An efficient Solid Phase Synthesis of 5’-phosphodiester and phosphoramidate monoester nucleoside analogues

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ELECTRONIC SUPPLEMENTARY INFORMATION:
Experimental details
EXPERIMENTAL

**General Method.** NMR spectra were recorded in CDCl₃, CD₃OD or D₂O with a Bruker WM 400 or an INOVA 500 Varian spectrometer. The chemical shifts (δ) are given in ppm with respect to the residual solvent signal (7.26, 3.31 and 4.80 ppm, respectively) and coupling constants (J) are in Hz.

³¹P NMR spectra were recorded at 161.98 MHz on a Bruker WM-400 spectrometer using 85% H₃PO₄ as external standard.

For the ESI MS analysis a Waters Micromass ZQ instrument – equipped with an Electrospray source – was used in the negative mode.

HPLC analyses were performed on a Beckman System Gold 126 instrument equipped with a UV detector module 166 and a Shimadzu Chromatopac C-R6A integrator. The crude material was analyzed by HPLC on a Nucleosil 100-5 C18 column (4.6x250 mm, 5 µm) eluted with a linear gradient of CH₃CN in H₂O, flow = 0.8 mL/min, detection at λ = 260 nm.

Tentagel®-NH₂ resin was purchased from Novabiochem. The functionalizations of the solid support were carried out in a short glass column (5 cm length, 1 cm i.d.), equipped with a sintered glass filter, a stopcock and a cap. The coupling reaction to obtain support 3 was performed using 5’-O-(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite-3’-O-(4,4’-dimethoxytriphenylmethyl)-thymidine as building block. The nucleotide, the activator solution (0.45 M tetrazole in CH₃CN) and the oxidizer solution (0.1 M iodine/THF/H₂O/pyridine) were all purchased from Applied Biosystems.

Conventional abbreviations are used.
**Functionalization of the support ans synthesis of 4**  
500.0 mg of Tentagel®-NH₂ HL (0.41 meq/g, 0.20 mmol) were reacted, at r.t. overnight, with a mixture of 382.0 mg (2.0 mmol) of 3-chloro-4-hydroxy-phenylacetic acid, 317 µL (2.0 mmol) of DIC, 352 µL of DIEA and 313.6 mg (2.0 mmol) of N-hydroxybenzotriazole (HOBt·H₂O) dissolved in 4 mL of anhydrous pyridine. After exhaustive washings with DCM, CH₃OH and Et₂O, the support was dried under reduced pressure. By Kaiser test, performed on weighed samples of the dried support, the incorporation of the linker was estimated almost quantitative. After capping of the unreacted amino functions with Ac₂O/Py (1:1, v/v) for 1 h at r.t., the support was treated with conc. aq. ammonia (28%) at 50 °C for 1 h.

\[
\text{NH}_2 \quad \text{OH} \quad \text{Cl} \quad \text{+} \quad \text{HOOC-C-Cl} \quad \text{OH} \quad \text{\rightarrow} \quad \text{NH} \\
\text{H} \quad \text{C} \quad \text{O} \quad \text{N} \quad \text{H} \quad \text{Cl} \quad \text{O} \quad \text{H} \quad \text{OH}
\]

4.5 mL (2.03 mmol) of a commonly used ‘activator solution’ (0.45 M tetrazole in CH₃CN) were added to 224 mg (0.31 mmol) of 5′-O-(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite-3′-O-(4,4′-dimethoxytriphenylmethyl)-thymidine (1) and 500.0 mg (0.41 meq/g, 0.20 mmol) of support 2. After 1 h the support was exhaustively washed with CH₃CN and treated with 5 mL of a commonly used ‘oxidizer’ solution (I₂/pyridine/H₂O/THF) for 5 min. After exhaustive washings with CH₃CN, DCM and Et₂O, resulting support 3 was dried under reduced pressure. The complete oxidation of the phosphite triester to phosphate triester leading to support 3 was monitored by ³¹P-NMR of the resin suspended in CDCl₃. Typically, a relevant upfield shift of the signal at 135 ppm to two signals centered at ca. 4 ppm was observed. Incorporation yields of nucleotide 1 onto 2 were always in the range 70-80% (0.28-0.32 meq/g), as determined by quantitative DMT test performed on dried and weighed samples of support 3.

