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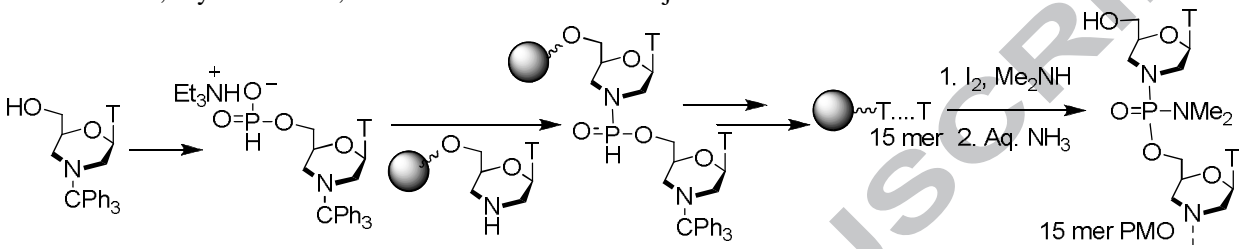

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Synthesis of phosphorodiamidate morpholino oligonucleotides by H-phosphonate method

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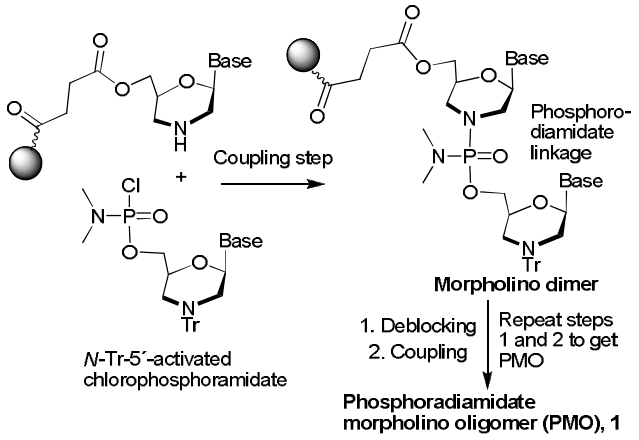
ABSTRACT

Synthesis of T-containing phosphorodiamidate morpholino oligomers (PMO) by H-phosphonate method on solid support has been reported for the first time. Initially, 5-mer then 15-mer of T-containing PMO using H-phosphonate-T monomer was synthesized on polystyrene resin bead. The phosphonamidate backbone was oxidized by iodine-dimethylamine mixture followed by the cleavage from solid support by aqueous ammonia to obtain 15-mer T-containing PMO.

Phosphorodiamidate morpholino oligos (PMO), **1**[1] (Scheme 1) are routinely used for gene silencing as antisense reagents when they are designed targeting 5´ – UTR of mRNA[2] or pre-mRNA splicing.[3] The extraordinary stability, lack of net charge on the internucleosidic linkage backbone, and total resistance to metabolic degradation has made this class of molecules worthy of consideration as potential therapeutics. Phosphorodiamidate morpholino oligos have entered into clinical trial unusually fast for the treatment of Duchenne muscular dystrophy and flaunted impressive safety and pharmacokinetics records during Phase I/II clinical trials.[4] Solid phase synthesis of PMO is reported by Gene Tools in their patent[5] using *N*-tritylated 5´-activated chlorophosphora-midate morpholino monomers to get the desired length of PMO (25 mer) for antisense properties (Scheme 1). The coupling time between two monomers was not efficient in the presence of large excess of *N*-ethylmorpholine, accordingly, Sekine, M. *et. al.* reported[6] another method to improve the coupling efficiency using LiBr as an additive and has shown its application only for the synthesis of 2-mer PMO in both solution and solid phase.

We have been working on the morpholino chemistry[7] for the past several years where we realized that the stability of activated 5´-chlorophosphoramidate morpholino monomer is poor in solution particularly in the presence of base. Our goal was to develop the solid supported synthesis of PMO using a chemistry which is compatible to a DNA synthesizer where coupling time is efficient and activated monomers are stable. H-phosphonate chemistry is well known for DNA synthesis[8] however, such chemistry has not been explored in the synthesis of PMO.



Scheme 1. Solid phase synthesis of phosphorodiamidate morpholino oligonucleotides (PMO).

In this communication, we wish to report the synthesis of T-morpholino H-phosphonate and its use in the synthesis of 15-mer of T-containing PMO on solid support.

