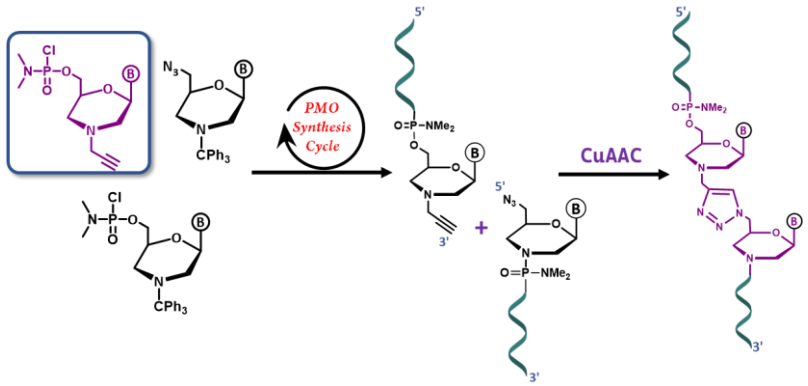
A Convergent Click Ligation Approach to the Synthesis of Triazole-incorporated PMOs and Evaluation of Hybridization Properties

Arpan Banerjee[1], Arnab Das[1], Atanu Ghosh[1], Abhishek Gupta[1], Surajit Sinha[1,*]

[1]School of Applied and Interdisciplinary Sciences, Indian Association for the Cultivation of Science, Kolkata – 700 032, INDIA



ABSTRACT: The synthesis of Phosphorodiamidate Morpholino Oligonucleotides (PMOs) incorporating single triazole rings in the backbone has been achieved via Cu(I) catalyzed Azide-Alkyne cycloaddition reaction (CuAAC). The synthetic approach implemented, is convergent, involving the ligation of a 5'-azide PMO fragment to 3'-alkyne fragment both in solution and on solid support. To access the 3'-alkyne PMO fragment, we synthesized 3'-N-propargyl chlorophosphoramidate morpholino monomers for all four nucleobases. The resulting triazole-incorporated PMOs (TL-PMOs) have exhibited comparable or improved binding affinity towards complementary DNA/RNA strands compared to its regular analogs.

Introduction:

Phosphorodiamidate morpholino oligonucleotide (PMO) is a nucleic acid analog where each subunit consists of a nucleobase connected to a methylenemorpholine ring and a neutral phosphorodiamidate inter-subunit linkage.[1,2] PMOs are generally utilized as gene silencing agents and have shown significant promise in the field of antisense oligonucleotide (ASO) therapeutics. Notably, four PMO-based drugs, namely Eteplirsen, Golodirsen, Viltolarsen, and Casimersen, have already received approval from the US Food and Drug Administration (FDA) for the treatment of various sub-types of Duchenne Muscular Dystrophy (DMD).[3]

Majority of PMO syntheses typically involves a linear synthetic protocol in which a monomer unit is coupled with a growing oligomer chain.[4-6] Linear synthetic protocol may yield the contamination with the formation of N-1, N-2 mer, which are single or double subunit shorter than the desired oligo. These shorter sequences are challenging to separate using standard purification methods for oligonucleotides due to their almost identical polarity, consequently impacting the purity of the desired PMO. To overcome this hurdle, the utilization of a fragment ligation approach might be effective. Moreover, it

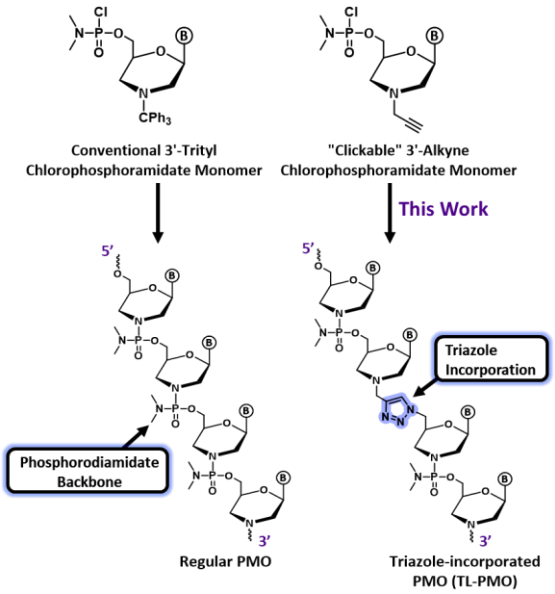
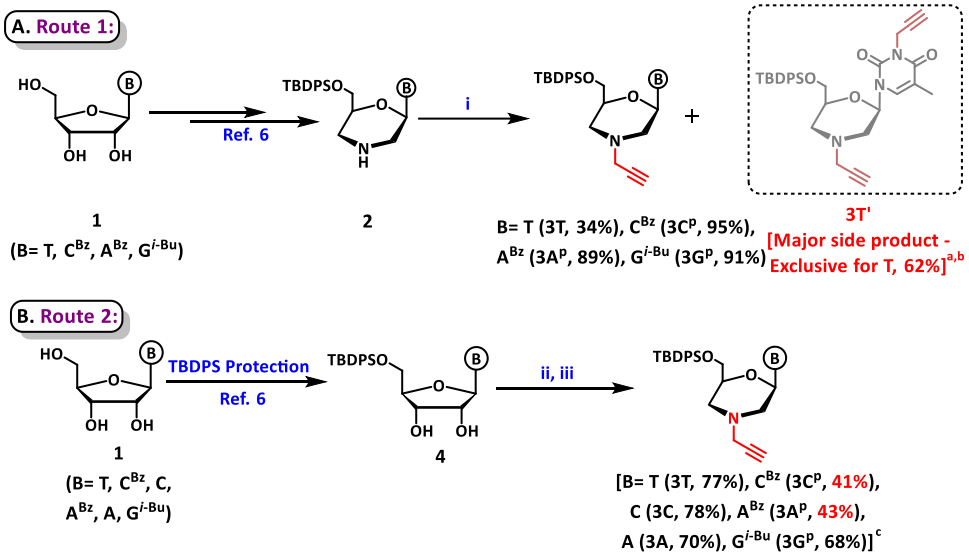


