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A Novel Solid Support for Synthesis of 3'-Phosphorylated Chimeric Oligonucleotides Containing Internucleosidic Methyl Phosphotriester and Methylphosphonate Linkages

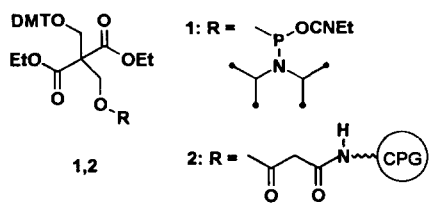
Andrei Guzaev* and Harri Lönnberg

Department of Chemistry, University of Turku, FIN-20014 Turku, Finland

Abstract: A novel solid phase synthesis of 3'-phosphorylated oligonucleotides is described. The chain assembly is carried out by phosphoramidite strategy on solid support **2**, which allows a mild and fast release of the oligonucleotide in solution. The applicability of the method is demonstrated by preparation of 3'-phosphorylated chimeric oligonucleotides containing methyl phosphotriester and methyl phosphonate internucleosidic linkages. © 1997 Elsevier Science Ltd.

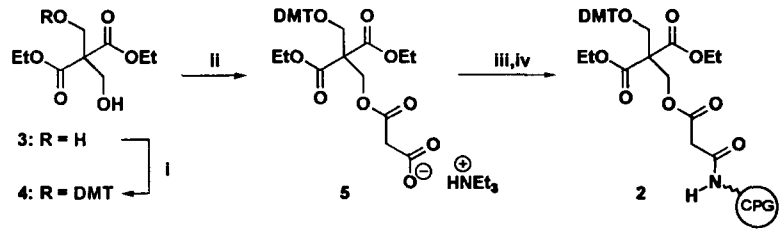
Oligoribonucleotides bearing a 3'-monophosphate group undergo metal-ion-promoted hydrolysis considerably faster than their dephosphorylated counterparts.[1,2] Evidently the 3'-monophopshate group offers a good primary coordination site for the metal ion that then interacts with one of the intrastrand phosphodiester bonds. To learn how stable this kind of macrochelates are, chimeric oligonucleotides that contain, in addition to a 5'-terminal ribonucleotide phophodiester bond and a 3'-terminal monophosphate group, only neutral noncoordinating internucleosidic linkages were required as model compounds. Oligonucleotides consisting of methyl phosphotriester and methylphosphonate bonds were chosen for the purpose. To obtain these structures, a novel method of 3'-phosphorylation had to be developed. Synthesis of 3'-phosphorylated oligonucleotides also is of wider interest, since the 3'-phosphate group allows chemical ligation[3-5] and conjugation of reporter groups at the 3'-terminus.[6]

Several methods for the synthesis of 3'-phosphorylated oligonucleotides have been reported. Usually orthogonal conditions are applied to cleave the oligonucleotide chain from a modified solid support, and the 3'-phosphate group is simultaneously released. Examples include 4,4'-diaminobenzidine[7] and allyl linkers[8] that require *i*-amyl nitrite and a Pd(0) complex as a cleaving reagent, respectively. Alternatively, direct condensation of phosphoramidite to aminoalkyl CPG gives, upon oxidation, a 3'-terminal nucleoside phosphoramidate, which may be hydrolysed to a 3'-terminal phosphate *via* prolonged treatment with 80% aq. acetic acid.[9] None of these methods has yet been applied to preparation of oligonucleotides having a modified backbone. Dithiodiethanol[6,10] or related linkers[11] can also be cleaved under very mild conditions, but the sulfide anion employed may be expected to demethylate methyl phosphotriester oligonucleotide analogues. Linkers based on 2-hydroxyethyl sulfonyl group are routinely used in 3'-phosphorylation of oligonucleotides.[1,7,12,13] In our hands, their stability towards ammonolysis is, however, somewhat higher than that of G^{ib} protection, which renders them incompatible with the preparation of base-labile oligonucleotide analogues. For the same reason, 2-(2-nitrophenyl)ethyl linker,[14] cleavable with DBU, does not appear attractive.



We have previously introduced a new method for chemical synthesis of oligonucleotide 5'-monophosphates,

which is based on phosphoramidite reagent 1.[15] Detritylation of the attached non-nucleosidic unit and subsequent treatment with a weak base release the 5'-phosphate. We now report on a closely related solid support 2 that extends the same phosphorylation strategy to the 3'-phosphates of oligonucleotides and their methylphosphonate and methyl phosphotriester analogues.



