Synthesis of Phosphorodiamidate Morpholino Oligonucleotides Using Trityl and Fmoc Chemistry-A New Method Amenable to Automated Synthesizer

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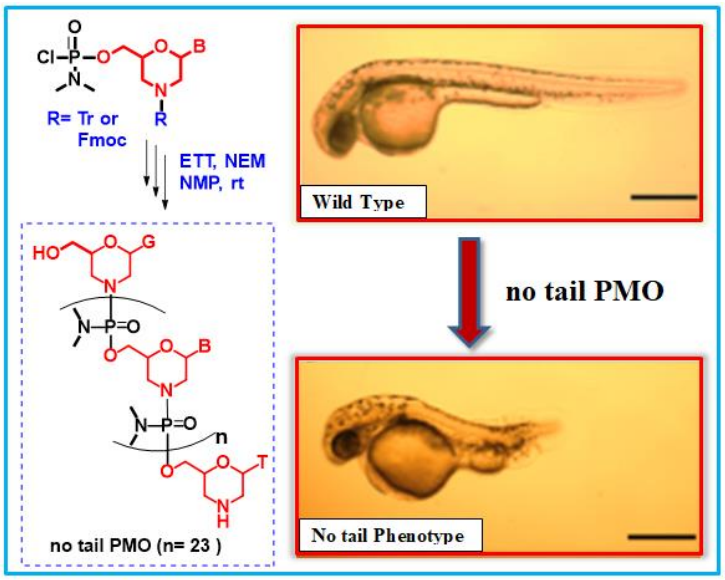
Abstract: Phosphorodiamidatemorpholino oligonucleotides (PMO) are routinely used for gene silencing and the recently developed PMO-based drug "Exondys51" has highlighted the importance of PMO as excellent antisense reagents. However, the synthesis of PMO has remained challenging. Here a method for the synthesis of PMO using either trityl or Fmoc-protected active morpholino monomers using chlorophosphoramidate chemistry in the presence of a suitable coupling agent on a solid support has been reported. After screening several coupling agents (tetrazole, 1,2,4-triazole, ETT, iodine, LiBr and dicyanoimidazole), ETT and iodine were found to be suitable for efficient coupling. Fmoc chemistry was not known for PMO synthesis because the preparation of Fmoc-protected chlorophosphoramidate monomers was not trivial. Synthesis of Fmoc-protected activated monomers and their use in PMO synthesis is reported for the first time. 25-mer PMO has been synthesized using both the methods and validated *in vivo* in the zebrafish model by targeting the *no tail* gene. Methods have been transferred in DNA synthesizer which has become user friendly for PMO synthesis and opened a new avenue to explore PMO for various applications. Fmoc chemistry could be suitable for scalable approach of PMO synthesis using peptide synthesizer as it is a neutral oligomer like peptide.



Introduction

Phosphorodiamidatemorpholino oligonucleotides (PMOs, Figure 1)[1,2] are very promising antisense

agents which are routinely used for gene silencing. They work through binding to the complimentary mRNA by virtue of Watson-Crick base pairing with very high sequence specificity.[3,4] Among the recently developed antisense reagents such as 2'-O-Methyl (2'-OMe),[5] 2'-O-Methoxyethyl (2'-O-MOE),[6] PMO,[1] locked nucleic acid (LNA),[7,8] peptide nucleic acid (PNA),[9] tricyclo-DNA (tcDNA),[10] unlocked nucleic

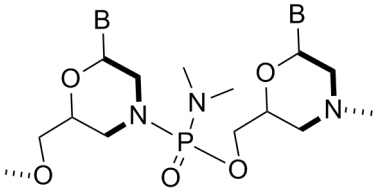


Figure 1: Structure of PMO

acid (UNA)[11] and 2'-OMe phosphorothioRNA,[12] PMO is considered as a better antisense agent because of the neutral backbone, water solubility, less toxicity and sufficient endonuclease stability.[13] Recently it has drawn attention to the scientific community because Exondys 51, a PMO based oligo has got clinical approval from FDA for the treatment of Duchenne muscular dystrophy (DMD).[14] PMO is globally supplied by Gene Tools[2] for research purpose. The existing protocol of PMO synthesis using chlorophosphoramidate chemistry involves a long coupling time which is a major concern in terms of stability of trityl-protected chlorophosphoramidate active monomers. Though, Sekine *et. al.* have reported[15] the improvement of coupling efficiency in presence of LiBr, however, large quantity of LiBr (6 to 20 equiv) was required when coupling was carried out on polymer support even that has also not reached to 100 % efficiency after 1 hr. Our report[16] on PMO synthesis using LiBr method took 2 hr for each coupling to complete the synthesis of 10-mer oligo and the coupling efficiency was monitored by trityl assay color. Therefore it became a problem for a longer oligomer synthesis particularly 25-mer, normally used for antisense application. To complete the 25-mer synthesis, 2 x 25 = 50 hrs is required only for coupling. Moreover, solubility of activated monomers is also a major concern. They are easily soluble in DMF or NMP (*N*-methyl-2-pyrrolidone) but at the same time stability was poor. In the case of CH_3CN, stability was better but solubility was poor especially when argon was purged into the solution. Therefore every time a freshly prepared solution was used for coupling in the solid phase synthesis which is not practical if the synthesis is done in automated synthesizer. Another important step is the deblocking of Tr group which takes longer time in 3% TCA in DCM. To overcome the problems, recently, a suitable condition has been developed for automated fast flow synthesis of PMO using LiBr chemistry at 90ºC with a hope to meet the demand of PMOs application for clinical research.[17] It is important to mention that active chlorophosphoramidate monomers used for the synthesis of PMO, are prepared by only two methods[1,18] in which our method of preparation[18] in presence of LiBr and DBU has become the only available method to the scientific community and also for commercial source of pharma companies. Due to the poor stability of Tr or MMTr-protected chlorophosphoramidate monomers, we then explored the H-phosphonate chemistry for the synthesis of PMO.[19] Recently, Marvin *et. al.*[20] has reported the PMO synthesis using phosphoramidite chemistry by DNA synthesizer. Though PMOs are oligonucleotides and unlike DNA, PMO is neutral molecule like peptide, however, to the best of our knowledge, nobody has reported the PMO synthesis using Fmoc chemistry.

With our continuous effort for developing a new and efficient method for PMO synthesis, we have found the gateway for the successful synthesis of PMO. Herein we report the solid phase synthesis of PMO by both Tr-chemistry and Fmoc chemistry using their corresponding chlorophosphoramidate monomers and improve the coupling efficiency in presence of ETT or iodine. For the validation of protocol in biological application, 25-mer antisense PMO targeting zebrafish *no tail* gene has been synthesized using both the methods and compared the phenotypic characterization and toxicity level of both the methods. Furthermore the protocol of Tr-chemistry has been transferred into DNA synthesizer for the synthesis of oligomers.