\[
\text{H-N} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{Thy} \quad \text{3 R'} = \text{DMT, R'' = CH₂CH₂CN} \\
\text{OR'} \quad \text{OR'} \\
\text{3 R'} = \text{Ac, R'' = H}
\]

DMT removal was achieved by treatment with 1% DCA in DCM solution. After standard capping procedure with Ac₂O/pyridine 1:1 (v/v), the phosphate deprotection from 2-cyanoethyl group was then carried out by treatment with Et₃N/pyridine solution (1:1, v/v) for 1 h at 50 °C,
giving support 4. The total deprotection was confirmed by a characteristic upfield shift in the signal of the $^{31}$P NMR spectrum of the solid support suspended in CDCl$_3$, from $ca. \delta = 4$ ppm to $\delta = -3$ ppm.
A general procedure for the solid phase synthesis of 5'-phosphodiester and 5'-phosphoramidate monoester nucleosides

A general procedure for the preparation of phosphodiesters 5 – 8

30 mg (0.28 meq/g, 0.0084 mmol) of dried support 4 were washed and swelled in anhydrous pyridine and then reacted with a mixture of 0.084 mmol of the chosen alcohol (see Table) and 25 mg (0.084 mmol) of 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT) in 300 µL of anhydrous pyridine for 12 h at r. t. After exhaustive washings with pyridine, CH₃OH, DCM and Et₂O, the target analogues were detached from the support by conc. aq. ammonia treatment at 50 °C for 5 h.

A general procedure for the preparation of phosphoramidate monoester 9 – 12

30 mg (0.28 meq/g, 0.0084 mmol) of dried support 4 were washed and swelled in anhydrous pyridine. The support was then treated with 1 mL of p-tosyl chloride solution (0.2 M p-TsCl, 0.4 M N-methyl imidazole in pyridine) for 15 min to r. t. to generate the active ester, followed by addition of 1 mL of the amine solution (0.45 M in pyridine), with appropriate washing steps in between. After exhaustive washings with pyridine, CH₃OH, DCM and Et₂O, the target analogues were detached from the support by conc. aq. ammonia treatment at 50 °C for 5 h.

Table 1. Alcohols and amines used for the synthesis of phosphodiester and phosphoramidate nucleoside analogues 5-12

<table>
<thead>
<tr>
<th>R-OH</th>
<th>R-NH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Diethylaminomethyl-protected nucleoside" /></td>
<td><img src="image2.png" alt="N,N-diethyl-protected nucleoside" /></td>
</tr>
<tr>
<td><img src="image3.png" alt="Cyclohexyl protected nucleoside" /></td>
<td><img src="image4.png" alt="2-O-Acetyl-4-deoxy-6-deoxy nucleoside" /></td>
</tr>
<tr>
<td><img src="image5.png" alt="4-O-Acetyl-2-Deoxy-6-deoxy nucleoside" /></td>
<td><img src="image6.png" alt="4-O-Acetyl-2-Deoxy-6-deoxy nucleoside" /></td>
</tr>
</tbody>
</table>

The crude released materials were analyzed by RP-HPLC. In all cases, the HPLC profiles of the detached nucleotides showed a major peak,^{2} typically accounting for more than 90% of the total integrated area. In a typical experiment, from 30 mg of resin 4, 2-4 mg of the pure target compounds could be obtained. Yields of target compounds 5 - 12, evaluated by quantitative UV analysis at 260 nm, were always in the range 70-80%, referred to the initial functionalization of the solid support.^{3}

\[ \text{ESI-MS (m/z)} \]
\[
\begin{array}{ll}
\text{found} & \text{Calcd. for M} \\
585.50 \text{[M-H]}^{-} & 586.21 \\
\end{array}
\]
Retention time: 11.5 min.