Scheme 1 i: DMT-Cl/Py; ii: malonic acid/DCC/Py; iii: H_2N-CPG/DIC/Py; iv: Ac_2O/*N*-methylimidazole/Py/THF.

For the preparation of 2, diethyl 2,2-bis(hydroxymethyl)malonate 3 was selectively dimethoxytritylated to 4, as reported previously[15] (Scheme 1). A malonyl linker, being more base-labile than the commonly used succinyl linker,[16] but less labile than an oxalyl linker,[17] was used to attach 4 to aminoalkylated CPG. Accordingly, 4 was acylated to 5 with malonic acid using *N,N'*-dicyclohexyl carbodiimide as a condensing reagent. After evaporation, the residue was dissolved in methylene chloride and washed with aqueous TEAA (pH 8.5) to remove unreacted malonic acid. At this step, no products of basic hydrolysis (4 or DMT-OH) was detected by TLC. Drying and evaporation gave the crude triethylammonium salt of 5 as a foam, which remained stable at +4°C for several weeks. 5 was immobilized without further purification on beads of long chain aminoalkyl CPG, using *N,N'*-diisopropyl carbodiimide (DIC) as a condensing agent. Varying the reaction time, two batches of solid support 2 were obtained, having a loading of 22 and 60 μmol g^{-1}, respectively (assayed by dimethoxytrityl response[16]). These supports were used in standard (0.2 to 1.0 μmol) and medium (20 to 40 μmol) scale syntheses, respectively.

The applicability of solid support 2 was first verified by running small scale syntheses of oligodeoxyribonucleotides. Two important observations are worth noting. First, initial detritylation of the solid support should be carried out with a solution of trifluoroacetic acid (2% in CH_2Cl_2) for 25-30 s. We found it more convenient to pass 3 to 5 mL of the acid solution manually from a syringe attached to the synthesis column, followed by washing with dry MeCN. Second, while the phosphite triester moiety obtained by coupling a nucleoside phosphoramidite to the detritylated support is moderately stable towards the capping mixture, the

Table 1. Time Required to Release the Oligonucleotide from Solid Support 2 and Deprotect the 3'-Terminal Phosphate.

Conditions	Time,(in min) required for		
	90% cleavage	95% cleavage	3'-phosphate deprotection
Conc. aq. NH_3-H_2O	10	20	20
0.05 M K_2CO_3 in MeOH	40	90	180
50% 1,2-ethanediamine in EtOH	nd	<10	600

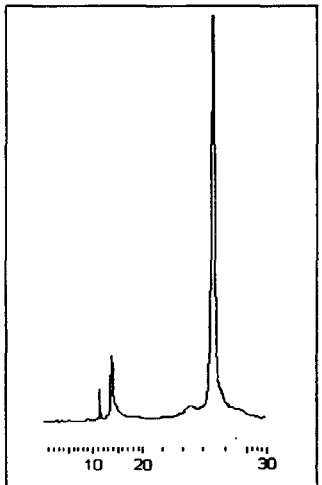
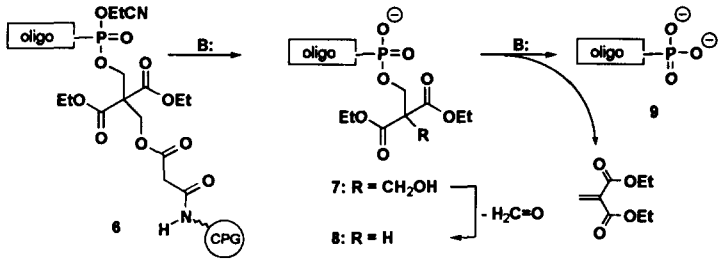


Figure 1. RP HPLC profile of a crude 3'-phosphorylated 27-nt. oligo.

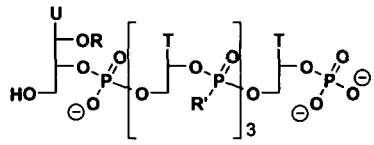
corresponding phosphate triester is completely stable toward preparation of a 15 to 40-nt. oligonucleotide. Consequently, the yield is improved when the first synthetic cycle is reprogrammed to have the oxidation step preceding the capping reaction rather than following it. After standard ammonolysis, the reaction mixtures were analysed by RP (DMT-On) and anion exchange (DMT-Off) HPLC. The main product was found to be identical with that obtained by using commercially available reagents.[13] Moreover, the quality of synthesis was very similar in both cases. As an example, an RP HPLC profile of a crude 27-nt. oligodeoxynucleotide (DMT-CAG TCT ACG ACC ATG ATG TTC GTT CAGp) synthesized on **2** is shown in Figure 1.