Figure 1. Conventional trityl monomer leading to Regular PMO (left), Clickable 3′-Alkyne monomer, leading to Triazole-incorporated PMO (TL-PMO) (Right)

reduces the number of reaction steps necessary to achieve the desired oligo. While convergent approach has been

Scheme 1: Synthesis of 5′-OTBDPS-3′-N-propargyl morpholino monomers from Ribonucleosides



Regents and Conditions: (i) Propargyl Bromide (1.1 equiv), K_2CO_3 (1.1 equiv), DMF, 0˚C-rt, 1h; **(ii)** $NaIO_4$ (1.2 equiv), Propargyl amine (1.2 equiv), MeOH, rt, 4h; **(iii)** $NaBH_3CN$ (2.2 equiv), AcOH (2.2 equiv), 4Å MS, 0˚C-rt, 4h

[a] Rection was performed at 0˚C, ice was maintained throughout the duration of the reaction, unlike other cases, [b] **3T'** formed as the major product of the reaction, i.e., N3 of thymine got propargylated along with morpholino -NH (yield 62%); [c] Benzoyl protected C and A ribonucleosides were partially de-benzoylated in presence of propargyl amine, resulting in low yield of the desired product (**3C^P/3A^P**). Unprotected ribonucleosides were used instead in such cases, which were benzoylated later (**Scheme 2A**).

demonstrated for synthesizing DNA[7], its application to PMOs is not explored much, except for a recent attempt by Wada et. al. via H-Phosphonate Chemistry.[8]

Triazole linked oligonucleotide backbones have proven to be good mimics of the phosphodiester linkages in oligonucleotides. They are reported to form stable duplexes with complementary counterparts[9-16]. They also respond to enzymatic read-through experiments and proved to have encouraging results in qPCR studies.[17-21] The results of triazole linking in DNA/RNA, are promising enough to have good biocompatibility. With these precedents in mind, we sought to investigate the impact of substituting the phosphorodiamidate linkage with a triazole ring within the PMO backbone. Herein, we present a novel Cu^I catalyzed azide-alkyne cycloaddition[22, 23] (CuAAC) mediated PMO (TL-PMO) synthesis approach, involving the convergent ligation of PMO fragments (4-6 mer) both on solid support and in solution. To create the 'Click'able terminal alkyne and azide containing PMO fragments, we first synthesized 3'-alkyne chlorophosphoramidate morpholino building blocks for all nucleobases (A, T, G, C). By exploiting this Click ligation approach, PMOs up to 12 mer were successfully achieved in solution, demonstrating excellent hybridization properties compared to their regular counterparts. Due to challenges in handling longer oligos in solution, we then transferred this convergent approach to semi-automated solid-phase synthesis. These triazole-incorporated PMOs exhibited favorable biophysical properties, including improved or comparable hybridization with complementary DNA/RNA strands and a consistent global conformation.

Results and Discussion:

Molecular design of triazole-incorporated PMO backbone, started out with the replacement of phosphorodiamidate linkage with 5-membered triazole ring in such a way, that it does not massively alter the inter-subunit distances. To achieve so, we prudently designed the 5′-azide and 3′-alkyne precursors, which would then be 'Clicked' via Cu(I) catalyzed [3+2] cycloaddition. Both fragments were synthesized by using previously reported trityl chemistry[6].