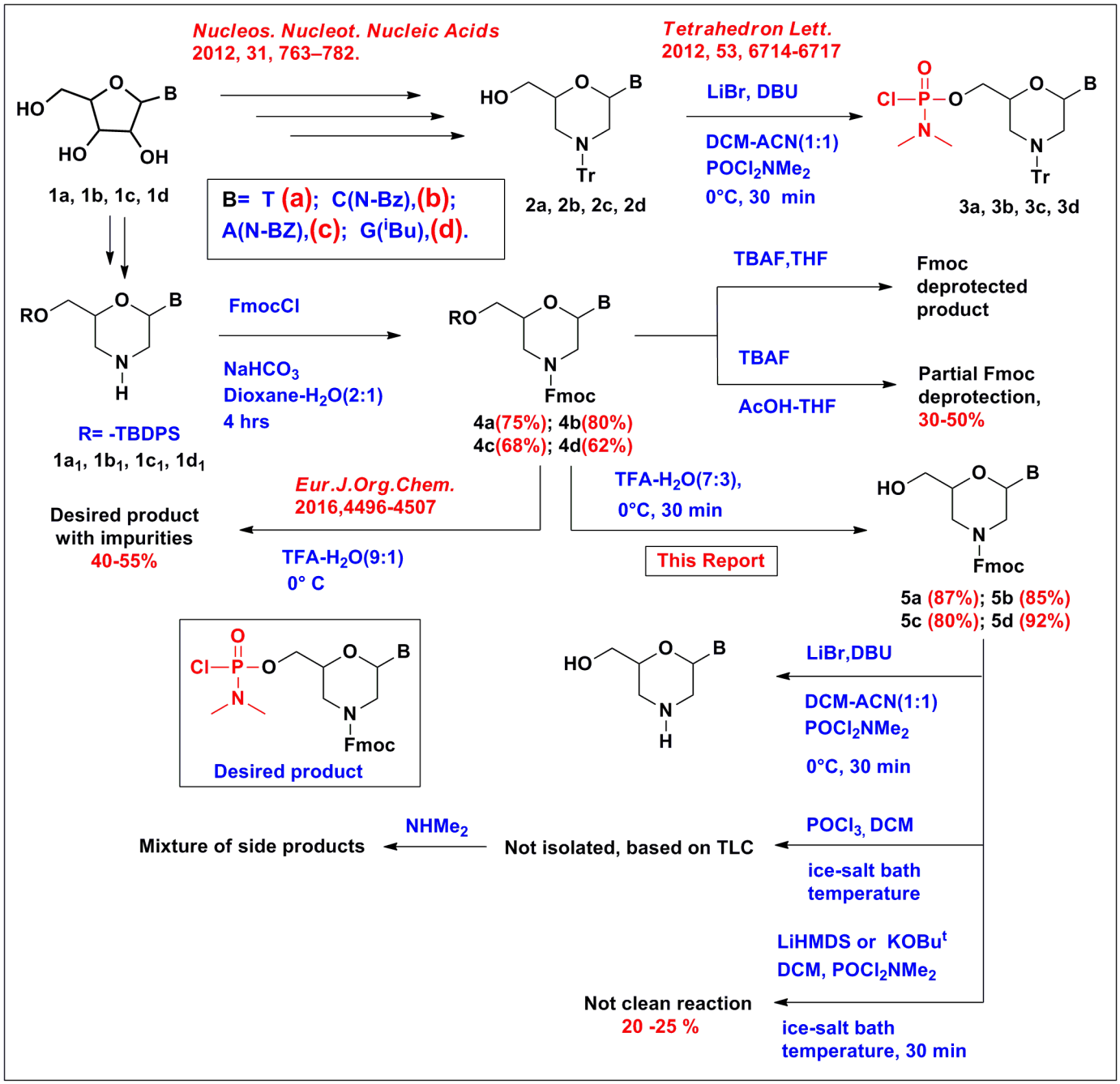
Results and Discussion

In order to synthesis the PMO, we required morpholino monomers. As per our previous report, we have synthesized Tr-protected monomers (**2a**, 2**b**, 2**c,** 2**d**)[21,22] and Tr-protected active monomers (**3a**, 3**b**, 3**c**, 3**d**)[18] (Scheme 1). Fmoc- and TBDPS-protected monomers **4a, 4b**, **4c** and **4d** were easily synthesized following our previously reported Tr-monomer synthesis procedure[21] (Scheme 1). However, TBAF-mediated selective removal of TBDPS group was a problem because Fmoc was also deprotected under this condition. There is a report where acetic acid was used as a buffer to reduce the basicity of TBAF[23] and under this condition, we obtained partially deprotected products in 30 to 50 % yields. We followed the protocol published by Collins *et. al.*[24] to use 9:1 mixture of TFA:H_2O and obtained the products in 40 to 55 % yields with the impurities. In the case of nucleobase A, this impurity was more. Purification of the product became difficult as the polarity of the impurity was very close to the desired product. We then standardized with 7:3 mixture of TFA:H_2O to obtain clean desired products **5a, 5b, 5c** and **5d** in 80–92% yields (Scheme 1). Prolong reaction time again gave side products. Reaction need to be monitored by TLC and was conducted at 0°C.

Optimization of Fmoc protected active monomer synthesis:

After having the Fmoc-protected monomers **5a**, **5b**, **5c** and **5d**, we then became interested to make the active monomers **6a**, **6b**, **6c** and **6d** in presence of DBU/LiBr using our previously reported protocol.[18] Unfortunately, we ended up with many undesired products with the deprotection of Fmoc group in the presence of DBU as its pKa is ~13.5. It is also important to mention that this method did not work earlier when morpholino ″N″ was protected with Fmoc like eletron withdrawing group benzoyl (Bz).[18] Our attempt to use other organic bases such as DIPEA, NEM was not successful to get the desired product. We then attempted to make these monomers starting from $POCl_3$, though a new spot was appeared in TLC, however after the addition of Me_2NH in the reaction mixture, we observed the deprotection of Fmoc group in TLC which led to the formation of several spots. Hence we could not isolate the desired product. We also tried the reaction with LiHMDS or KOBu^t except in the case of LiHMDS, we could isolate the product in 20 to 25% yield and in the case of Fmoc-morpholinocytidine (**5b**), the yield was 51% though the reproducibility of the reaction was poor.

We then envisioned that if we increase the reactivity or electrophilicity of phosphorous center of *N,N*-dimethyl-phosphoramic dichloride perhaps product the addition of CF_3COOAg however, no active monomer was formed.



