An efficient solid phase synthesis of 5′-phosphodiester and phosphoramidate monoester nucleoside analogues†

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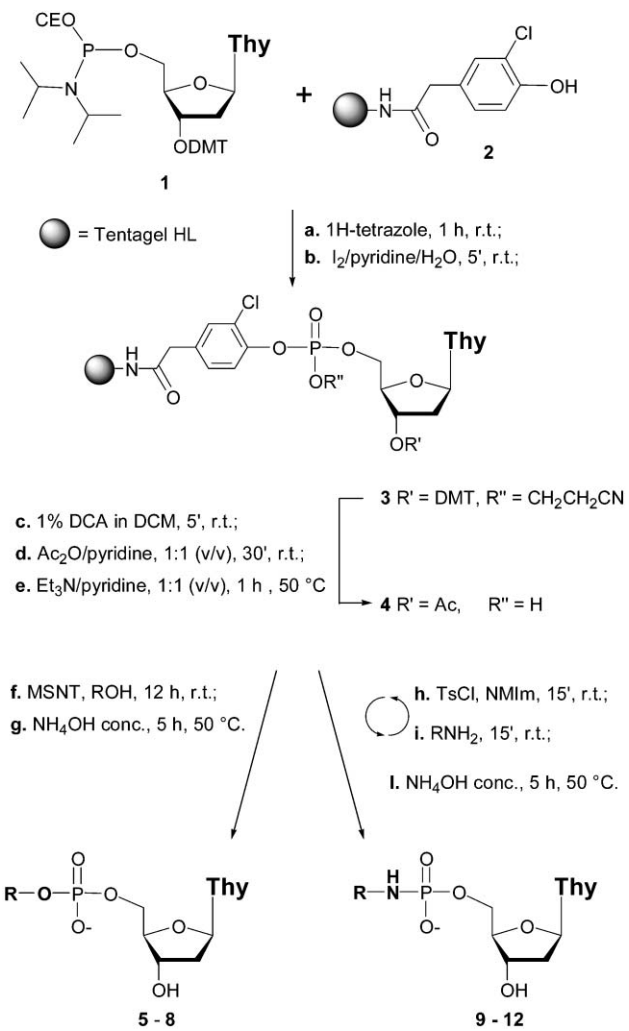
An easy and efficient strategy to obtain libraries of 5′-phosphodiester and 5′-phosphoramidate monoester nucleoside analogues in a highly pure form has been developed, starting from a new nucleoside based solid support. The nucleoside scaffold has been anchored through a 5′-phosphodiester linkage to Tentagel® HL resin, functionalized with a 3-chloro-4-hydroxyphenylacetic linker. The solid phase synthesis of small libraries of 5′-phosphodiester and 5′-phosphoramidate monoester thymidine analogues is also reported.

Nucleoside and nucleotide analogues are a class of molecules with a broad spectrum of biological properties.[1]

The biological activity of most of these analogues requires intracellular metabolism to 5′-mononucleotides by a kinase-mediated phosphorylation. The development of drug resistance or toxicity has however limited the effectiveness of these agents.[2] In order to overcome these problems, many research groups have developed a pro-drug approach to deliver biologically active nucleosides into cells using masked, charged monophosphate derivatives.[3–5]

For this purpose, a variety of 5′-phosphorothioate and 5′-phosphodiester nucleoside analogues have been prepared by classical solution or solid phase synthesis.[6] In contrast, only a limited number of nucleoside 5′-phosphoramidate derivatives have been prepared and evaluated for their biological activity.[3,4] In addition, the known phosphoramidate analogues have only been synthesized by solution methods, generally involving many steps and requiring tedious chromatographic separation. In association with combinatorial chemistry methods, the solid phase approach to the synthesis of small organic molecule libraries[7] offers a good pathway to a large number of these analogues.

