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A New Deprotection Strategy for Automated Oligonucleotide Synthesis Using a Novel Silyl-Linked Solid Support

Anne Routledge, Mark P. Wallis, Kenneth C. Ross and William Fraser *

Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham, B4 7ET, UK

Abstract: Automated solid phase synthesis of 2'-deoxyribose oligonucleotides; $d(Tp)_7T$, $d(Tps)_7T$ and $d(Tp)_3(C^{Bz}p)_2(Tp)_2T$, was achieved directly in 99% overall yields using a novel silyl linkage to a CPG silica support cleavable within seconds at room temperature using TBAF.

Intracellular delivery of antisense oligonucleotides remains an inefficient process. There is much current interest in the development of techniques to improve the cellular uptake of antisense oligonucleotides, in particular, nuclease resistant phosphorothioates[1]. One approach towards improving the intracellular delivery of oligonucleotides has involved the use of acyloxymethyl protecting groups to mask the negatively charged phosphorothioate centres giving prooligonucleotides[2]. Once internalised, carboxyesterase-catalysed hydrolysis of the prooligonucleotide yields the parent phosphorothioate. The accessibility of oligonucleotides with base sensitive functional groups has relied on solution chemistry[1,2] which can often be time consuming, indirect, low yielding and therefore impractical for expeditious synthesis of multiple sequence analogues for biological evaluation. While automated solid phase synthesis remains the most rapid and direct way to make oligonucleotides[3], removal of oligonucleotides from commercially available succinamide-linked controlled pore glass (CPG) silica supports requires treatment with concentrated aqueous ammonia for several hours at room temperature[4] or for shorter times at elevated temperature[5]. In either case, such conditions are incompatible with oligonucleotides containing base sensitive functional groups such as ester and amide. The use of a photolabile linker has been reported recently but a major drawback to the general applicability of this strategy is the formation of thymine-thymine photodimers during cleavage of the oligonucleotide from the solid support[6].

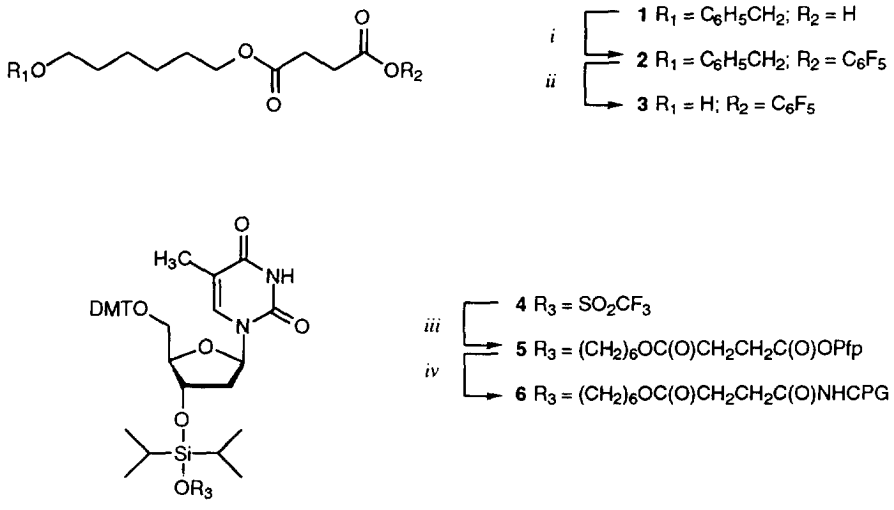
Our aims were to: (i) introduce a fluoride-labile silyl linker group for attachment of a nucleoside to a CPG silica support, (ii) investigate the compatibility of the silyl linker with conventional automated solid phase oligonucleotide synthesis protocols, and (iii) achieve post synthetic deprotection and cleavage of oligonucleotides from the silyl-linked solid support without use of concentrated aqueous ammonia.

Silyl protecting groups have already found uses in masking amino[7], phosphate[8] and 2'-OH functions[9] during solid phase synthesis of oligonucleotides. The diphenylsilyl group has been used recently to link a sugar to a polystyrene support in solid phase synthesis of oligosaccharides[10]. Cleavage of the diphenylsilyl

linkage in this case required treatment for 4 h with tetra-*n*-butylammonium fluoride (TBAF) in tetrahydrofuran (THF) buffered with acetic acid. We reasoned that use of the diisopropylsilyl group to link a nucleoside to a CPG solid support for solid phase synthesis of oligonucleotides might allow a more rapid post synthetic cleavage under similarly mild deprotection conditions[11].