Scheme 1: Synthesis procedure of monomers

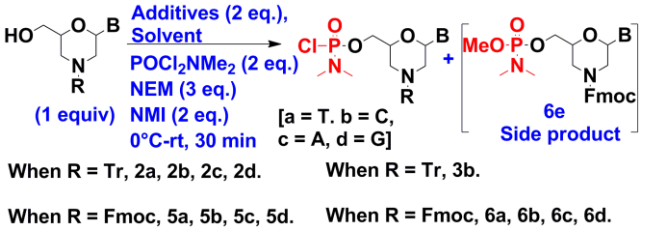
could be formed under a mild condition. Our attempt to use silver trifluoroacetate (CF_3COOAg) hypothesizing that Ag^+ will have affinity to the Cl of phosphorous reagent which will facilitate the formation of active monomer in presence of milder base. Though we observed a white precipitation immediately after

We then moved away a bit and decided to check the efficiency of few coupling agents having protonation property which are generally used in oligo synthesizer. Our initial standardization started with Tr-protected C monomer **2b**. Accordingly, *N,N*-dimethylphosphoramic dichloride (2 eq) was added to

a solution of monomer **2b** (1 eq) in (1:1) DCM-CH_3CN in presence of *N*-ethylmorpholine (NEM, 3 eq) and ETT (2 eq). There was no formation of active monomer even after 15 minutes stirring of the reaction. Then we added 2 eq of *N*-methylimidazole (NMI). Interestingly, we observed the disappearance of the starting material **2b** along with the formation of active monomer **3b** within 15 minutes after the addition of NMI and isolated yield of **3b** was 71% (Entry 1 in Table 1) with the contamination of phosphorous reagent as an impurity (Section VII, Spectral data), which was confirmed by ^{1}H and ^{13}C NMR. There was also extra peak (δ 0.43, 0.73) in ^{31}P NMR except desired peak of **3b** at 18.24 and 18.61. Interestingly, the contamination of ETT with **3b** did not create any problem in dimer synthesis (vide infra). Significant amount of **2b** was unreacted as per TLC when less equivalent of ETT (1.1) and phosphorous reagent (1.25 eq) were added keeping NMI and NEM same. 1,2,4-Triazole was inferior than ETT as ~50 % starting morpholino OH was left (TLC) (Entry 2). With the standardized conditions, we became encouraged to use this protocol on Fmoc-protected monomers (**5a-d**). Unfortunately, trace amount of products from 5 to 20% were obtained in presence of ETT when (1:1) DCM-CH_3CN solvent was used (Entry 3). Yield was not improved when tetrazole was used (Entry 4). In contrast to earlier reaction, a clean product from 40 to 60% yields were obtained when the reaction was carried out in DCM only (Entry 5). A slightly polar side product (**6e**) was formed as a major product in case of CH_3CN-DCM solvent. However **6e** was minimized when DCM was used as a solvent (TLC, Figure S1).

This minor side product **6e** was properly characterized (Section VII, spectral data). Unlike **3b**, the reagent contamination was removed during column purification of **6a-d**. All the monomers showed a single peak in HRMS, except **6d** a 100% peak at 639.1973 appeared along with the molecular ion peak $[M + H]^+$ at 684.2101. The 639.1973 peak corresponded to the fragment of $[M\text{-}NMe_2]$. In presence of other additives triazole, DCI (dicyanoimidazole) and iodine (I_2), the yield was not improved (Entries 6 to 9). Excess of NMI (3 equiv) was detrimental for the reaction where desired product was decomposed. In case of iodine, the product was formed in less than 15 minutes in the presence of only 5 equiv of NMI without using any NEM (Entry 9). DCM was found to be the best solvent than DCM-CH_3CN mixture to obtain the highest yields for Fmoc protected active monomers (Entry 5).

Table 1: **Screening the reaction conditions for active monomer synthesis using trityl and Fmoc chemistry**

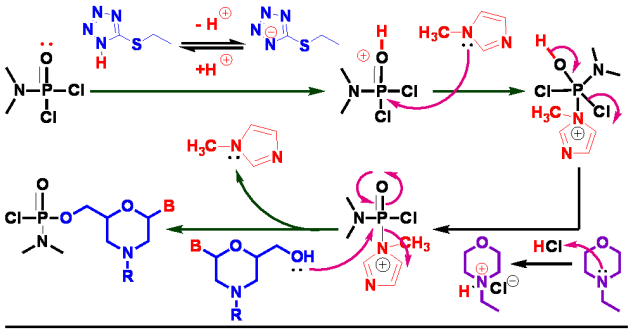


Entry	Additives	Solvent	Product (% of Yield)
1	ETT	DCM-ACN(1:1)	3b (61)[a]
2	1,2,4-Trizole	DCM-ACN(1:1)	3b (34)[a]
3	ETT	DCM-ACN(1:1)	6a, 6b, 6c, 6d (5-20)[b]
4	Tetrazole	DCM-ACN(1:1)	6a, 6b, 6c, 6d (5-10)[b]
5	ETT	DCM	6a(54)[a], 6b(60)[a], 6c(46)[a], 6d(40)[a]
6	Tetrazole	DCM	6a(18)[a], 6b(20)[a], 6c(10)[a,b], 6d(9)[a,b]
7	DCI	DCM	6a, 6b, 6c, 6d (8-10)[b]
8	1,2,4-Triazole	DCM	6a, 6b, 6c, 6d (5-10)[b]
9	I_2	DCM	6a (26)[a,c], 6b(28)[a,c], 6c, 6d (10-20)[b,c]

Table 1: Entries **1, 2,** starting material **2b** was used. Entries **3** to **9**, starting materials **5a**, **5b**, **5c** and **5d** were used.[a)] Isolated yield**,** based on NMR, **3b** was

61%. [b]approximate yield based on TLC, [c]NMI 5equiv, No NEM was used.

A plausible mechanism has been proposed in scheme 2.



Scheme 2: Plausible mechanism for the ETT-NMI mediated activation of Fmoc protected monomer.

Solubility and stability of the active monomers:

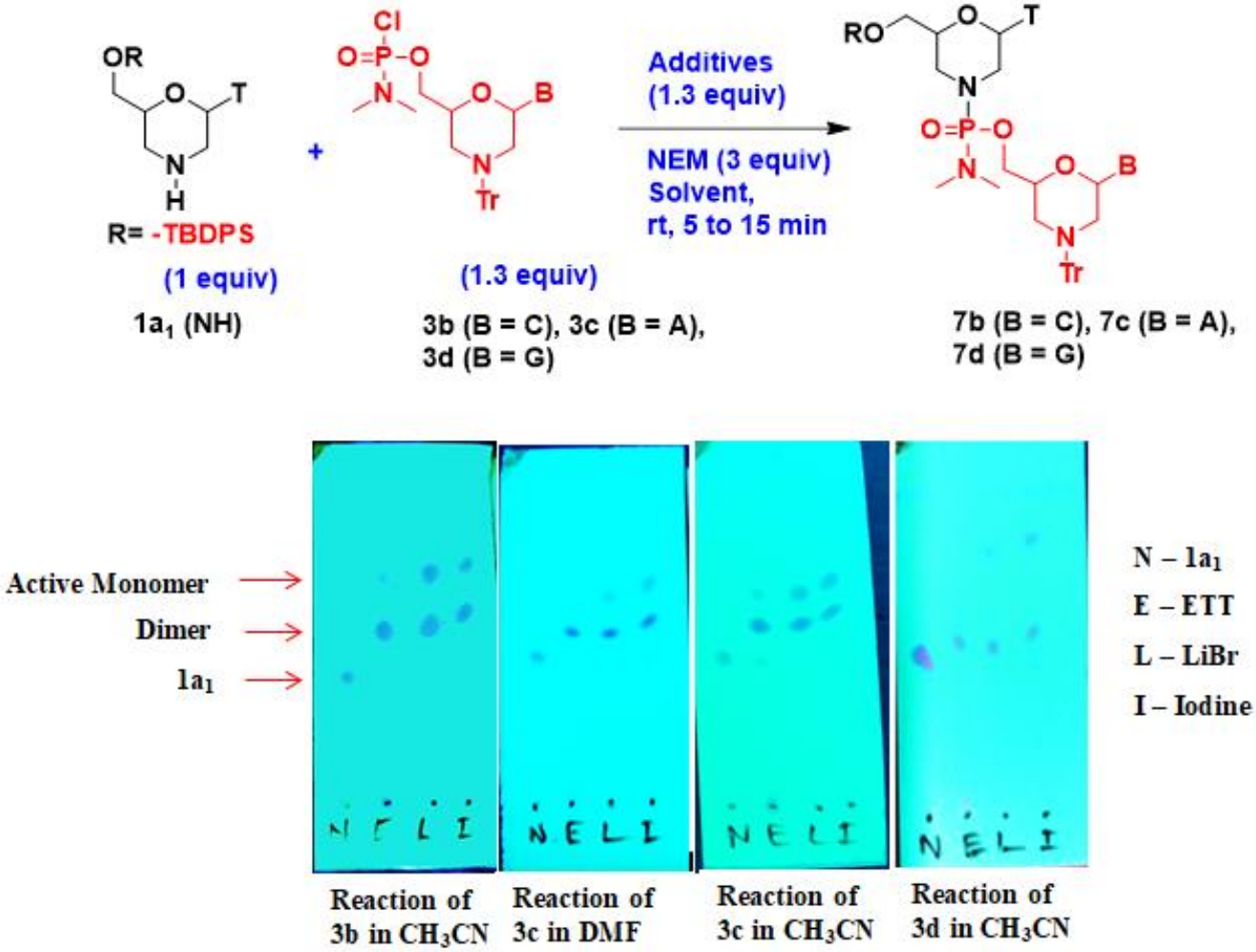
In order to check the solubility and stability of the active monomers, we have chosen CH_3CN and DMF or NMP because these solvents are commonly used in oligonucleotide synthesis. Trityl-protected monomers T (**3a**), C (*N*-Bz, **3b**) and A (*N*-Bz, **3c**) were at 0.05 M concentration in CH_3CN. However precipitation was observed when the monomer solution was purged with argon. Interestingly, Fmoc-protected monomers were clearly soluble at 0.1 M concentration in CH_3CN (Figure S2). The solubility is important in oligonucleotide synthesis, allowing for higher effective concentrations of active monomers during the coupling step in solid phase synthesis.