T-morpholino monomer **2** was synthesized using our previously reported method.[7a] Monomer **2** was then converted to H-phosphonate **3** according to the procedure reported for nucleosides[8] by treatment with PCl_3 and 1,2,4-triazole followed by quenching with triethylammonium bicarbonate buffer (TEAB, pH 8.5)[9] **(Scheme 2)**. The compound was purified by silica gel

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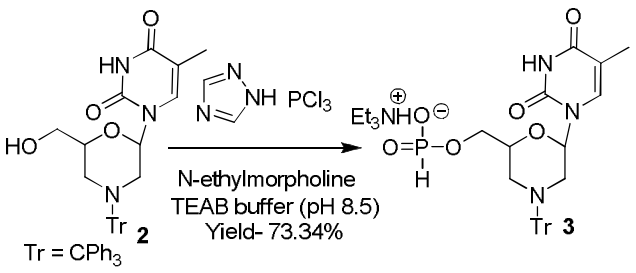
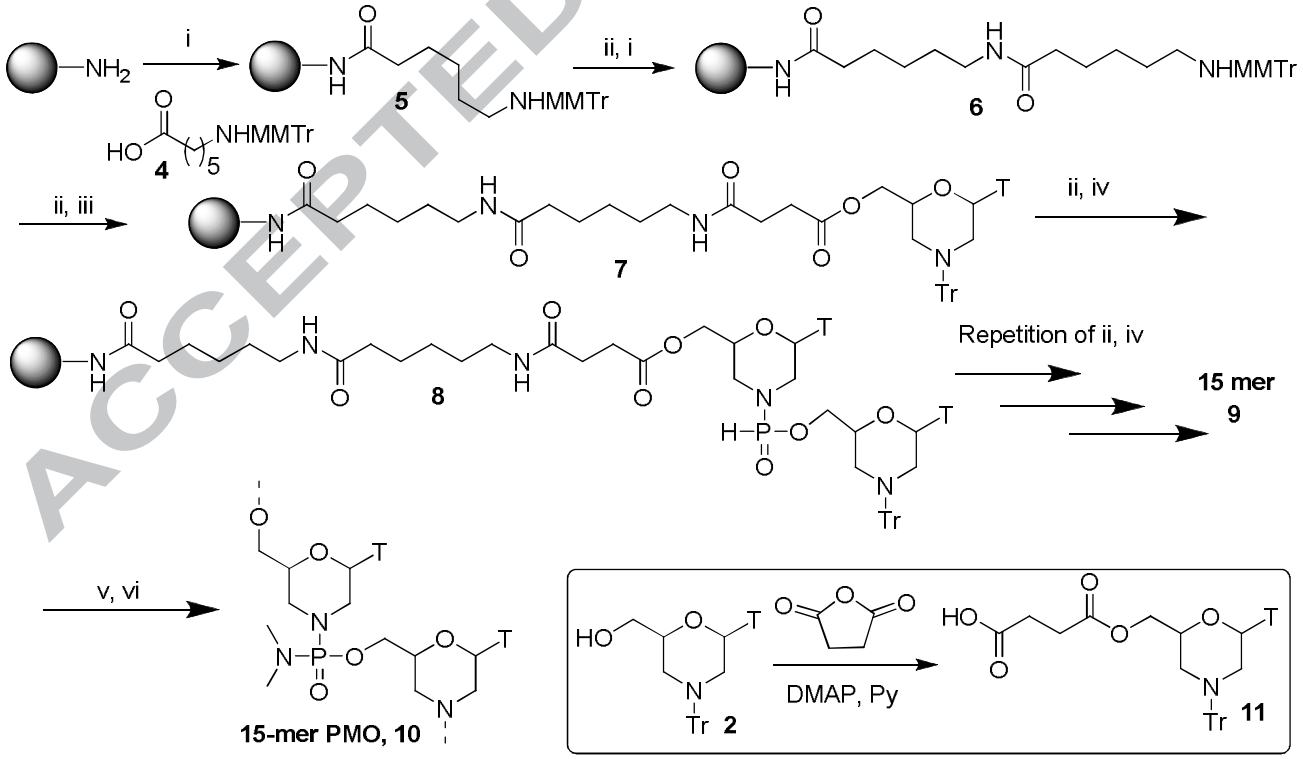
column chromatography and characterized by spectroscopic techniques.



Scheme 2. Synthesis of H-phosphonate T monomer.