Construction of conventional succinamide-linked CPG silica supported nucleosides normally involves ring opening of succinic anhydride by the nucleophilic 3'-OH group of a suitably protected nucleoside[4]. This is usually followed by transformation of the intermediate succinate into an activated *p*-nitrophenyl ester which is then reacted with CPG silica to give the solid-supported material for use in automated solid phase synthesis of oligomers. Our decision to use the known 2'-deoxythymidine (dT) nucleoside[12] **4** with its electrophilic 3'-*O*-diisopropylsilyl triflate group, prompted synthesis of the succinate spacer molecule **3** as an appropriate nucleophile for reaction with **4** (Scheme). Succinate **1** was prepared by monobenzylation of 1,6-hexanediol, followed by reaction of the protected alcohol with succinic anhydride[13]. The pentafluorophenyl (Pfp) ester **3** was synthesised in two steps from succinate **1** *via* **2** as shown in the scheme.

Scheme



Reagents: *i* PfpOH, 1,3-dicyclohexylcarbodiimide, 1,4-dioxane, 2,6-lutidine; *ii* H_2, 10% palladium on charcoal, ethanol (aq.); *iii* imidazole, **3**, acetonitrile; *iv* controlled pore glass (CPG) silica, *N*,*N*-dimethylformamide, 2,6-lutidine.

The extended silyl-linked nucleoside adduct **5** was produced by displacement of the triflate group of **4** by ester **3**. Commercially available CPG silica[14] was derivatised with silyl-linked adduct **5** giving the solid-supported material **6**. The efficiency of the coupling reaction was determined by measuring the amount of

dimethoxytrityl (DMT) cation released on treatment of **6** with mild acid following the standard method[4]. The DMT assay indicated that an acceptable loading of 27 μmol g^{-1} had been achieved. In order to investigate cleavage efficiency of the silyl linkage, a portion of the derivatised solid support **6** was treated with a 1 M solution of TBAF in THF for 60 seconds at room temperature. After removal of the CPG silica, the remaining solution was treated with 70% perchloric acid in methanol. Analysis of the released DMT cation indicated that quantitative cleavage of 2'-deoxythymidine from the solid support had been achieved.

Encouraged by the ease with which dT could be cleaved from the solid support, we used **6** in the automated synthesis of the phosphodiester-linked octamer $d(Tp)_7T$ on a 0.7 μmol scale using commercially available dT cyanoethylphosphoramidite[15]. Oligonucleotide synthesis was carried out using an Applied Biosystems 392 automated DNA synthesiser following the standard protocol recommended by the manufacturer[5]. Excellent coupling efficiencies were achieved giving the silyl-linked oligonucleotide in greater than 99% overall yield as indicated by trityl assays[4]. Deprotection was carried out using 1M TBAF in THF. Although quantitative cleavage of the oligonucleotide from the solid support was achieved in seconds, removal of the cyanoethyl protecting groups on phosphorus required heating at 50°C during 2 h. The crude product was analysed by reversed phase HPLC (Figure 1). The HPLC profile[16] of a crude sample of $d(Tp)_7T$ made using a standard succinamide-linked CPG silica solid support[15], cleaved with concentrated aqueous ammonia during 16 h at 55°C, is shown in Figure 1 (a). The HPLC profile of a crude sample of the $d(Tp)_7T$ sequence made using our silyl-linked CPG silica support **6** is shown in Figure 1 (b).

Figure 1

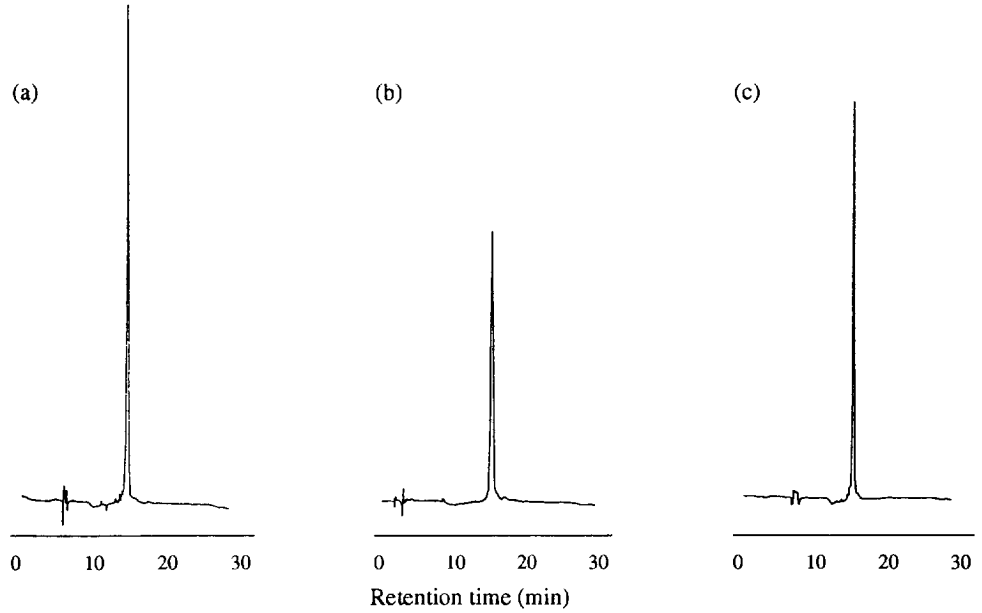


Fig. 1 HPLC elution profiles[16] of (a) $d(Tp)_7T$ made using a standard succinamide-linked CPG silica support[15], (b) $d(Tp)_7T$ made using the fluoride-labile silyl-linked CPG silica support **6**, and (c) co-injection of (a) and (b).