As the solubility of Tr-protected monomers were poor in CH_3CN, hence, the stability of both Tr and Fmoc protected T and G-monomers was checked in NMP solvent at 0.1 M concentration after 12 hrs. As per ^{31}P NMR (Figure S3), Tr- protected **3a** and **3d** and Fmoc-protected **6a** and **6d** were stable though in the case of **3d** some percentage of Tr-deprotection was observed in TLC. As we discussed earlier, Fmoc-protected monomers (**6a**, **6b**, **6c** and **6d**) were freely soluble in CH_3CN, hence we evaluated their stability after 12 hrs by ^{1}H and ^{31}P NMR (**Figure S4**). In this case, we could record ^{1}H NMR because CH_3CN was removed before the sample was recorded in $CDCl_3$ which was not possible in the case of NMP. We observed there was no decomposition of Fmoc-monomers even after 12 hrs as confirmed by both ^{1}HNMR and ^{31}P NMR.

Coupling in presence of ETT for the preparation of dimer in solution using trityl chemistry:

After having the active monomers in hand, we put our effort to standardize the synthesis of dimer in solution phase. Reaction was screened in presence of different additives (ETT, LiBr and iodine) and bases (NEM, DIPEA, lutidine and pyridine) in CH_3CN solvent. The dimer formation was performed with 1.3 equiv of active chlorophosphoramidate monomer **3b** and 1.3 equiv of additive (ETT or LiBr or I_2) w.r.t free amine $\mathbf{1a_1}$ in CH_3CN. As per TLC, reaction was completed within 5 min (**Scheme 3, Figure S5**) with the disappearance of free amine $\mathbf{1a_1}$. Progress of the reaction was monitored by HPLC using C-18 column (Xbridge RP18 10 mm I.D x 250 mm). After 5 minutes, reaction mixture was injected into HPLC column and observed the formation of dimer [TBDPS-T-C(Bz)Tr, **7b**] at 17 min. The peak at 14.6 min corresponded to the free NH $\mathbf{1a_1}$ was almost disappeared (**Table 2, HPLC chromatograms, Figure S6**). Using the same protocol formation of dimer [TBDPS-T-A(Bz)Tr, **7c**] and [TBDPS-T-G(iBu)Tr] **7d** with active monomers **3c** and **3d** was performed, respectively. As per TLC, all the additives were found to work almost equally well (**Scheme 3, TLC**). Coupling efficiency was better in DMF than CH_3CN

as per TLC (Scheme 3, TLC) as free amine **1a$_1$** was completely disappeared within 5 min in the reaction.



Scheme 3. Formation of dimers using Tr-protected active monomers. Dimer formation monitored by TLC in 5% MeOH-DCM, Under UV light, reaction time 5 min.

Table 2: HPLC analysis for the formation of dimer **7b**.

Entry	Additives	Solvent	% Area ratio of 7b to 1a$_1$
1	ETT	ACN	49.28
2	LiBr	ACN	39.71
3	I_2	ACN	41.56

HPLC of TBDPS-T-C(N-Bz)-Tr (**7b**). Ratio of dimer **7b** : free NH **1a$_1$**

with A active monomer **3c** to give a dimer **7c**. All the dimers were characterized by HRMS. 0.5 Equiv of additives (ETT, I_2 or LiBr) were used for the formation of dimer [TBDPS-T-C(N-Bz)Tr, **7b**]. Significant amount of free amine **1a$_1$** was left in all the cases (TLC, Figure S7) even after 30 minutes of the reaction.

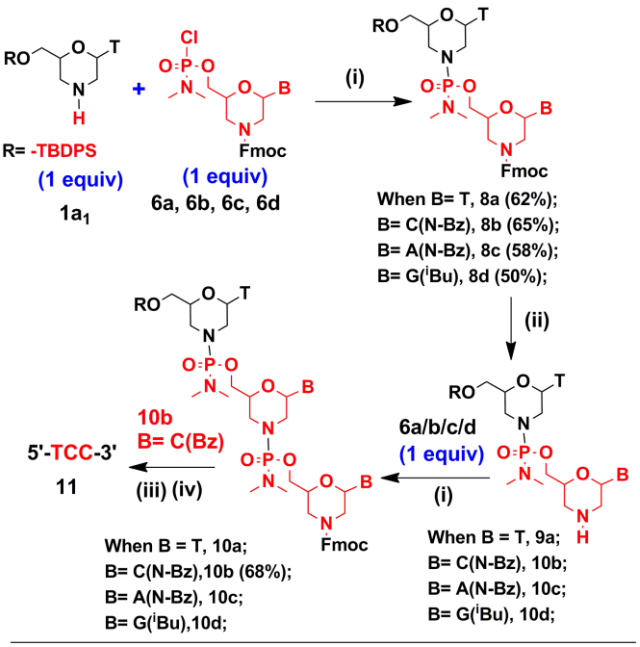
Unlike active monomer synthesis **6a-d** (Table 1, entry 9), I_2 was as effective as ETT in coupling reaction. We have noticed that reaction didn't proceed in the absence of base. We screened different bases like NEM, DIPEA, lutidine and pyridine. In case of pyridine the active monomer was decomposed very fast.