\[ \text{1H NMR (D}_2\text{O, 400 MHz): 7.78 (1H, s, H-6 T), 6.39 (1H, dd, J = 7.2 and 7.2 Hz, H-1' T), 4.63 (1H, m, H-3' T), 4.21 (1H, m, H-4' T), 4.13 (2H, m, H2-5' T), 4.05 (2H, m, CH}_2\text{OP0}_3, 3.87-3.67 (22H, overlapped signals, OCH}_3 \text{hexaethylene glycol residue ), 2.42 (2H, m, H2-2' T), 1.97 (3H, s, CH}_3 \text{T).}
\]

\[ \text{31P NMR (D}_2\text{O, 161.98 MHz): 3.3.} \]

\[ \text{ESI-MS (m/z)} \]
\[
\begin{array}{ll}
\text{found} & \text{Calcd. for M} \\
403.42 \text{[M-H]}^{-} & 404.13 \\
\end{array}
\]
Retention time: 14.2 min.

\[ \text{1H NMR (D}_2\text{O, 400 MHz): 7.79 (1H, s, H-6 T), 6.41 (1H, dd, J = 6.8 and 6.8 Hz, H-1' T), 4.66 (1H, m, H-3' T), 4.22 (1H, m, H-4' T), 4.11 (3H, overlapped signals, CH}_2 \text{cyclohexanol and H2-5' T), 2.46 (2H, m, H2-2' T), 1.99 (3H, s, CH}_3 \text{T), 1.73 (4H, m, 2CH}_2\text{-CH-O), 1.44-1.24 (6H, overlapped signals, other 3CH}_3 \text{protons in cyclohexanol residue).} \]

\[ \text{31P NMR (D}_2\text{O, 161.98 MHz): 2.7.} \]

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^{2} As expected, in the case of entry 7, two thymidine derivatives were obtained. They were easily identified as 7a and 7b, i.e. the 6- and 4-phosphodiester linked regioisomers of mannose.

^{3} In all cases, \( \varepsilon \) for the nucleotide adduct was assumed to be the same as the one reported in the literature for thymidine-monophosphate (8800 cm\(^{-1}\)M\(^{-1}\) at 25 °C in H\(_2\)O), neglecting the contribution of the alcoholic or amino portion. In the specific case of aromatic amine adducts 11 and 12, this led to underestimate the \( \varepsilon \) value for the target compounds by no more than 2 \%, which is well taken into account in the range of yields reported.
ESI-MS (m/z)

<table>
<thead>
<tr>
<th></th>
<th>found</th>
<th>Calcd. for M</th>
</tr>
</thead>
<tbody>
<tr>
<td>7a</td>
<td>537.39 [M-H]</td>
<td>538.16</td>
</tr>
</tbody>
</table>

Retention time: 12.3 and 12.8 min.

\[ ^1H \text{NMR (D}_2\text{O, 400 MHz)} \]

7a and 7b as a mixture: 7.84 and 7.82 (s’s, H-6 T), 6.41 (dd, J = 6.8 and 6.8 Hz, H-1’ T), 5.06 and 5.03 (s’s, H-1 mannose), 4.65 (m, H-3’ T), 4.31 – 3.74 (overlapped signals, H-4’, H-5’ T and H-2, H-3, H-4, H-5 and H-2-6 mannose), 3.49 and 3.46 (s’s, 1-OCH\textsubscript{3} mannose), 2.43 (m, H-2-2’ T), 1.99 and 1.98 (s’s, CH\textsubscript{3} T), 1.58, 1.55, 1.45 and 1.42 (s’s, CH\textsubscript{3} acetonide).

\[ ^3P \text{NMR (D}_2\text{O, 161.98 MHz)} \]: 3.5, 2.7.

ESI-MS (m/z)

<table>
<thead>
<tr>
<th></th>
<th>found</th>
<th>Calcd. for M</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>689.78 [M-H]</td>
<td>690.40</td>
</tr>
</tbody>
</table>

Retention time: 24.8 min.

\[ ^1H \text{NMR (CD}_3\text{OD, 400 MHz)} \]

7.79 (1H, s, H-6 T), 6.35 (1H, dd, J = 6.4 and 6.4 Hz, H-1’ T), 5.32 (1H, m, H-6 cholesterol residue), 4.50 (1H, m, H-3’ T), 4.04-3.90 (3H, overlapped signals, H-4’ and H-5’ T), 3.65 (1H, s, H-3 cholesterol residue), 2.30 (2H, m, H-2-2’ T), 2.20-0.65 (complex signals of cholesterol residue), 1.94 (3H, s, CH\textsubscript{3} T).

\[ ^3P \text{NMR (CD}_3\text{OD, 161.98 MHz)} \]: 2.6.
**ESI-MS (m/z)**

<table>
<thead>
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<th>Molecule</th>
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<th>Calcd. for M</th>
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</thead>
<tbody>
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<td>9</td>
<td>376.34</td>
<td>377.14</td>
</tr>
<tr>
<td>10</td>
<td>456.35</td>
<td>457.20</td>
</tr>
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</table>

Retention time: 11.5 min. 15.6 min.

