triester intermediates **4** or **5** treated with 100 mM I_2 in the presence of 0.5 M amine. Reduced yields of the phosphoramidates were generally obtained using lower amine concentrations or in the presence of DMF. Removal of the 2-cyanoethyl moieties from decamers bearing terminal phosphoramidates derived from primary amines (**6c**–**f**) and their simultaneous cleavage from the support was effected using standard conditions. Attempted deprotection of the 5′-morpholidate-terminated oligomer **6g** using these conditions gave rise principally to the phosphate monoester **7a**. We therefore adapted the procedure of Ohkubo et al.[14] to effect initial decyanoethylation using a tertiary amine prior to removal from the support.

Analysis of the crude products following deprotection was performed using RP-HPLC. Characterisation was either by MALDI-MS or by coinjection with standards. In addition to the desired product, two major side-products were observed: **7a** and an oligonucleotide conjugate, which we tentatively assign to the 9-anthracenemethyl phosphate diester based upon its UV-absorption profile. Due to the large absorbance of the anthracenyl moiety, the levels of the side-product are over-estimated using absorbance at 260 nm.

In conclusion, we have prepared a novel phosphoramidite and demonstrated its utility for the rapid derivatisation of support-bound oligomers using standard phosphoramidite methodology. Decathymidylates bearing 5′-phosphoramidate-linked lipophilic, fluorescent and cationic moieties were thus prepared in a fashion amenable to split-bead, parallel synthesis of nucleic acid analogues. This will thereby directly complement methodologies developed in the laboratories of Richert and Gait for the solid-phase functionalisation of modified bases or sugars.[15] During the preparation of this manuscript Fabio and co-workers described the preparation of highly pure 5′- and 3′-labelled decanucleotides including phosphoramidates on solid support via phosphate triester methodology;[16] we believe that the novel phosphoramidite methodology described here provides access to a greater diversity of phosphate diester analogues.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2005.11.098.

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