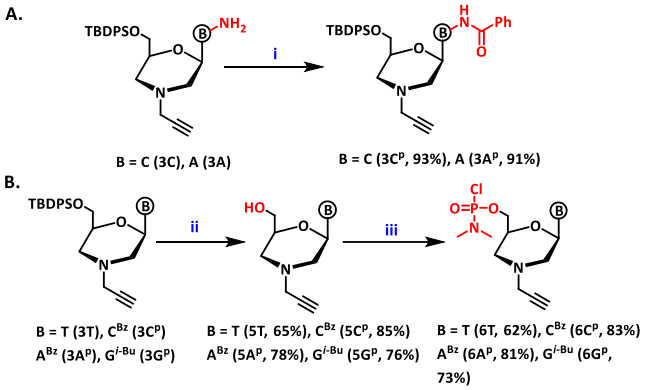
3′-N-Propargyl Morpholino Chlorophosphoramidate Monomer Synthesis:

The 3′-N-propargyl morpholino monomers were synthesized from commercially available ribonucleosides. At first, the 5′-OH of the ribonucleosides were silyl protected by TBDPS-Cl to form 5′-OTBDPS ribonucleoside (**4**) (**Scheme 1**).[24,25] The synthesis then diverges into two major routes. In the first route, **4** was subjected to 1,2-diol cleavage using $NaIO_4$ in presence of ammonium biborate tetrahydrate, followed by reductive amination by $NaBH_3CN$ in presence of acetic acid in methanol to form the morpholine ring (**2**).[24,25] Thereafter, **2** is treated with propargyl bromide in DMF in presence of K_2CO_3 at room temperature to give 3′-*N*-propargyl morpholino monomers (**3**) in excellent yields (89-95%) for all nucleobases (**Scheme 1, Route 1**), except thymine, where N3 position of thymine got propargylated, along with morpholino -NH (**3T'**). It was isolated as major product (62%) and characterized by NMR-spectroscopy. The desired product **3T** was obtained only in 34% yield. Therefore, we were in search of an alternative route to synthesize 3′-*N*-propargyl morpholino thymidine monomer.

In the second route (**Scheme 1, Route 2**), 5′-OTBDPS protected ribonucleoside was subjected to 1,2-diol cleavage by

$NaIO_4$ in presence of propargylamine, followed by reductive amination by $NaBH_3CN$/acetic acid in methanol. This results in direct formation of 5′-OTBDPS-3′-*N*-propargyl morpholino monomers (**3**), in 68-78% yield for different nucleobases, including thymine with no major side products. It is worth mentioning here that, partial benzoyl deprotection in the exocyclic amine of adenine and cytosine was observed in presence of propargyl amine. Therefore, for A & C, we started out with unprotected ribonucleosides, to achieve optimum yields. The unprotected morpholino monomers of C (**3C**) and A (**3A**) were benzoylated later using a mild benzoylating agent, (phenyl(1H-tetrazol-1-yl)methanone)[26], in presence of DMAP in acetonitrile at 65˚C, which gave exclusively the monobenzoylated products (in > 90% yield) **3C^{P}** and **3A^{P},** respectively (**Scheme 2A**), conforming to our previous report[27].

Scheme 2: (A) Benzoylation of C and A **(B)** Synthesis of 3'-N-propargyl Chlorophosphoramidate Morpholino Monomer



Reagents and conditions: (i) Phenyl(1H-tetrazol-1-yl) methanone (1.5 equiv), DMAP (1 equiv), MeCN, 1.5h, 65˚C; **(ii)** TBAF (1M in THF) (1.5 equiv), NH_4Cl, THF, 4h, 0˚C-rt, **(iii)** LiBr (5 equiv), 1,1,3,3-tetramethylguanidine (4 equiv), $POCl_2NMe_2$ (4 equiv), DCM-MeCN (1:1), 30 min, 0˚C

Propargylated morpholino monomers (**3**) were then undergone silyl deprotection using tetrabutyl ammonium fluoride (TBAF) in presence of NH_4Cl, in THF to get the respective 5′-OH monomers (**5**). These monomers were then converted to respective chlorophosphoramidate morpholino monomers (**Scheme 2B, Compound 6**), following previously reported LiBr-TMG protocol[27] and obtained in 62-83% yield for different nucleobases.

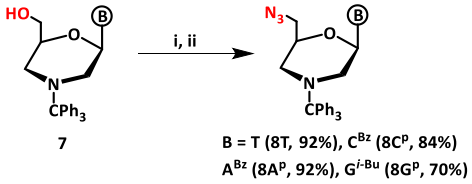
5′-Azide Morpholino Monomer Synthesis:

5′-Azide morpholino monomers were synthesized from 5′-OH morpholino monomers (**7**), following our previously reported mesyl chloride-sodium azide protocol[28] (**Scheme 3**).