In order to perform the solid supported synthesis, Novasyn TG-amino resin (2 mg, 0.52 µmol, 0.26 mmol/g, pore size 130µm) was suspended in *N*-methyl-2-pyrrolidone (NMP) and allowed to swell for 12 hrs. It was then washed five times with NMP. Then a solution of MMTr protected amino caproic acid **4** (5 equiv.), HOBT (5 equiv.), HBTU (5 equiv.), *N*-ethylmorpholine (NEM, 15 equiv.) in NMP (50 µL) was added. The resin was suspended in this solution and vortexed for 12 hrs. The resin was then washed with NMP to remove the excess starting material/reagents and capped with a freshly prepared solution of 10% Ac_2O and 10% DIPEA in NMP for 2 min with vortexing. Resin beads were then washed with NMP. To evaluate the coupling yield, resin was washed with 1.5 ml of CYPTFA[6] (3-cyano pyridine 300 mg, TFA 100 uL, CF_3CH_2OH 3.5 ml, DCM 15 ml) for 2–3 min. All the flow through resin was collected, diluted 10-fold with concentrated methanesulphonic acid and the yield was calculated based on the measurement of the absorbance of MMTr cation by UV-VIS spectrophotometer (λ= 472 nm, ε = 51,100 cm^{-1} M^{-1}).

The first linker was attached in 99 % yield. Similarly the second linker was attached to obtain **6** in 99 % yield. The *N*-Tr-T-morpholino monomer **2** was functionalized with succinic anhydride to obtain the succinic ester **11**. Compound **11** was purified by silica gel column chromatography to obtain 66% yield. **11** was then loaded on the functionalized polystyrene resin beads **6** after MMTr deprotection using HOBt (5 equiv.), HBTU (5 equiv.), NEM, 15 equiv.) in NMP (50 µL) and vortexed for 12 hrs. The unreacted amine of the beads was capped with acetyl group by treatment with acetic anhydride. After trityl deprotection, the loading yield was calculated and found to be 96 %. Free amine of **7** was used for coupling with H-phosphonate T **3**. For the coupling, 50 µL solution of **3** (20 equiv.), *p*-nitrophenol (20 equiv.), 2,4,6-trichlorophenol (20 equiv.) and pivaloyl chloride (20 equiv.) in 1:1 mixture of pyridine-acetonitrile was added to trityl deprotected resin **7** and vortexed for 10 min. The resin was then washed with acetonitrile (4 ml) to eliminate the excess reagents and capped with capping reagent (1 ml). The resin was again washed with DCM (2 ml) and deblocked using deblocking reagent (1.5 ml, 2–3 min). The first coupling of T (dimer) was obtained in 77 % yield. It was then washed with 30 % pyridine-acetonitrile (2 ml) and the cycle was repeated to get initially the desired 5 mer (TTTTT) with phosphonamidate backbone. This oligomer was oxidized to PMO using 0.1 M I_2/ 2 M Me_2NH in THF (5 min vortexed). After that the resin was washed with THF (2 ml) and water (2 ml). Finally PMO was cleaved from the solid support using 33 % aqueous NH_3 (0.5 ml) at 40 °C for 12 hrs.[10] NH_4OH solution (liquid phase) was lyophilized to obtain the desired PMO and the pentamer phosphorodiamidate oligomer was characterized by ESI mass. Employing the same protocol, we synthesized the longer 15-mer T-containing oligomer **10**. The lyophilized crude product was dissolved in water and six volume excess chilled acetone was added, mixed well and a precipitate was formed which was centrifuged to get a pellet.



Scheme 3. Solid phase synthesis of PMO. Reagent and conditions: (i) **4**, HOBT, HBTU, NEM, NMP; (ii) 3-Cyanopyridine, TFA, TFE, DCM; (iii) **11**, HOBT, HBTU, NEM, NMP; (iv) **3**, *para*-nitrophenol, 2,4,6-trichlorophenol, pivaloyl chloride, pyridine, acetonitrile; (v) 0.1 M I_2 / 2 M Me_2NH in THF; (vi) Aqueous NH_3