In case of lutidine, dimer was formed but the reaction was not clean. Some others side products were generated along with the dimer. In NEM and DIPEA there was no significant difference in terms of formation of dimer.

Dimer and trimer synthesis with Fmoc-protected active monomers:

We then extended the reaction for dimer synthesis with Fmoc-protected monomers in TLC scale. Formation of [TBDPS-T-T-Fmoc] **8a** was completed within 5 min in presence of ETT with the complete consumption of $\mathbf{1a_1}$. Using the same protocol the dimer formation [TBDPS-T-C(N-Bz)Fmoc] **8b** and [TBDPS-T-A(N-Bz)Fmoc] **8c** was carried out using additives (1.3 eq) and active chlorophorphoramidate monomers (1.3 eq) and NEM (3 eq) w.r.t $\mathbf{1a_1}$, the reaction was completed within 5 to 15 minutes (**TLC, Figures S8–10**). In the case of coupling with A monomer, LiBr was inferior as the reaction was not completed even after 30 min where ninhydrin active spot of $\mathbf{1a_1}$ was observed (**TLC, Figures S9, S10**). Comparing with the Tr-protected dimer **7c** synthesis, LiBr was less effective in the case of Fmoc-protected dimer synthesis **8c**. This observation again supports our earlier report for active monomer synthesis where LiBr/DBU method did not work when electron withdrawing group was attached with morpholino *N*.[18]

Using this method, we were interested to see the progress of the reaction in trimer synthesis (**Scheme 4**). Initially, dimers **8a-d** were synthesized and purified by silica gel column chromatography. Reaction was completed within 30 min with 1 eq, of active monomers in presence of ETT (2 eq.) which was not possible earlier using LiBr method.[15] Purity was checked by HPLC and characterized by ^{1}H, ^{13}C, ^{31}P and mass analysis. There was no problem for Fmoc deprotection using 20% piperidine/DMF while making trimmers **10a-d**. Compound **10b** was purified by silica gel column chromatography, characterized by NMR and mass and purity was checked by HPLC. Remaining trimmers **10a, 10c** and **10d** were purified by preparative HPLC and purity was checked in HPLC and characterized by HRMS.

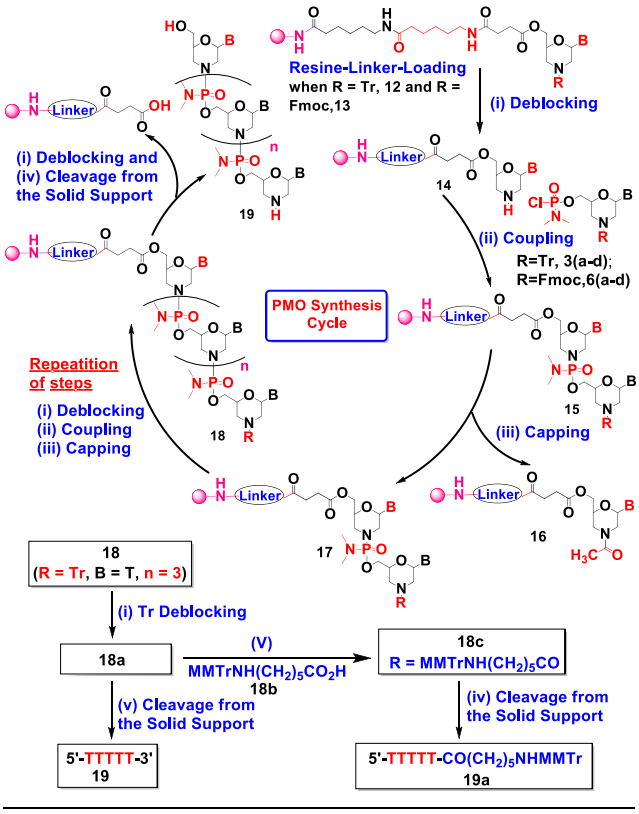


Scheme 4: Synthesis procedure of Fmoc protected Dimer and Trimer. Reagents and conditions (i) ETT (2 equiv), NEM (3 Equiv), DMF, rt, 30 min; (ii) 20% Piperidine in DMF, rt, 10 min; (iii) 1 Molar TBAF-THF solution, rt, 6 hrs; (iv) 30% NH_3-H_2O solution, 55°C, 16 hr.

Solid phase synthesis of PMO using Tr chemistry:

Encouraging by the results, we then transferred the protocol on solid phase synthesis using ETT as a coupling additive. Accordingly, Novasyn TG-amino resin (2 mg, 0.26 mmol /gm, pore size 130 µm) was swelled in *N*-methyl-2-pyrrolidone (NMP) for 12 h. Resins were first functionalized with 6-aminocaproic

acid (**12**) two times followed by the loading with succinic ester of Tr or Fomc-morpholino monomer as per our reported protocol.[16,19] After Tr-deprotection of **14**, the free amine (Scheme 5) was found to be 450 nmol



Scheme 5: Solid phase synthesis of PMOs. Reagents and conditions **(i) Deblocking:** CYPTFA (3-cyano pyridine, TFA, CF_3COOH, DCM) (when **R= Tr**) or 20% piperidine-DMF (when **R= Fmoc**), 5 x 1 min = 5 min; **(ii) Coupling:** Active monomer (3 equiv), ETT (6 equiv) and NEM (6 equiv), CH_3CN or NMP, no. of couplings (3 x 15 min = 45 min); **(iii) Capping:** (1:1)-10% Ac_2O-CH_3CN and 10% DIPEA- CH_3CN (when solvent used CH_3CN) or (1:1) 10% Ac_2O-NMP and 10% DIPEA-NMP (when solvent used NMP) (5 x 1 = 5 min); **(iv) Cleavage from the solid support:** 30% aq NH_3, 55°C, 16 h (For T-oligomer, RT); **(v)** HOBT, HBTU and NEM (3 equiv), NMP.

which was used for coupling with Tr-T active monomer **3a** (3 equiv w.r.t loading yield) in presence of coupling reagent ETT (2 quiv) and base *N*-ethyl morpholine (3 equiv) w.r.t **3a**. The first coupling of T (dimer, **15**, B = T, R = Tr) was obtained in 85% yield (382.50 nmol). It was then washed with NMP (2 ml) and the cycle was repeated to get the desired 5 mer T (**18**, B = T, n = 3) with phosphorodiamidate backbone. The Tr of the TTTTT-Tr oligomer was deblocked and then washed with DCM. The resin was divided into two parts. To one part, MMTr protected aminocaproic acid [$MMTrNH(CH_2)_5CO_2H$] was added along with HOBT and HBTU in presence of NEM in NMP for the 3'-modification of the synthesized 5-mer T PMO. Both parts of the resin were washed with water and then transferred into a 1.5 ml eppendorf tube and left with aq. NH_3 for 16 h to cleave the oligomer from solid support. The aq. NH_3 was lyophilized and the residue was re-dissolved in water and quantified by UV-VIS spectrophotometry using the absorbance at 260 nm (total **19** and **19a** 302 nmol, 67% with respect to loading in solid support). Then the pentamer T **(19)** and 3'-modified pentamer T **(19a)** were characterized by HRMS and purity was checked by reverse phase HPLC (RP-HPLC). PMO **19** was eluted at 5.99 min with almost a single peak. Due to the presence of MMTr group, **19a** was eluted at 28.56 min with a minor peak at 9.115 min. It indicated that coupling efficiency was almost quantitative in presence of ETT as there was no other truncated peak in HPLC. Extra broad peak at R_t = 9.115 min was characterized as MMTr deprotected **19a [19a-**MMTr] by MALDI-TOF and HRMS analysis. In HRMS, calculated mass of $[M\text{-MMTr} + H]^+$ was 1674.6275 and found 1674.6371. The same compound was recorded in MALDI-TOF, mass obtained was 5 units more [1679.878] than HRMS data. Frac-

tion collected at 9.115 min was again injected in HPLC and interestingly, it gave a clean single peak at 6.998 min.