The HPLC profile shown in Figure 1 (c) corresponds to a co-injection of a mixture of the crude $d(Tp)_7T$ made by both methods. The HPLC results demonstrate that $d(Tp)_7T$ made using our new deprotection strategy was identical to $d(Tp)_7T$ made by the conventional method.

Having established the compatibility of the diisopropylsilyl linkage with solid phase synthesis conditions, we were very interested in using **6** for the synthesis of the corresponding phosphorothioate octamer $d(Tps)_7T$. Thus, automated oligonucleotide synthesis was carried out as before but with a commercially available sulphurising agent composed of tetraethylthiuram disulphide in MeCN in place of the usual oxidising solution (I_2/THF/H_2O). Again, coupling efficiencies in excess of 99% were achieved giving the silyl-linked phosphorothioate oligonucleotide $d(Tps)_7T$. Deprotection was carried out during 2 h at 50°C using 1M TBAF in THF. Figure 2 (a) shows the HPLC profile[16] corresponding to a crude sample of the phosphorothioate $d(Tps)_7T$ made using a standard succinamide-linked CPG silica solid support[15] whereas Figure 2 (b) shows the crude $d(Tps)_7T$ sequence made using our silyl-linked CPG silica support **6**. Co-injection of a mixture of $d(Tps)_7T$ made by both methods gave a single peak Figure 2 (c), again showing both deprotected oligonucleotides to be identical.

Figure 2

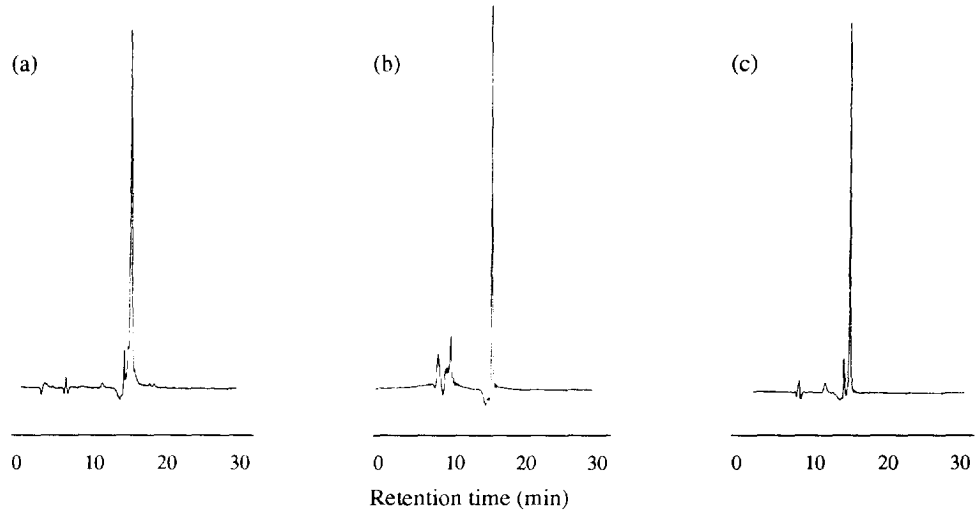


Fig. 2 HPLC elution profiles[16] of (a) $d(Tps)_7T$ made using a standard succinamide-linked CPG silica support[15], (b) $d(Tps)_7T$ made using the fluoride-labile silyl-linked CPG silica support **6**, and (c) co-injection of (a) and (b).

In order to determine whether solid support **6** in combination with the TBAF deprotection strategy could be compatible with oligonucleotides containing base sensitive functional groups, we synthesised the phosphodiester $d(Tp)_3(C^{Bz}p)_2(Tp)_2T$ octamer containing benzamide (Bz) protecting groups at the exocyclic amino group of C. Automated oligonucleotide synthesis was carried out using **6** as before giving the crude $d(Tp)_3(C^{Bz}p)_2(Tp)_2T$ sequence in 99% overall yield. It was possible to achieve efficient cleavage of