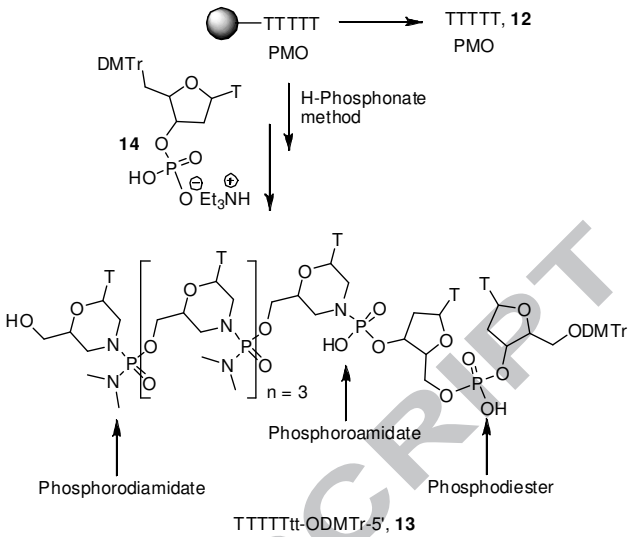
The resulting pellet containing product **10** was re-dissolved in water and transferred into a dialysis bag with a 600 MWCO which was gently shaken in water for 12 hrs. The dialyzed product **10** was lyophilized to get final 15mer PMO as a white powder and was characterized by ESI mass. The yield was measured by UV-VIS spectrophotometer at absorbance 263 nm to obtain 0.27 µmol (from 0.52 µmol resin beads) (Scheme 2).
Except first coupling on solid support, the coupling yield from 3rd to 15th mer was 98 to 100% using H-phosphonate method. To the best of our knowledge, we are the first to report the synthesis of PMO by H-phosphonate method. Gene Tools synthesis of PMO using chlorophosphoramidate method is their patented technology[5] and PMO is only commercially available with them for biological applications.
15-Mer PMO was characterized by ESI mass and analyzed by their fragmentation pattern. In every peak of mass spectrum, there is a loss of one $N(Me)_2$ from the $[M + 4Na]^{4+}$ (Table 1) which is supported by the literature[11] when compared with the analysis of ESI mass spectral data of phosphoramidate.

Table 1. Positive-ion ESI-MS data of 15-mer PMO

Entry	Mol. formula $(M + Na)^+$	Observed mass (%)[a]
1	$C_{178}H_{281}N_{59}O_{74}P_{14}Na_4$	1230.3219 (22)[b]
2	$C_{176}H_{275}N_{58}O_{74}P_{14}Na_4$	1186.2678 (22)
3	$C_{174}H_{269}N_{57}O_{74}P_{14}Na_4$	1142.1805 (32)
4	$C_{172}H_{263}N_{56}O_{74}P_{14}Na_4$	1098.1792 (40)
5	$C_{170}H_{257}N_{55}O_{74}P_{14}Na_4$	1054.0815 (53)
6	$C_{168}H_{251}N_{54}O_{74}P_{14}Na_4$	1010.0498 (55)
7	$C_{166}H_{245}N_{53}O_{74}P_{14}Na_4$	966.0043 (52)
8	$C_{164}H_{239}N_{52}O_{74}P_{14}Na_4$	921.9444 (68)
9	$C_{162}H_{233}N_{51}O_{74}P_{14}Na_4$	877.8767 (78)
10	$C_{160}H_{227}N_{50}O_{74}P_{14}Na_4$	833.8475 (95)
11	$C_{158}H_{221}N_{49}O_{74}P_{14}Na_4$	789.8019 (100)
12	$C_{156}H_{215}N_{48}O_{74}P_{14}Na_4$	745.7612 (98)
13	$C_{154}H_{209}N_{47}O_{74}P_{14}Na_4$	701.7156 (58)
14	$C_{152}H_{203}N_{46}O_{74}P_{14}Na_4$	657.6720 (57)
15	$C_{150}H_{197}N_{45}O_{74}P_{14}Na_4$	613.6161 (51)

[a] *m/z* with relative abundance (%) in parentheses. Calculated mass for $C_{178}H_{281}N_{59}O_{74}P_{14}Na_4$ 1238.6489