With this encouraging result, we then synthesized 5-mer C (**20**) and 25-mer mix sequence of PMO (**21**). To validate the synthetic protocol, antisense PMO (**21**) was synthesized for biological screening which targets the mRNA of *no tail* gene of zebrafish[25,26] (*vide infra*). To compare the coupling efficiency of LiBr, **20** was attempted to synthesis using LiBr (2 equiv) for a total coupling time of 30 min (3 x 10 min). We could not complete the synthesis of 5-mer C as per Trityl assay because no yellow color was observed after 4th coupling (**Figure S11**). 25-mer PMO (**21**) was >97% pure as per HPLC with the appearance of a single peak at 19.98 min. **20** and **21** were characterized by MALDI TOF mass. In the case of **20**, masses of all $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ were obtained with 5 unit more as it was observed in the case of **19a**-MMTr compound. Mass of **21** was obtained at 8238.342 which corresponded to $[M-2NMe_2+Na]^+$. Liberation of NMe_2 group is common while recording mass of PMO which was observed in the case of monomer **6d** earlier.

Table 3: Yield of PMOs

PMOs	Loading yield (nmol)	Crude Yield[a] (nmol)	% of purity based on HPLC
5'-TTTTT-3' (**19**) and with linker **19a**	450	117 185	72.3 75.6
5'-CCCCC-3' (**20**)	248	145	97.9
5'-GACTTGAGGC-AGACATATTTC-CGAT-3' (**21**)	794	405	100[b]

[a] After ammonia deprotection, measured at 260 nm. [b] After chilled acetone precipitation and 3000 Mwt cut off filter unit 251 nmol was obtained from 405 nmol.

Table 4: ESI and MALDI TOF Mass analysis

PMO	Molecular formula $[M + Na/H]^+$	Calculated mass	Observed mass
19	$C_{58}H_{92}N_{19}O_{24}P_4Na$	1585.3598	1585.8270
	$C_{58}H_{92}N_{19}O_{24}P_4H$	1563.3780	1563.9174
19a	$C_{84}H_{118}N_{20}O_{26}P_4Na$	1970.8389	1977.041[a]
	$C_{64}H_{102}N_{20}O_{25}P_4H$*	1674.6275	1674.6371
	$C_{64}H_{102}N_{20}O_{25}P_4H$*	1674.6275	1679.878[a]
20	$C_{53}H_{86}N_{24}O_{19}P_4Na$	1510.2842	1515.683[a]
21	$C_{289}H_{443}N_{141}O_{99}P_{24}Na$**	8238.7565	8238.342[a]

*(M-MMTr).[a]MALDI TOF mass. **$[M-2NMe_2+Na]^+$

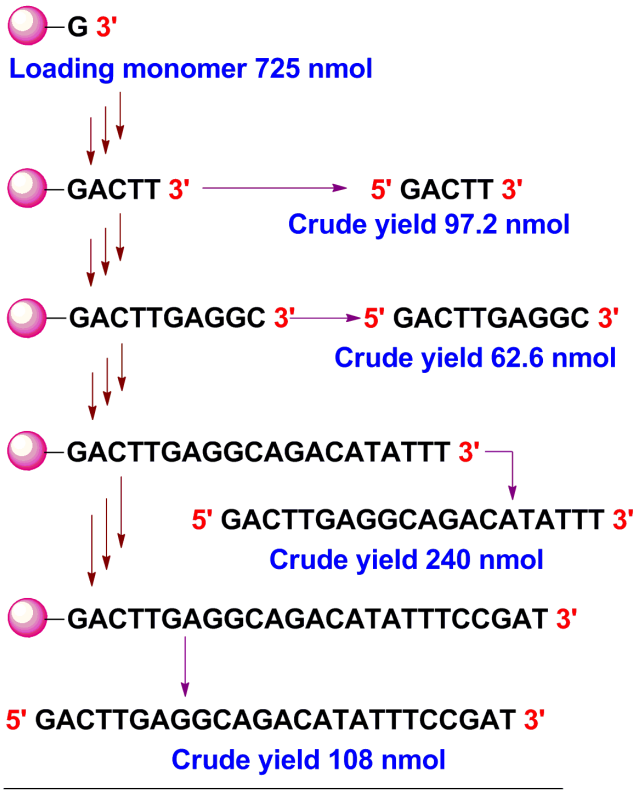
PMO synthesis using Fmoc chemistry:

After the successful synthesis of dimers and trimers in solution phase using Fmoc chemistry, we then became interested to explore the protocol using solid phase synthesis (**Scheme 5**). In order to confirm the progress of synthesis, first we synthesized a trimer 5'-TCC-3', **11** as it was synthesized earlier using solution phase method (**Scheme 3**). Then HPLC chromatograms of trimer (5'-TCC-3', **11**) were compared and found to have almost same Rt values 2.6 min (from solution phase) and 2.3 min (synthesized in solid phase) and characterized by HRMS.

Next we have synthesized 5 mer 5'-TATAT-3' (**22**), using Fmoc chemistry (**22,** crude yield 206 nmol from 352 nmol loading resin, 96 % pure as per HPLC, R_t 6.4 min). **22** was characterized by both MALDI-TOF and HRMS analysis. In MALDI-TOF, peak obtained at 1618.503 [calc. 1618.5303] which corresponded to $[M+K]^+$. In HRMS, many fragments were obtained

which matched with trimer $[TAT+Na]^+$, [M-A (nucleobase) + $H]^+$ and [M-T (nucleobase)]$^+$.

Next, few more sequences of PMOs (**21, 23–25**) were synthesized from the same solid support through splitting of resins starting from G-loading monomer (Scheme 6). Sequence of PMO **21 (21-Fmoc)** was same as PMO **21** was synthesized earlier by Tr-chemistry **(21-Tr)**, designed for the evaluation of antisense efficacy targeting z-*no tail* gene. For control experiment another 25-mer PMO **26** of mismatched sequence was synthesized (**26**-control).



Scheme 6: Synthesis of PMOs with different sequences from same solid support through splitting of resins using Fmoc chemistry. Procedure was same as discussed in scheme 5.