Triazole-linked Morpholino Dimer Synthesis via CuAAC:

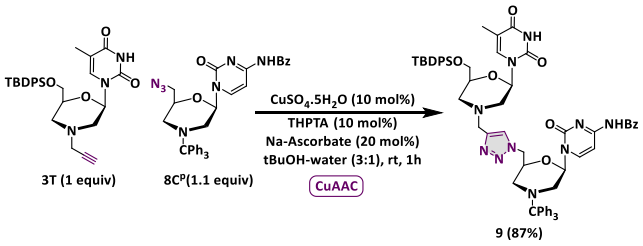
Having synthesized the desired terminal alkyne and azide monomers, we first coupled them via CuAAC to form a triazole linked dimer, before applying it to morpholino oligomers. 5′-OTBDPS-3′-*N*-propargyl morpholino thymidine monomer (**3T**) has been ligated to 5′-azide morpholino cytidine complement (**8C^{P}**) in presence of $CuSO_4.5H_2O$ (10 mol %), sodium ascorbate (20 mol%) and THPTA (10 mol %) [29] as a ligand, in ^{t}BuOH-water (3:1). The reaction was completed within 1 h at room temperature, and the triazole linked TC dimer (**9**) was isolated in 87% yield (**Scheme 4**).

Scheme 3: Synthesis of 5′-Azide morpholino monomers



Reagents and Conditions: (i) Mesyl Chloride (1.2 equiv), Et_3N (1.5 equiv), DMAP (0.3 equiv), 0°C, 30 min., **(ii)** NaN_3 (3 equiv), DMF, 65°C, 4h

Scheme 4: TC Dimer formation via CuAAC



Convergent PMO Synthesis *in solution via* CuAAC:

Next, we concentrated on synthesizing a full length PMO *in solution*, by using CuAAC mediated PMO block ligation approach. To achieve so, we initially choose a 11 mer-T sequence for initial standardizations. The retro synthesis was divided into two fragments: a hexamer alkyne and a pentamer azide fragment. The hexamer and pentamer PMO fragments were synthesized in solution following our previously reported protocol[6], followed by chromatographic purification (**Scheme S1**). For, 3′-propargyl PMO fragment, we commenced with a 5′-OTBDPS protected T monomer (**2T**) and the last coupling has been performed using chlorophosphoramidate monomer **6T**. Whereas, synthesis of 5′-azide fragment initiated with the 5′-azide T monomer, and for both cases, all intermediate couplings have been performed using *N*-trityl T chlorophosphoramidate monomers (**Scheme 5**). 4-6 mer PMO fragments (**Scheme 5**) are characterized by ESI-MS (**Figures S1-6**).

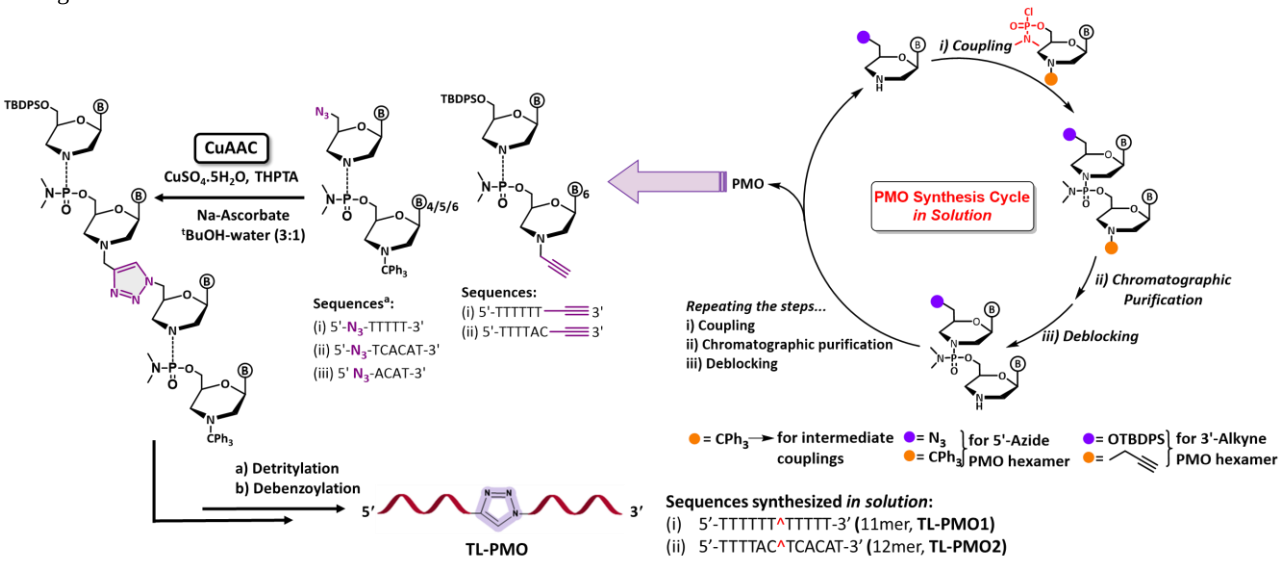
After synthesizing the terminal alkyne and azide PMO fragments, they were ligated via Cu(I) catalyzed Click reaction, in ^{t}BuOH-water (3:1), using $CuSO_4.5H_2O$ (0.5 equiv), Na-ascorbate (1 equiv.), in the presence of THPTA ligand (0.5 equiv.). The 11 mer PMO was then detritylated in AcOH (10% in MeOH-water). RP-HPLC profile of the crude reaction mixture has shown almost complete conversion (**Figures S7-9**). The 11 mer T PMO (**TL-PMO1**) has been characterized by MALDI-TOF MS (**Figure S10**). After initial standardizations, we targeted a custom hetero 12-mer PMO sequence, 5′-TTTTAC^TCACAT-3′ (**TL-PMO2**) *in solution* (**Figures S11-13**) to study hybridization properties; divided into two hexamer PMO fragments to be ligated later via CuAAC (triazole linkage between 6th and 7th subunit). The synthesis has been