$d(Tp)_3(C^{Bz}p)_2(Tp)_2T$ from the CPG support and to remove the cyanoethyl protecting groups from phosphorus during 2 h at 50°C using 0.5 M TBAF in THF buffered with one equivalent of acetic acid. The HPLC profile[16] of a crude sample of $d(Tp)_3(Cp)_2(Tp)_2T$ made using a standard succinamide-linked CPG silica solid support[15], cleaved with concentrated aqueous ammonia during 16 h at 55°C, is shown in Figure 3 (a). Under the ammonia deprotection conditions the benzamide groups were removed from C giving the fully deprotected $d(Tp)_3(Cp)_2(Tp)_2T$ sequence. The HPLC profile of a crude sample of the benzamide-protected sequence $d(Tp)_3(C^{Bz}p)_2(Tp)_2T$ made using our silyl-linked CPG silica support **6** is shown in Figure 3 (b). Figure 3 (c) corresponding to co-injection of products $d(Tp)_3(Cp)_2(Tp)_2T$ and $d(Tp)_3(C^{Bz}p)_2(Tp)_2T$ made by the two methods and indicates two major peaks implying that the benzamide protecting groups had remained intact.

Figure 3

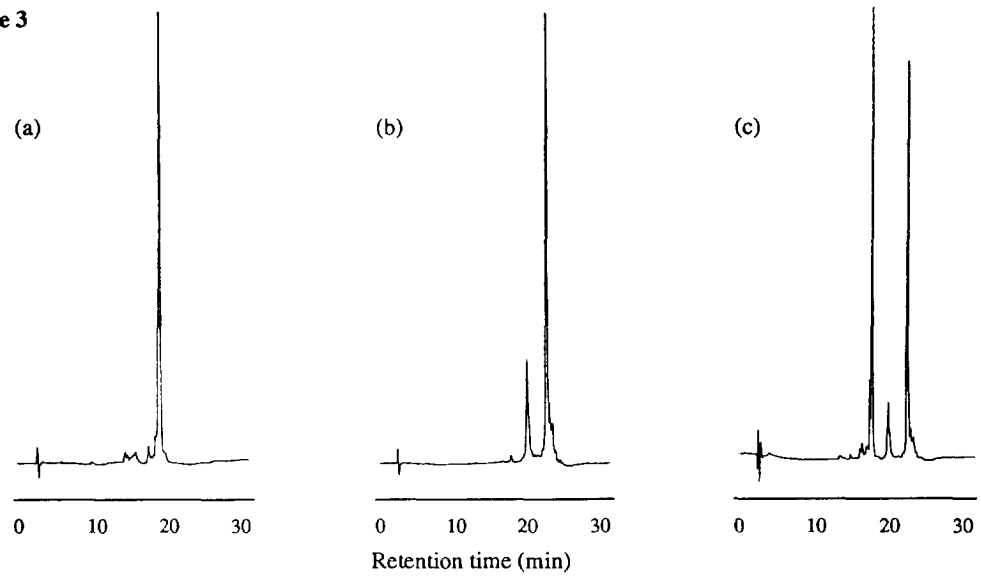


Fig. 3 HPLC elution profiles[16] of (a) $d(Tp)_3(Cp)_2(Tp)_2T$ made using a standard succinamide-linked CPG silica support[15], (b) $d(Tp)_3(C^{Bz}p)_2(Tp)_2T$ made using the fluoride-labile silyl-linked CPG silica support **6**, and (c) co-injection of (a) and (b).

It was important to unambiguously establish that the benzamide protecting groups in $d(Tp)_3(C^{Bz}p)_2(Tp)_2T$ were indeed intact as expected after the deprotection step. The crude sequence was therefore purified by HPLC and subjected to MALDI-TOF mass spectrometric analysis[17]. A molecular ion peak was observed at m/z 2548 which was identical to the molecular weight calculated for $d(Tp)_3(C^{Bz}p)_2(Tp)_2T$ corresponding to molecular formula $C_{92}H_{111}N_{18}O_{54}P_7$.

The deprotection strategy which we have described is direct, efficient and compatible with base sensitive benzamide substituents. We are currently applying our new deprotection strategy to solid phase synthesis of oligonucleotides containing other base sensitive groups for antisense applications.

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13. All new compounds reported gave satisfactory TLC, ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, mass spectral, and elemental analysis data, in full agreement with their assigned structures.
14. Fractosil 500 CPG silica was purchased from the Sigma Chemical Company.
15. Cyanoethylphosphoramidites (dT, dC^{Bz}) and dT succinamide-linked CPG silica support were purchased from Cruachem.
16. HPLC using gradient elution was performed using an HPLC Technology C18 Reversed Phase Column (250 x 46 mm) with solvent system A mixed with solvent system B (0-35%) during 20 min. then with B (50%) during a further 10 min. where A was composed of 1 M aqueous triethylammonium acetate (TEAA, 10%) and MeCN (2%) at pH 7 and B was composed of 1 M aqueous TEAA (10%) and MeCN (80%) at pH 7.
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