Table 5: Yield of PMOs from total 725 nmol G-loading support

PMOs	Crude Yield[a] (nmol)	% of purity based on HPLC
5'-GACTT-3' (**23**)	97.2	98.8
5'-GACTTGAGGC-3' (**24**)	62.6	99.1
5'-GACTTGAGGCAGAC-ATATTT-3'(**25**)	240	99.2[b]
5'-GACTTGAGGC-AGACATATTTCCGAT-3' (**21**)	108	100[b]

[a] After ammonia deprotection, measured at 260 nm. [b]After chilled acetone precipitation and 3000 Mwt cut off 196 nmol of **25**, and 76 nmol of **21** were obtained. Total crude yield 508 nmol (70%) from 725 nmol.

The mismatched sequence of *z-no tail* gene 5'-GA**G**TTGA**C**G**G**A**G**A**G**ATATTTC**G**GAT-3'(**26**) was obtained 354 nmol as a crude yield from 623 nmol loading. After acetone precipitation and passed through 3000 Mwt cut off filter unit, 229 nmol was obtained with a single peak in HPLC.

Commercially available PMOs are usually purified by acetone precipitation method and directly used for biological applications. Using this method, longer PMOs **21, 25-26** have been purified and purity was checked by HPLC. All the PMOs have been eluted with a single peak in HPLC. Both 25-mer PMOs of same sequence **21** though have been synthesized in two different methods; however, they have been eluted almost in same time at 19.98 and 20.2 min, respectively. For biological application, these 25-mer PMOs were further purified through filtration using 3000 Mwt cut off filter and HPLC.

Thus from 725 nmol G-loading support, total combined yield of crude PMOs (**21**, **23-25**) was 508 nmol (70%).

Table 6: Mass analysis of PMOs synthesized by Fmoc chemistry

PMO	Molecular formula	Calculated mass	Observed mass
22	$C_{58}H_{89}N_{25}O_{20}P_4K$	1618.5303	1618.503
23	$C_{57}H_{88}N_{26}O_{20}P_4Na$	1603.5516	1601.568
24*	$C_{112}H_{166}N_{57}O_{39}P_92H$	1607.0265	1607.140
25*	$C_{233}H_{357}N_{114}O_{79}P_{19}$	6603.2359	6601.244
21	$C_{293}H_{453}N_{143}O_{99}P_{24}Na$	8329.1091	8328.018
26	$C_{296}H_{455}N_{149}O_{99}P_{24}K$	8467.3086	8468.016

*24: [M-2NMe_2+2H]; *25: [M-NMe_2]

All the sequences were characterized by MALDI TOF mass analysis in **Table 6**.

As per trityl and Fmoc assay, we observed the coupling efficiency was close to 99.95% except first coupling. After deblocking by ammonia treatment at 55ºC for 16 hrs, the crude yield was 51 % for 25-mer PMO **21** in trityl chemistry, 70 % for PMOs (**21**, **23-25,** 508 nmol from 725 nmol) in Fmoc chemistry and 56.8 % (354 nmol from 623 nmol) for control PMO **26**. After acetone precipitation, Millipore filter column and HPLC, final pure yield was 59 % for normal sequence with Fmoc and 65 % for control sequence with Fmoc.

In MALDI TOF mass, like **6d** and **21 (Tr method)**, the major peaks of **24** and **25** were found with the loss of NMe_2 group whereas PMO **21 (Fmoc method)** was characterized with $[M+Na]^+$ peak which has been synthesized from the same resin supports of **24** and **25**. Interesting observation was both **21-Tr** and **21-Fmoc** were eluted almost at the same time in HPLC. It indicated that under the reaction conditions, the phosphorodiamidate backbone was stable. Loss of NMe_2 group was occurred under mass analysis. Mismatched control sequence **26** was characterized with $[M+K]^+$ peak.

Evaluation of antisense efficacy of 25-mer no tail PMO in zebrafish for validation of synthetic protocol:

After synthesizing 25-mer PMOs, **21** [5'-GACTTGAGGCAGACATATTTCCGAT-3'] by both **Tr** chemistry and **Fmoc** chemistry, we then evaluated the antisense efficacy in zebrafish model targeting *no tail* gene because zebrafish is an ideal model organism for PMO's application.[27] We have targeted *no tail* gene because no tail-dependent phenotypes such as no notochord formation with U-type somites are clearly visible with high reproducibility.[28] 25-mer PMOs synthesized by both Fmoc (**21-Fmoc**) and Tr (**21-Tr**) chemistry gave no tail-dependent phenotypes which was consistent with no tail mutant phenotypes[29,30] (Figure 2 C, D) and >95% of embryos have shown no tail dependent phenotypes with a negligible mortality rate (**Section XX, Table S1**). As a control experiment, standard mis-matched control PMO from Gene tools and a mis-matched sequence 25-mer **26** were also injected where no phenotype was observed (Figure 2A, B). Our protocol for the synthesis of PMO now has been validated in biological application. There is a report on the use of shorter than 25-mer PMO for antisense application.[31] We were then interested to test 20-mer **PMO 25** which also targets *no tail* gene but 5 nucleotide shorter than **21** from 3'-end. However, we observed a high mortality rate with no phenotypes. It indicated that one should be careful while designing the PMO for antisense application.

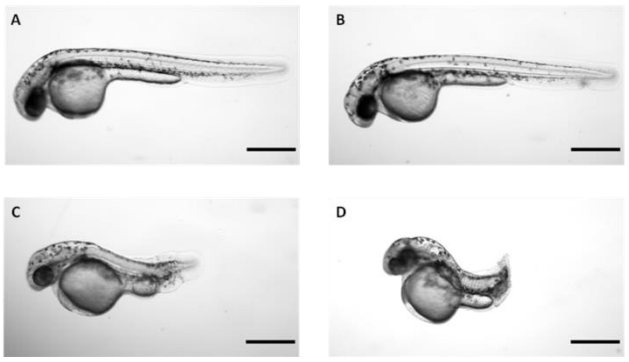


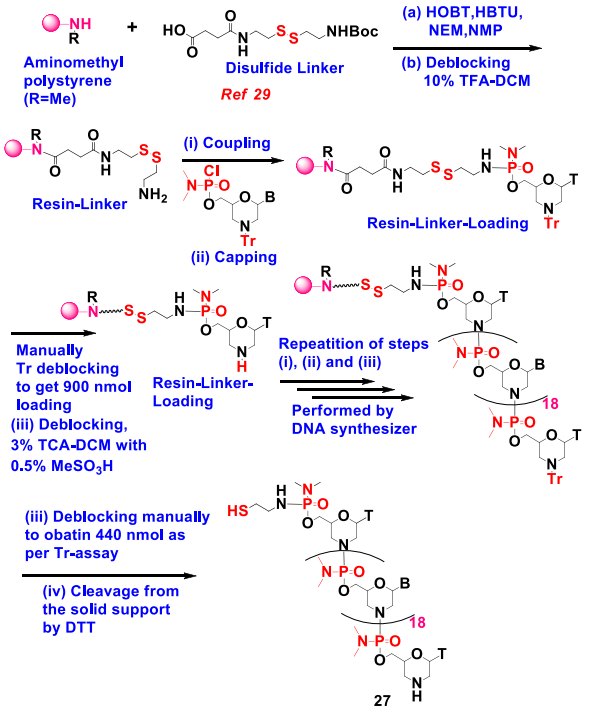
Figure 2: A) Control, mis-matched PMO from Gene Tools; B) Control, mismatched **26**; C) PMO, 21-Fmoc; D) PMO, 21-Tr injected phenotypes. Scale bar is equivalent to 500 µm.