performed following the same protocol discussed above (**Scheme 5**).

Combined Solid and Solution Phase Convergent synthesis of PMO and Hybridization properties:

Since longer oligos are difficult to handle in solution, due to various concerns, we thought of transferring this convergent protocol to semi-automated solid phase synthesis, a relatively more standardized platform for PMO synthesis in general. The goal was to scale this protocol to the synthesis of longer PMOs (18-25 mer), suitable for antisense applications.

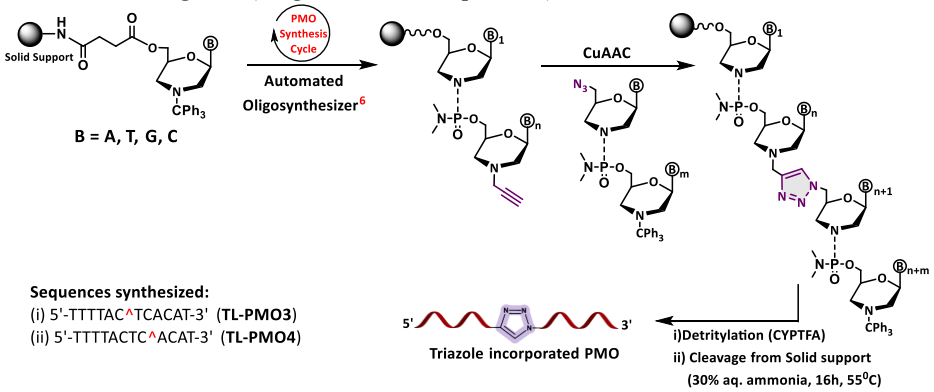
Scheme 5. Synthesis of PMO fragments *in solution* having alkyne and azide terminal, and subsequent Cu(I) Click (CuAAC) ligation of the PMO fragments



Reagents and conditions: (i) coupling: chlorophosphoramidate monomer (1.2 equiv), ETT (1.2 equiv), NEM (2 equiv), DMF, rt, 45 mins.; **(ii) Deblocking/ Detritylation:** 10% AcOH in TFE (MeOH-water for the 12mer PMO detritylation), rt, 1h; **(iii) CuAAC:** $CuSO_4.5H_2O$ (0.5 equiv), THPTA (0.5 equiv), Na-Ascorbate (1 equiv), ^{t}BuOH-Water (3:1), rt, 6h; **(iv) Debenzoylation:** 30% aq. ammonia, 16h, 55˚C. '^' indicates position of the inter-subunit triazole linkage.

[a] Sequence **i** was exclusively used for CuAAC in solution, **ii** was used both in solution and combined solid and solution phase approach, **iii** was required for **TL-PMO4** in combined solid and solution phase approach.

Scheme 6: Semi-automated Solid Phase Synthesis of 3′-Alkyne PMO fragment and subsequent Cu(I) Click (CuAAC) ligation (*on solid support*) with 5′-Azide PMO fragment (Single triazole incorporation),



Reagents and conditions (i) **Deblocking:** CYPTFA, 25s, four times; (ii) **Coupling:** chlorophosphoramidate monomer (3 equiv.), ETT (3 equiv.), and NEM (5 equiv.), NMP, 10 min, 3 times (4 times, for 3′-N-propargyl chlorophosphoramidate) (iii) **Capping:** 1:1 10% Ac_2O-NMP and 10% DIPEA-NMP, 30 s, three times; (iv) **cleavage from the solid support:** 30% aq. NH_3, 55°C, 16 h. **Cu-AAC:** $CuSO_4.5H_2O$ (5equiv), THPTA (5 equiv.), Na-ascorbate (10 equiv), NMP-water (4:1) (6h x 3times). '^' indicates position of the inter-subunit triazole linkage.