Scheme 2

Next, the release of a model oligonucleotide, DMT-$(Tp)_6$, was studied under various conditions compatible with the synthesis of modified oligonucleotides. The results in Table 1 suggest that: (i) in comparison to a succinyl linker, the use of its malonyl analogue results in considerably faster cleavage of oligonucleotide material from the solid support, and (ii) the subsequent degradation of **7** to 3'-phosphorylated **9** (Scheme 2) proceeds considerably less readily than with its 5'-counterpart: the product assigned as **8** appeared as the main peak in all reaction mixtures at early stages of deprotection. Formation of **9** proceeded as fast as cleavage from the solid support in conc. ammonia, took twice as long time in K_2CO_3/MeOH, and required 10 h in 1,2-ethanediamine/EtOH.

Nevertheless, the data obtained show that solid support **2** is suitable for preparation of both methyl phosphotriester[18] and methylphosphonate[19-21] oligonucleotide analogues. Two oligonucleotides, **10** and **11**, were synthesized in 0.2 and 40 μmol scale, and the 5'-terminal DMT protection was removed on the instrument. To obtain the chimeric triester analogue **10**, *tert*-butyl hydroperoxide was employed as an oxidizer,[17] and the capping step was omitted. After chain assembly, the solid support was treated with 0.05 M K_2CO_3 in MeOH for 3 h at room temperature.[18] The methanolic solution was neutralized with Dowex 50 (H^+), which also resulted in removal of the 2'-O-Fpmp protection. The reaction mixture was separated by RP HPLC to give, after desalting, oligonucleotide **10** in 55% yield.[22]



10: R = H, R' = CH_3O; **11**: R = Fpmp, R' = CH_3

Synthesis of methylphosphonate analogue **11** was carried out as recommended in *Ref.*19. Two deprotection schemes were evaluated. A small portion of solid support was treated with 1,2-ethanediamine in EtOH (1:1) for 10 h.[20] Another aliquot was deprotected with conc. aqueous ammonia for 20 min.[21] As no nucleic base deprotection was required, the second step consisting of treatment with 1,2-ethanediamine was omitted. No difference between these two reaction mixtures was found by HPLC analysis, and brief ammonolysis was chosen for deprotection in 40 μmol scale. The product was isolated in 71% yield by HPLC.[22]

The structures of chimeric oligonucleotides **10** and **11** were established by ^{31}P NMR and MALDI/TOF mass spectrometry,[23] using 3-hydroxypicolinic acid as a matrix.[24]

In summary, solid support **2** allows efficient synthesis of 3'-phosphorylated oligonucleotides and their methyl phosphotriester and methylphosphonate analogues.