As a part of our continuing research effort towards the synthesis of new solid supports, useful for generating nucleoside and nucleotide analogue libraries,[8] we present here the preliminary results of a new and efficient synthetic procedure for obtaining a wide variety of 5′-phosphate diester and phosphoramidate monoester nucleoside analogues in highly pure forms. 5′-Phosphoramidite thymidine derivative **1** (Scheme 1) was chosen as the model compound and anchored to a Tentagel® HL support by exploiting classical phosphoramidite chemistry. To do this we used a slightly modified form of the procedure described by Pedroso *et al.*,[9] originally developed for the solid phase synthesis of cyclic oligonucleotides. The key point to our strategy is the derivatization of the solid support with a 3-chloro-4-hydroxyphenylacetic linker, leading to **2**, onto which the nucleotide is attached through a phosphite triester linkage. The complete oxidation of the phosphite to the phosphate triester **3** was achieved by treatment with the commonly used 'oxidizing reagent' I_2 in a pyridine–H_2O–THF mixture and monitored by ^{31}P NMR spectroscopic analysis of the support, suspended in $CDCl_3$. Typically, the signal at 135 ppm disappeared and two new signals, centered at *ca.* 4 ppm, were observed. The loading of the nucleotide, determined by quantification of the 4,4′-dimethoxytrityl (DMT) cation



Scheme 1

† Electronic Supplementary Information (ESI) available: General procedure for the synthesis of **5–12**. ^1H NMR, ^{31}P NMR and ESI–MS data for **5–12**. See http://www.rsc.org/suppdata/cc/b5/b501043h/

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released from weighed amounts of support **3** upon acidic treatment, was always above 0.28 mequiv. g^{-1}.

After DMT removal (using 1% dichloroacetic acid (DCA) in dichloromethane (DCM)) and capping of the 3′-hydroxy function (using acetic anhydride in pyridine), treatment with triethylamine in pyridine (1 : 1 v/v, 1 h, 50 °C) led to phosphate deprotection, and the formation of support **4**. The presence in the resin's ^{31}P NMR spectrum of a single signal at *ca.* −3 ppm confirmed the total conversion of the phosphotriester to phosphodiester function.

To test the efficiency of this support in the synthesis of phosphodiester and phosphoramidate monoester analogues of thymidine, we followed two different methods, both exploiting classical phosphotriester chemistry.

To obtain phosphate diester analogues **5–8** (Scheme 1 and Table 1), support **4** was reacted with 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT) and the chosen alcohol in pyridine at rt for 12 h.

Detachment from the support was achieved by treatment with concentrated aqueous ammonia (5 h, 50 °C) and the crude released material analysed by RP–HPLC.

The HPLC profiles of the detached nucleotides showed a major peak, typically accounting for more than 90% of the total integrated area (*e.g.* Fig. 1). ^{1}H and ^{31}P NMR analysis of the crude materials confirmed the presence of a single product in each reaction mixture.† Such elevated purity is obtained because only the nucleoside linked to the support through a phosphotriester linkage is easily removed, whereas the nucleoside anchored through a phosphodiester bond, *i.e.* the unreacted material, is not cleaved from the resin upon treatment with ammonia.

Indeed control experiments carried out by treating support **4** with ammonia under the same conditions showed no traces of nucleosidic material in the eluate by either ^{1}H NMR or UV analysis.

In order to prepare phosphoramidate analogues **9–12** (Scheme 1), a published procedure for introducing the amidate linkage in oligonucleotides was followed.[10] Support **4** was treated with *p*-tosyl chloride in pyridine for 15 min and after the appropriate washing steps, reacted with the appropriate amine dissolved in pyridine for a further 15 min.