The intended method will essentially be a combination of solid and solution phase since the azide fragment (4-6mer, **Scheme 5**) is being synthesized in solution. Initially, we standardized this protocol on the same custom 12 mer PMO sequence, 5′-TTTTAC^TCACAT-3′ (**TL-PMO3**), with hexamer azide PMO fragment being ligated to alkyne fragment on solid support via CuAAC. After monomer loading on solid support, up to terminal alkyne chlorophosphoramidate monomer coupling has been performed on automated oligosynthesizer to obtain 5′-TTTTAC-propargyl (**Scheme S2**), as per our previously

reported protocol[6]. Thereafter, Click ligation of the azide PMO fragment (5′-N_3-TCACAT-3′) was conducted manually on solid support in NMP-water (4:1) system, using similar conditions used in solution (**Scheme 6**). The optimized CuAAC conditions have shown almost quantitative conversion as per RP-HPLC profile (**Figures S14-15**). After Click ligation, the TL-PMO is detritylated using CYPTFA, followed by cleavage from solid support in 30% aq. ammonia. The TL-PMOs are purified by RP-HPLC and characterized using MALDI-TOF MS (**Figures S16-18**). These PMOs are then subjected to

Table 1. Sequences and Thermal Melting Temperatures (T_m) of Duplexes of PMOs and Triazole-incorporated PMOs (TL-PMOs) with DNA and RNA[a]

Sl. No.	PMO Sequence [b]	T_m with DNA (˚C)	ΔT_m (˚C)[d]	T_m with RNA (˚C)	ΔT_m (˚C)[d]
PMO1[6]	5′-TTTTACTCACAT-3′	26.0	-	24.0	-
TL-PMO2[c]	5′-TTTTAC^TCACAT-3′	25.8	-0.2	31.6	+7.6
TL-PMO3	5′-TTTTAC^TCACAT-3′	24.8	-1.2	30.6	+6.6
TL-PMO4	5′-TTTTACTC^ACAT-3′	28.3	+2.3	34.5	+10.5

[a]**Conditions:** 40 mM phosphate buffer (pH 7), with concentration of 2 μM (each strand). T_m values reported are the averages of two independent experiments that were within ±1.0°C. [b] '^' indicates position of the inter-subunit triazole linkage. [c] **TL-PMO1** is 5′-OTBDPS protected. [d] ΔT_m values are calculated w.r.t. melting temperatures of corresponding regular analogue (**PMO1**).

hybridization studies with complementary DNA/ RNA, and melting temperatures were compared with regular analogues (**Figures S19-24**). The only difference between the previously synthesized solution phase PMO (**TL-PMO2**), and this PMO (**TL-PMO3**) lies in the 5′ end. The 5′-OH end of **TL-PMO2** is TBDPS protected, unlike the case of this one (**TL-PMO3**). Therefore, differential analysis of their hybridization properties of these two TL-PMOs is an important parameter to consider. Both, **TL-PMO2** and **TL-PMO3** were subjected to form duplex with their complementary DNA/ RNA, and have shown almost similar melting temperature, which is slightly higher than that of regular PMO. Hence, we can conclude that the designed triazole linkage is a good fit inside the PMO backbone, and does not negatively affect its hybridization properties (**Table 1**). Also 5′-OTBDPS protection has virtually no role in effecting Watson-Crick base pairing in PMO-DNA/ PMO-RNA duplexes, which is comprehensible since 5′ end stays far away from Watson-Crick base pairing.

Both in **TL-PMO2** and **TL-PMO3,** triazole linkage is exactly at the middle of the sequence. Hence, the effect of shifting the triazole towards termini in its hybridization properties needs to be evaluated. PMO synthesis rolls in 5′→3′ direction, unlike phosphoramidite chemistry of DNA/RNA. Therefore, in case of the PMOs, the 3′-end is more accessible for triazole incorporation via convergent method. Hence, we had shifted the triazole to the 3′ terminal (between 8th and 9th subunit) in the same sequence (**TL-PMO4**), hybridized with complementary DNA/RNA, and found to have even higher binding affinity than both **PMO1** and **TL-PMO2/3** (**Table 1**). Therefore, possibly shifting the triazole into 3′-terminal is positively affecting the Watson-Crick base pairing, which is indeed a great result to consider for longer PMO synthesis.

Conclusion:

In summary, we have synthesized a novel triazole incorporated backbone of PMO, leveraging the robustness of Cu^I catalyzed Click chemistry[22,23], and 'Click'able terminal alkyne monomer building blocks, required for the synthesis of it. The synthetic strategy reported here is convergent, with 4-6 mer PMO fragments being Click ligated at 3′-end. Such approach is important for PMOs for its efficacy as an antisense agent, since it can easily rule out N-1, N-2 mers, one or two units shorter than the desired oligo, which are virtually inseparable under standard purification conditions. In addition, incorporation of a single triazole unit, is perfectly fitted inside the backbone, with almost same or even higher duplex stability with complementary strands than its regular version. The selection of a flexible triazole linkage with methylene groups at both ends has positively affected the Watson-Crick base pairing, as evident in hybridization studies. Further evaluation of the secondary structures of the duplexes is required to fully understand the effect of triazole incorporation. While duplex stability is an inherent parameter for the therapeutic use of ASO-based drugs, there are other equally crucial parameters to consider, including metabolic stability, cell permeation, liberation from endolysosomal compartments, and target specificity. Based on the findings presented over here, it is imperative to conduct further assessments to evaluate the gene-silencing and splice-modulating abilities of triazole incorporated PMOs, to fully understand their potential for therapeutic use as an antisense agent. To achieve so, synthesis of full-length triazole-incorporated PMOs (20-25mer) are required exploiting this approach, to evaluate its antisense efficacy. Nevertheless, to the best of our knowledge, this is the second attempt to the convergent synthesis of PMOs, after a preliminary attempt by Wada *et. al.*[8] This Click ligation approach would facilitate for the synthesis of full length PMO and scaling up of PMO, while retaining favourable biophysical properties will be reported in due course.