According to Gene Tools, PMO is purified by precipitation method which is good enough for biological applications. However Tang *et al*[12] purified the caged PMO by HPLC using Dionex DNAPac column, though elution time is in below 4 min. Again Sekine *et al*[6] purified the dimer PMO using reversed-phase HPLC though solvent gradient was not mentioned. In order to know the purity of our PMO, accordingly, we have synthesized both 5-mer PMO and a PMO-DNA chimer of a sequence, TTTTT **12** and TTTTTtt-ODMTr **13**, respectively. First 5-mer PMO was synthesized using our method.[10] After, trityl deprotection, H-phosphonate of T-deoxyribonucleoside **14** was coupled using Froehler's method[8] to get a 7-mer of PMO-DNA chimer **13** in which last two linkages were phosphoramidate and phosphodiester (Scheme 4). The compound was cleaved from the resin by 33% ammonium hydroxide solution at 40°C for 5 hr. The material was lyophilized and washed with acetonitrile to get a white powder. The 7-mer chimer **13** synthesis indicated that in PMO synthesis, the sequence can be modified as per need.



Scheme 4: Synthesis of PMO and PMO-DNA chimer using H-phosphonate method

To check the purity of PMO, we used the C-18 column. Interestingly, 5-mer PMO **12** was eluted in 5.75 min when 5 to 40% acetonitrile in 0.1 M NH_4OAc, pH 7.2, 25 min was used as a solvent gradient (Figure 1). To confirm the retention time with the length of PMO, we then injected Gene Tools 25 mer PMO (5'-GTGATTCGATTCAAGATGCCGTCTC-3') as a control in C-18 column under the same conditions where it was eluted in 30 min.

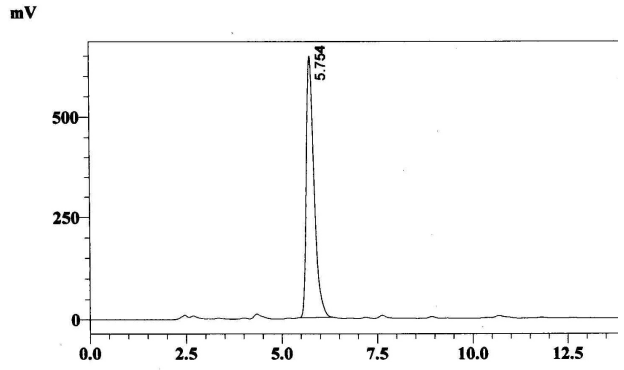


Figure 1: HPLC Chromatogram of PMO-5 mer **12**. C-18 analytical column (4.6 mm × 250 mm, 5 µm), run by acetonitrile 5 to 40% in 0.1 M NH_4OAc, 25 min, 1 mL/min. λ = 260 nm. Retention time = 5.75 min.

In case of PMO-DNA chimer **13**, the best possible HPLC chromatogram was obtained when solvent gradient 20 to 50% acetonitrile in 0.1 M NH_4OAc buffer, pH 7.2, 30 min was used (Figure 2).

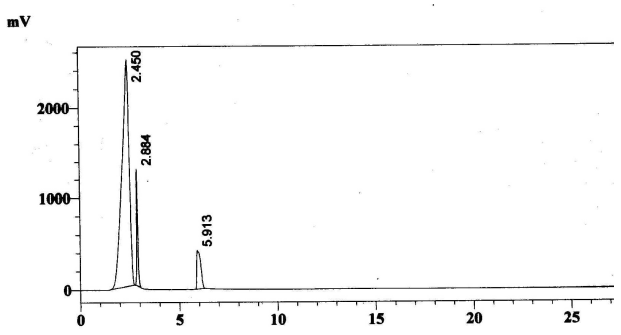


Figure 2: HPLC Chromatogram of **13**. Flow rate 1 mL/min. λ = 260 nm. Retention time = 2.45 min.

The peak at 5.913 min was the truncated 5-mer PMO **12** and the major fraction at 2.45 min was liophilized and ESI mass indicated the desired product **13**. ESI mass analysis has shown the masses of **13** with DMTr: *m/z* = 1235.29 (15%), calcd for $C_{99}H_{137}N_{23}O_{40}P_6$ $(M + 2H)^{2+}$: 1236.9; 1195.54 (20%), calcd for $C_{95}H_{125}N_{21}O_{40}P_6$ $(M - 2NMe_2 + 2H)^{2+}$: 1192.84; 656.88 (100%), calcd for $C_{69}H_{94}N_{19}O_{38}P_6$ $(M - 4NMe_2 + 3H)^{3+}$: 660.81 and 469.54 (98%), calcd for $C_{91}H_{116}N_{19}O_{40}P_6$ $(M - 4NMe_2 + 5H)^{5+}$: 460.12, respectively.