Transfer the synthesis protocol to DNA synthesizer:

The manually synthesized protocol on solid support has been transferred to automated DNA synthesizer. All the activated monomers were connected to the port of corresponding amidite bottles. Mixture of activator either ETT or iodine with NEM was connected to the port of activator bottle. Similarly, deblocking and capping reagent bottles were connected as per the specification of machine (Section XXI, Supporting information). As the coupling was monitored by Tr-assay (**Figures S12 and S13**) hence, deblocking reagent was changed from CYPTFA (**used in scheme 5**) to 3% TCA in DCM with 0.5% $MeSO_3H$. In later case, Tr color was observed whereas in CYPTFA, no color was observed due to the presence of TFE.

Initially 5-mer T (**19**) was synthesized using iodine as an activator and coupling time was 3x10 min. Active monomer was used (3x3 equiv). As per Tr-monitor (**Figure S12**), quantitative coupling was obtained in each step. After ammonia deprotection, 345 nmol was obtained as a crude yield from 500 nmol loading. Tr-assay was confirmed by HPLC to obtain a single peak at 6.14 min and characterized by HRMS (**Table 7**).

During iodine-mediated coupling, washing step was complicated as it required proper washings to remove the iodine color. As ETT is routinely used for DNA synthesis hence, a longer PMO was synthesized using ETT as an activator. There was also a partial cleavage of succinimide ester linkage from the solid support in presence of deblocking reagent. Accordingly, we changed the solid support where ester linkage was replaced by disulfide linkage (**Scheme 7**). The disulfide linkage was synthesized as per literature report.[32] After loading monomer, Tr-was deblocked manually to get the loading yield 900 nmol. Then Tr-deprotected resins were used in DNA synthesizer to make a 20-mer PMO keeping the last Tr-group intact at the *N*-terminal of PMO. The last Tr-group was deblocked again by manually to measure the final coupling yield (440 nmol based on Tr assay). Solid support was cleaved from resin by DTT treatment and obtained the 5'-thiol functionalized PMO **27** in 250 nmol after dialysis. Purity was checked by HPLC where a sharp single peak at 19.11 min and a small broad peak at 10.7 min were obtained. The peak at 19.11 min was characterized by MALDI TOF mass and identified as **27 (Table 7).** 5'- SH functionalized PMO could be useful for conjugation chemistry



Scheme 7. Synthesis of PMO by DNA synthesizer.

The final product after ammonia deprotection was characterized by HPLC and mass. A clean mass with a single peak indicated that the chemistry worked well.

Table 7: Mass analysis of PMOs synthesized by DNA synthesizer using Tr chemistry

PMO	Molecular formula	Calculated mass	Observed mass
19	$C_{58}H_{91}N_{19}O_{24}P_4H$	1563.3780	1564.5616[a]
27	$C_{239}H_{374}N_{114}O_{83}P_{20}SK$	6862.9695	6862.570[b]

[a] HRMS, [b] MALDI TOF

Conclusion

In conclusion, we are the first to disclose the synthesis of PMO using Fmoc chemistry and third report by DNA synthesizer using Tr chemistry. We have improved the coupling efficiency using either ETT or iodine as an additive. For Tr-deprotection, we have used 0.5% $MeSO_3H$ in 3% TCA in DCM for effective Tr-deblocking for the first time. PMO is quite stable in acidic conditions, perhaps 3 % TFA in DCM could be alternative deblocking reagent. As PMO is a neutral molecule hence Fmoc chemistry is suitable for its synthesis which can be easily transferred to the peptide synthesizer. It was necessary to develop the synthesis protocol for automated synthesizer (DNA or peptide) which could be user friendly for PMO synthesis to the scientists. Finally, we have validated the synthesis protocol *in vivo* using the PMO in zebrafish where we could recapitulate the *no tail* phenotype. Synthesis of various sequences in gram scale is under study.

EXPERIMENTAL SECTION:

SECTION:

After the loading monomer was added into the solid support the following synthetic cycle was followed for the solid phase synthesis of PMOs.

Step 1: Washing of monomers and reagents with NMP (300 µL for 3 times in 30 s interval with vortexing at 600 rpm.

Step 2 (Capping): Un-reacted free NH was capped with 1:1 mixture of 10 % Ac_2O-NMP-10% DIPEA-NMP (300 µL for 3 times in 1 min interval with vortexing at 600 rpm). All the excess reagents were washed out with 10 % DIPEA-DCM (300 µL for 3 times in 30s interval with vortexing at 600 rpm) followed by DCM (300 µL for 3 times in 30s interval with vortexing at 600 rpm).

Step 3 (Deblocking): Deblocking with deblocking cocktail CYPTFA (for trityl) or 20 % Piperidine-NMP (for Fmoc) (300 µL for 5 times in 1 min interval with vortexing at 600 rpm) followed by washing of the deblocking reagents with 10 % DIPEA-NMP (300 µL for 3 times in 30s interval with vortexing at 600

rpm) and NMP (300 µL for 3 times in 30s interval with vortexing at 600 rpm).

Though, Tr color was not observed in CYPTFA, due to the presence of TFE, however, after deblocking we added few drops $MeSO_3H$ to the flow through resin deblocking solvent to get color of Trityl cation. The trityl was then quantified by UV-VIS spectrophotometry using the absorbance at 260 nm to obtain the coupling yield

Step 4 (coupling): 3 Equiv of active monomer (0.05 M) was used for each coupling. This was repeated for two more times. Total coupling time was 3x15 min. All the excess reagents were washed out with NMP (300 µL for 3 times in 30s interval with vortexing at 600 rpm)

Step 5 (Solid support deprotection): Resins were treated with aq. NH_3 at 55°C for 16 hrs (except T-containing homo oligomer**s**).

Zebrafish experimental procedures: Wild-type Indian strain of zebrafish were housed in a circulating water aquarium at 28.5 °C under a 14 h light and 10 h dark cycle. Adult fish under the age of 10 months were crossed to obtain fertilized embryos. The embryos were injected at 1 cell stage with 2 nL of the respective doses of morpholinos. The injected embryos were raised in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM MgSO4, 0.33 mM CaCl2) supplemented with methylene blue at 28.5°C under a 14 h light and 10 h dark cycle. After manual dechorionation using hypodermic needles, 36 hpf (hours post fertilization) embryos were transferred to 4% methylcellulose solution in E3 with 0.01% MS-222 (Sigma-Aldrich) and imaged using an Olympus MVX10 microscope at 2.5x magnification. All protocols were approved by the Institutional Animal Ethics Committee of IISER Pune.

Author Contributions

SS: Conceived the idea, designed the hypothesis and wrote the manuscript, JK and UG: Synthesized morpholino monomers and oligomers. AG and AD: helped during synthesis of PMO in DNA synthesizer. DN and AG: Carried out the biological experiments in zebrafish embryos.

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Notes

The authors declare no competing financial interest.

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ASSOCIATED CONTENT

Supporting Information. Detailed experimental procedures and characterization data including the spectra for all new compounds can be found in the supporting information.

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