EXPERIMENTAL SECTION

General method for the synthesis of 3′-*N*-propargyl-chlorophosphoramidate monomer (6): To a stirred solution of **5** (1 equiv) in dry (1:1) MeCN-DCM at 0°C were added Lithium Bromide (5 equiv.), 1,1,3,3-tetramethylguanidine (4 equiv) and $POCl_2NMe_2$ (4 equiv). Then the reaction mixture

was left for 30 min at 0˚C. TLC showed complete consumption of the starting material. After completion of the reaction (TLC analysis), the reaction mixture was quenched with saturated NH_4Cl solution maintaining ice bath. The solvent was removed under reduced pressure and re-dissolved in chilled EtOAc. The organic layer was washed with water for 2 times and finally with saturated NH_4Cl. The collected organic layer was dried over anhydrous Na_2SO_4 and solvent was evaporated *in vacuo*. The crude product was purified immediately by silica gel (100-200 mesh) column chromatography eluting with Acetone-DCM to obtain the compound **6** in 62-83% isolated yield for different nucleobases.

((2S,6R)-6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-(prop-2-yn-1-yl)morpholin-2-yl)methyl dimethylphosphoramidochloridate (6T): Compound **6T** was synthesized from **5T** (291mg) and obtained as white solid**.** Yield: 261mg, 62%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 9.59 (d, *J* = 4.9 Hz, 1H), 7.31 – 7.21 (m, 1H), 5.82 (dd, *J* = 9.8, 2.7 Hz, 1H), 4.26 – 4.13 (m, 2H), 4.08 (ddd, *J* = 11.7, 4.7, 2.4 Hz, 1H), 3.39 (d, *J* = 2.4 Hz, 2H), 2.97 – 2.82 (m, 3H), 2.82 – 2.68 (m, 6H), 2.66 (d, *J* = 0.9 Hz, 4H), 2.37 – 2.19 (m, 3H), 1.89 (t, *J* = 1.5 Hz, 3H). 13C{1H}NMR (75 MHz, $CDCl_3$) δ (ppm) 163.9, 163.8, 150.1, 135.6, 135.5, 111.1, 79.6, 77.6, 77.4, 77.2, 77.0, 76.7, 74.7, 74.3, 74.1, 67.2, 67.1, 54.3, 51.4, 46.4, 40.5, 38.7, 36.8, 36.7, 12.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm) 18.56, 18.19. HRMS (ESI) m/z $[M + H]^+$ **:** Calculated for $C_{15}H_{22}N_4O_5PCl$ was 405.1094; found 405.1096.

((2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1(2H)-yl)-4-(prop-2-yn-1-yl)morpholin-2-yl)methyl dimethylphosphoramidochloridate (6C^P): Compound **6C^P** was synthesized from **5C^P**(516mg) and obtained as white solid**.** Yield: 574mg, 83%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.05 – 7.82 (m, 3H), 7.63 – 7.40 (m, 4H), 5.90 (dd, J = 9.5, 2.6 Hz, 1H), 4.25 (ddd, J = 15.3, 7.0, 4.0 Hz, 2H), 4.19 – 4.06 (m, 1H), 3.42 (d, J = 2.4 Hz, 2H), 3.16 (d, J = 10.9 Hz, 1H), 2.71 (d, J = 13.9 Hz, 7H), 2.39 (td, J = 10.9, 7.3 Hz, 1H), 2.28 (d, J = 2.5 Hz, 1H), 2.15 (td, J = 10.1, 3.1 Hz, 1H). 13C{1H}NMR (75 MHz, $CDCl_3$) δ (ppm) 162.5, 144.9, 144.7, 133.3, 132.9, 129.0, 127.9, 97.2, 81.5, 77.6, 77.1, 77.0, 76.7, 74.6, 74.6, 74.5, 67.3, 67.2, 54.9, 51.3, 46.4, 36.8, 36.7. ^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm) 18.64, 18.16. HRMS (ESI) m/z $[M + H]^+$ **:** Calculated for $C_{21}H_{26}N_5O_5PCl$ was 494.1360; found 494.1362.