**1H NMR (CD3OD, 400 MHz)**:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Shifts</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>7.89 (1H, s, H-6 T), 6.35 (1H, dd, J = 6.4 and 6.4 Hz, H-1' T), 4.52 (1H, m, H-3' T), 4.03 (1H, bs, H-4' T), 3.96 (2H, m, CH2-NH), 2.86 (2H, m, CH2-NH), 2.33-2.18 (2H, m, H2-2' T), 1.95 (3H, s, CH3 T), 1.46 (2H, m, CH3-CH2-CH2-CH2-NH), 1.32 (2H, m, CH3-CH2-CH2-CH2-NH), 0.89 (3H, t, J = 7.2 Hz, CH3 butyl residue).</td>
</tr>
<tr>
<td>10</td>
<td>7.89 (1H, d, J=1.2 Hz, H-6 T), 6.35 (1H, dd, J = 8.0 and 6.0 Hz, H-1’ T), 5.29 (1H, t, J = 7.6 Hz, olefinic CH-CH2-NH), 5.08 (1H, apparent t, J = 1.2 and 1.6 Hz, olefinic CH-CH2-CH2), 4.51 (1H, m, Hz, H-3’ T), 4.02 (1H, m, H-4’ T), 3.97 (2H, apparent t, J = 4.4 and 2.8 Hz, H2-5 T), 3.48 (2H, t, J = 8.0 Hz, CH2-NH), 2.33-2.15 (2H, m, H2-2’ T), 2.06 (2H, t, J = 7.6 Hz, CH2-CH2), 1.98-1.93 (5H, overlapped signals, CH3 T and CH2-CH2), 1.65, 1.61, 1.58 (3H each, s’ s, 3xCH3).</td>
</tr>
</tbody>
</table>

**31P NMR (CD3OD, 161.98 MHz)**: 11.5. 11.2.
ESI-MS (m/z)

<table>
<thead>
<tr>
<th>found</th>
<th>Calcd. for M</th>
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<tbody>
<tr>
<td>523.47</td>
<td>524.20</td>
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</table>

Retention time: 12.8 min.

**Retention time:**

11

**1H NMR** (CD$_3$OD, 400 MHz): 7.85 (1H, s, H-6 T), 7.36-7.24 (5H, m, aromatic protons Phe), 6.33 (1H, dd, J = 6.0 and 6.0 Hz, H-1' T), 4.60 (1H, m, H-3' T), 4.05 (1H, m, CH$_2$ Phe), 3.95 (1H, m, H-4' T), 3.93 (2H, m, H$_2$-5' T), 3.63 (2H, CH$_3$-Phe), 3.10 (2H, m, CH$_2$-NH), 2.65 (2H, m, CH$_2$-CH$_2$NH), 2.30-2.15 (2H, m, H$_2$-2' T), 1.91 (3H, s, CH$_3$ T), 1.60 (2H, m, CH$_2$-CH$_3$), 0.90 (3H, t, J = 7.2 Hz, CH$_3$).

**31P NMR** (CD$_3$OD, 161.98 MHz): 6.6.

12

**1H NMR** (CD$_3$OD, 400 MHz): 7.84 (1H, s, H-6 T), 7.36-7.17 (5H, m, aromatic protons Phe), 6.32 (1H, dd, J = 8.0 and 6.0 Hz, H-1' T), 4.65 (2H, AB system, CH$_2$-Ph ), 4.41 (1H, m, H-3' T), 3.98 (1H, m, H-4' T), 3.93 (2H, m, H$_2$-5' T), 2.25-2.13 (2H, m, H$_2$-2' T), 1.93 (3H, s, CH$_3$ T).

**31P NMR** (CD$_3$OD, 161.98 MHz): 10.8.