In summary, the synthesis of H-phosphonate of T morpholino monomer and its use in phosphorodiamidate morpholino oligomer (PMO) synthesis on solid support has been shown for the first time. This method is unique in comparison to Gene Tools chlorophosphoramidate method because it can be transferred to the DNA synthesizer where H-phosphonate chemistry is already used for DNA synthesis. H-phosphonate T morpholino monomer **3** is stable in solution and unlike ribonucleoside H-phosphonate, **3** is not hygroscopic in nature. The coupling was done in acetonitrile-pyridine solvent which is normally used during DNA synthesis. Except Gene Tools chlorophosphoramidate chemistry, this is a second method for PMO synthesis. We are now exploring this method to the synthesis of PMO with mixed sequences of A, G, C, T and will be published in due course.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at

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9) *Preparation and characterisation of H-Phosphonate T* **3***:* To a stirred solution of PCl_3 (10.34 mmol) and *N*-ethyl morpholine (103 mmol) in dry DCM was added 1, 2, 4-triazole (35.04 mmol) and stirred at room temperature for 30 min. DCM solution of **2** (2.06 mmol, dried by co-evaporation from CH_3CN) was then added dropwise at 0° C. The reaction mixture was stirred at 0° C for 10 min (until TLC showed complete consumption of starting material). The reaction mixture was poured into 80 ml of 1M TEAB buffer (pH 8.57) and stirred for 5 min and then separated in a separating funnel. The aqueous layer was extracted with DCM (2 × 100 ml). The combined organic part was dried over Na_2SO_4 and concentrated under reduced pressure. Crude product was purified by column chromatography in 100–200 mesh silica gel packing with 2% Et_3N – DCM and eluting with 2% Et_3N/ 8% MeOH–DCM. Yield: 73.34% (980 mg), R_f = 0.45 (10% MeOH–DCM). IR (neat/$CHCl_3$): *v* 3444, 2979, 2677, 2493, 1699, 1471, 1392, 1259, 1217, 1054 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.23 (9H, t, *J* = 7Hz), 1.29–1.39 (2H, m), 1.70 (3H, s), 2.96 (6H, q, *J* = 7Hz), 3.06 (1H, d, *J* = 11.5Hz), 3.23 (1H, d, *J* = 11Hz), 3.44 (1H, q, *J* = 11Hz), 3.73–3.80 (2H, m), 4.24–4.26 (1H, m), 5.74 (1H, d, *J* = 359.5Hz), 6.02 (1H, d, *J* = 8.5Hz), 6.96 (1H, s), 7.07–7.08 (3H, m), 7.17–7.20 (6H, m), 7.36 (6H, br s), 8.03 (1H, s), 9.45 (1H, bs), 11.82 (1H, br s). ^{13}C NMR (125 MHz, $CDCl_3$): δ 9.4, 12.2, 45.6, 49.2, 51.7, 52.6, 64, 76.6, 80.1, 110.2, 126.3, 127.6, 128.99, 135.3, 146.5, 150, 163.99. ^{31}P NMR (200 MHz, $CDCl_3$): δ 4.56 (PPh_3 –4.802). HRMS (ESI) $(M + H)^+$ calculated for $C_{35}H_{46}N_4O_6PH^+$ = 649.32 found 649.3147
10) *Solid phase synthesis cycle:* Morpholino H- Phosphonate (**3**) was dried by co evaporating with dry CH_3CN and dissolved in anhydrous Pyridine/CH_3CN (1/1). Synthesis was performed in plastic frit using the following protocol.

Synthesis Cycle (Scheme 2) (ii, iv)

1) *Washing* – dry CH_3CN (2 ml)
2) *Capping* – 10% Ac_2O- CH_3CN / 10% Pyridine CH_3CN (1:1, 1 ml).
3) *Deblocking* – CYPTFA (1.5 to 2 ml)
4) *Coupling* – **3** (20 equiv), *para*-nitrophenol (20 equiv), 2,4,6-trichlorophenol (20 equiv), pivaloyl chloride (20 equiv) in CH_3CN- Pyridine (1/1) (10 min)
5) Repeated above steps 1- 4 until the desired oligonucleotide sequence was completed.
6) *Oxidization* – 0.1 M I_2 / 2 M Me_2NH in THF for 5 min.
7) *Deprotection* – Aqueous NH_3, 40°C, 12 hrs.

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