((2S,6R)-6-(6-Benzamido-9H-purin-9-yl)-4-(prop-2-yn-1-yl)morpholin-2-yl)methyl dimethylphosphoramicdochloridate (6A^P): Compound **6A^P** was synthesized from **5A^P**(459mg) and obtained as white solid**.** Yield:490mg, 81%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.70 (s, 1H), 8.21 (d, *J* = 7.3 Hz, 1H), 8.03 – 7.91 (m, 2H), 7.59 – 7.35 (m, 3H), 6.03 (dd, *J* = 9.9, 2.6 Hz, 1H), 4.28 – 4.11 (m, 3H), 3.44 (d, *J* = 2.4 Hz, 2H), 3.14 (d, *J* = 11.0 Hz, 1H), 2.84 (d, *J* = 11.4 Hz, 1H), 2.68 (d, *J* = 3.6 Hz, 3H), 2.63 (s, 3H), 2.44 (t, *J* = 10.7 Hz, 1H), 2.31 (d, *J* = 2.4 Hz, 1H). 13C{1H}NMR (75 MHz, $CDCl_3$) δ (ppm) 165.1, 152.4, 151.2, 149.7, 140.7, 140.6, 133.4, 132.5, 128.5, 127.9, 122.9, 79.6, 79.6, 77.6, 77.4, 77.2, 76.9, 76.7, 74.7, 74.2, 74.2, 74.1, 74.0, 66.9, 66.8, 54.9, 51.4, 46.2, 36.5, 36.4. ^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm) 18.51, 18.32. HRMS (ESI) m/z $[M+Na]^+$ **:** Calculated for $C_{22}H_{25}N_7O_4PClNa$ was 540.1292; found 540.1294.

((2S,6R)-6-(2-Isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)-4-(prop-2-yn-1-yl)morpholin-2-yl)methyl dimethylphosphoram-idochloridate (6G^P): Compound **6G^P** was synthesized from **5G^P** (450 mg) and obtained as white solid**.** Yield: 438mg, 73%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 12.26 (s, 1H), 10.85 (s, 1H), 7.81 (d, *J* = 8.9 Hz, 1H), 5.66 (dd, *J* = 9.8, 2.6 Hz, 1H), 4.13 (dq, *J* = 16.0, 6.9, 5.7 Hz, 3H), 3.51 – 3.30 (m, 2H), 2.99 (d, *J* = 10.6 Hz, 1H), 2.91 – 2.73 (m, 3H), 2.64 (d, *J* = 1.6 Hz, 3H), 2.60 (d, *J* = 1.7 Hz, 3H), 2.43 – 2.34 (m, 1H), 2.32 (q, *J* = 2.3 Hz, 1H), 1.73 – 1.49 (m, 1H), 1.32 (h, *J* = 7.3 Hz, 1H), 1.12 (dd, *J* = 6.9, 2.8 Hz, 6H), 0.88 (t, *J* = 7.3 Hz, 1H). 13C{1H}NMR (75 MHz, $CDCl_3$) δ (ppm) 180.2, 155.9, 148.4, 148.1, 136.7, 120.3, 79.3, 77.6, 77.4, 77.2, 76.7, 76.6, 75.2, 73.7, 67.3, 67.1, 58.7, 54.77, 51.4, 51.2, 46.2, 36.6, 36.5, 35.8, 23.8, 19.6, 18.98, 18.9, 13.5. ^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm) 18.55, 18.37. HRMS (ESI) m/z $[M + Na]^+$: Calculated for $C_{19}H_{27}N_7O_5PClNa$ was 522.1398; found 522.1399.

ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information**.**

Supporting Information

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

AUTHOR INFORMATION

Corresponding Author

* **Surajit Sinha** − School of Applied and Interdisciplinary Sciences, Indian Association for the Cultivation of Science, Kolkata 700032, India; Email: ocss5@iacs.res.in.

Authors

Arpan Banerjee - School of Applied and Interdisciplinary Sciences, Indian Association for the Cultivation of Science, Kolkata 700032, India

Arnab Das- School of Applied and Interdisciplinary Sciences, Indian Association for the Cultivation of Science, Kolkata 700032, India

Atanu Ghosh- School of Applied and Interdisciplinary Sciences, Indian Association for the Cultivation of Science, Kolkata 700032, India

Abhishek Gupta- School of Applied and Interdisciplinary Sciences, Indian Association for the Cultivation of Science, Kolkata 700032, India

Author Contributions

S.S. conceived the idea, designed the hypothesis and edited the manuscript. A.B. synthesized the chlorophosphoramidate monomers, PMOs and characterized them and carried out all the biophysical studies. A.D. synthesized the 5-mer N_3-T_5 fragment and 11-mer T. A. Ghosh synthesized the Nanaog PMOs in automated synthesizer. A. Gupta synthesized 6-mer 5′-OTBDPS-T_6-alkyne fragment. A.B.

and A.D. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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