REFERENCES AND NOTES

1. Kuusela, S.; Azhayev, A.; Guzaev, A.; Lönnberg, H. *J. Chem. Soc. Perkin Trans. 2* **1995**, 1197.
2. Kuusela, S.; Guzaev, A.; Lönnberg, H. *J. Chem. Soc. Perkin Trans. 2* **1996**, 1895.
3. Prakash, G.; Kool, E.T. *J. Am. Chem. Soc.* **1992**, *114*, 3523.
4. Wang, S.; Kool, E.T. *Nucleic Acids Res.* **1994**, *22*, 2326.
5. Dolinnaya, N.G.; Blumenfeld, M.; Merenkova, I.N.; Oretskaya, T.S.; Krynetskaya, N.F.; Ivanovskaya, M.G.; Vasseur, M.; Shabarova, Z.A. *Nucleic Acids Res.* **1993**, *21*, 5403.
6. Asseline, U.; Bonfils, E.; Kurfurst, R.; Chassignol, M.; Roig, V.; Thuong, N.T. *Tetrahedron* **1992**, *48*, 1233.
7. Markiewicz, W.T.; Wyrzykiewicz, T.K. *Nucleic Acids Res.* **1989**, *17*, 7149.
8. Zhang, X.; Jones, R.A. *Tetrahedron Lett.* **1996**, *37*, 3789.
9. Gryaznov, S.M.; Letsinger, R.L. *Tetrahedron Lett.* **1992**, *33*, 4127.
10. Gupta, K.C.; Sharma, P.; Kumar, P.; Sathyanarayana, S. *Nucleic Acids Res.*, **1991**, *19*, 3019.
11. Kumar, P.; Bose, N.K.; Gupta, K.C. *Tetrahedron Lett.* **1991**, *32*, 967.
12. Reintamm, T.; Möller, U.; Oretskaya, T.S.; Shabarova, Z.A.; Lomakin, A.I. *Bioorg. Khim.* **1990**, *16*, 524.
13. Applied Biosystems, Division of The Perkin-Elmer Corporation. *User Bulletin* **1994**, 86.
14. Eritja, E.; Robles, J.; Fernandez-Forner, D.; Albericio, F.; Giralt, E.; Pedroso, E. *Tetrahedron Lett.* **1991**, *32*, 1511.
15. Guzaev, A.; Salo, H.; Azhayev, A.; Lönnberg, H. *Tetrahedron* **1995**, *51*, 9375.
16. Pon, R.T. in "*Protocols for Oligonucleotides and Analogs*", Agrawal, S. (Ed.); *Methods in Molecular Biology*, Humana Press, Totowa, N.J., **1993**, *20*, 465.
17. Alul, R.H.; Singman, C.N.; Zhang, G.; Letsinger, R.L. *Nucleic Acids Res.* **1991**, *19*, 1527.
18. Kuijpers, H.A.; Huskens, J.; Koole, L.; van Boeckel, C.A.A. *Nucleic Acids Res.* **1990**, *18*, 5197.
19. Hogrefe, R.I.; Reynolds, M.A.; Vaghefi, M.M.; Young, K.M.; Riley, T.A.; Klem, R.E.; Arnold, Jr., L.J. in "*Protocols for Oligonucleotides and Analogs*", Agrawal, S. (Ed.), *Methods in Molecular Biology*, Humana Press, Totowa, N.J., **1993**, *20*, 143.
20. Reddy, M.P.; Farooqui, F.; Hanna, N.B. *Tetrahedron Lett.* **1996**, *37*, 8691.
21. Agrawal, S. *Tetrahedron Lett.* **1989**, *30*, 7025.
22. Analysis of oligodeoxyribonucleotides was carried out on a Nucleosil 300-5C18 column, using 0.1 M NH_4OAc as buffer A, 60 % aq. MeCN as buffer B, and a linear gradient from 10 to 70 % B in 30 min; chimeric oligonucleotides were analyzed on a DNASep™ column (Sarasep Inc., CA), using 0.1 M TEAA pH 5.5 as buffer A, 0.1 M TEAA in 60 % aq MeCN as buffer B, and a linear gradient from 10 to 70 % B in 20 min at a flow rate 0.75 mL min^{-1}. Preparative isolations were performed on LiCrospher® 100 RP-18 (10×250 mm, 5 μm: buffer A 0.05 M NH_4OAc; buffer B 60 % aq. MeCN; a linear gradient from 5 to 50 % B in 25 min at a flow rate 3 mL min^{-1}. Desalting was performed by injecting to the same column, then washing with 0.1 M NaOAc (12 min), water (12 min) and eluting **2-4** (Na salts) with 60 % aq. MeCN at a flow rate 3 mL min^{-1}.
23. **10**: ^{31}P NMR (D_2O) δ (H_3PO_4) 0.58 (1P, terminal monophosphate), -1.04 (4P, phosphotriester and -diester). MALDI/TOF: $(M-H)^-$ 1582.4, calcd for $C_{52}H_{70}N_{10}O_{37}P_5$ 1582.04. **11**: ^{31}P NMR (D_2O) δ (H_3PO_4) 35.63 (3P, methylphosphonate), 0.28 (1P, terminal phosphate), -1.15 (1P, phosphodiester); ^{19}F NMR (D_2O) δ (TFA) -123.9. MALDI/TOF: $(M-H)^-$ 1741.3, calcd for $C_{64}H_{84}N_{11}O_{35}P_5$ 1741.29; $(M-Fpmp)^-$ 1533.9, calcd for $C_{52}H_{70}N_{10}O_{34}P_5$ 1534.04
24. Wu, K.J.; Steding, A.; Becker, C.H. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 142.

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