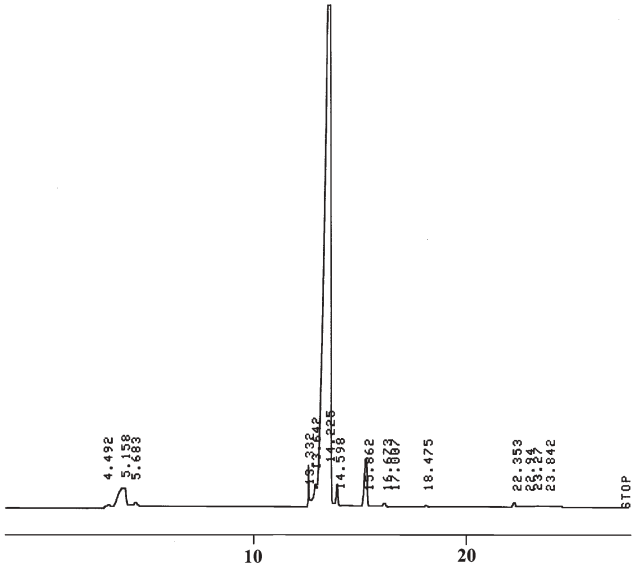


Fig. 1 The HPLC profile of crude, detached **6** (Nucleosil 100–5 C18 column, 4.6 × 250 mm, 5 μm), eluted with a linear gradient from 0 to 100% *B* in 30 min. *A* = H_2O, *B* = CH_3CN, detection at λ = 260 nm, flow rate = 0.8 mL min^{-1}.

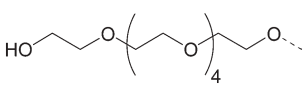
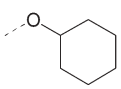
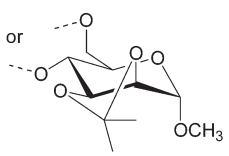
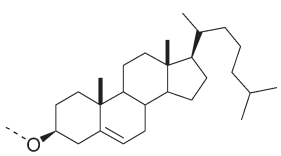
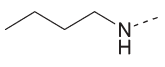
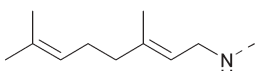
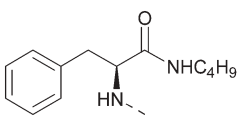
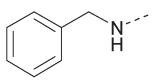
By iterating the described synthetic procedure 3 times, more than 80% of the starting diester could be converted into the desired amidate, as determined by ^{31}P NMR analysis of the resin.

The detachment from the support was achieved by treatment with concentrated aqueous ammonia (5 h, 50 °C) and the crude released material was analyzed by RP–HPLC. The HPLC profiles showed a single major peak in each of these cases.

The identity of **5–12** were ascertained by ^{1}H NMR, ^{31}P NMR and ESI–MS experiments carried out directly on the crude detached material. Yields of target compounds **5–12** were determined by quantitative UV analysis at 260 nm,† and were consistently in the range 70–80%, in reference to the initial functionalized support.

In conclusion, we have reported a simple solid phase methodology to obtain phosphodiester and phosphoramidate

Table 1 The structure of R groups in the synthesized 5′-phosphodiester and 5′-phosphoramidate monoester compounds **5–12** (Scheme 1) and their ^{31}P NMR chemical shifts.

RO–				
Entry	**5**	**6**	**7a**, **7b**	**8**
δ ^{31}P (ppm)	3.3	2.7	3.5 and 2.7	2.6
RNH–				
Entry	**9**	**10**	**11**	**12**
δ ^{31}P (ppm)	11.5	11.2	6.6	10.8

monoester nucleoside analogues by using standard phosphotriester chemistry. To demonstrate the feasibility of our synthetic approach, we prepared a small library of thymidine analogues, conjugated at the 5′-position with a set of representative alcohols and amines. Starting from 30 mg of support **4**, all the target nucleotides could be recovered as discrete compounds in 2–4 mg quantities in a highly pure form.

In principle, this methodology could easily be extended to all the natural nucleosides, both in the *ribo*- and *deoxyribo*- series, whose 5′-phosphoramidites are either commercially available or easily synthesized through standard, well known chemistry[6,11–13] and show the same high reactivity as their corresponding thymidine derivatives. In addition, since the sole prerequisite of the proposed strategy is the availability of the 5′-phosphoramidite analogue of the starting scaffold, a large number of nucleoside analogues could therefore be included as useful templates in this protocol. Further studies are currently in progress to scale-up our methodology to produce libraries with a high degree of molecular diversity in tens of mg quantities, built upon modified nucleosides that show relevant and well known anti-cancer or antiviral activities.

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