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Supplemental information

Feasibility of cRGD conjugation at 5′-antisense strand of siRNA by phosphodiester linkage extension

Xinyang Zhou, Yufei Pan, Lijia Yu, Jing Wu, Zheng Li, Huantong Li, Zhu Guan, Xinjing Tang, and Zhenjun Yang

Table S1. The single strands sequence and Mass

No	Name	Sequence (5'-3')	Calcd.	Found
siMB3	S	GCU ACA GAG AAA UCU CGA Udtdt	6677	6679
	As	AUC GAG AUU UCU CUG UAG Cdtdt	6608	6608
1	L0As	L_0-AUC GAG AUU UCU CUG UAG Cdtdt	6789	6789
2	L1As	L_1-AUC GAG AUU UCU CUG UAG Cdtdt	6965	6967
3	RL0As	cRGD-L_0-AUC GAG AUU UCU CUG UAG Cdtdt	7374	7374
4	RL1As	cRGD-L_1-AUC GAG AUU UCU CUG UAG Cdtdt	7553	7553
5	RL2As	cRGD-L_2-AUC GAG AUU UCU CUG UAG Cdtdt	7732	7735
6	RL3As	cRGD-L_3-AUC GAG AUU UCU CUG UAG Cdtdt	7913	7915
7	RL4As	cRGD-L_4-AUC GAG AUU UCU CUG UAG Cdtdt	8093	8095

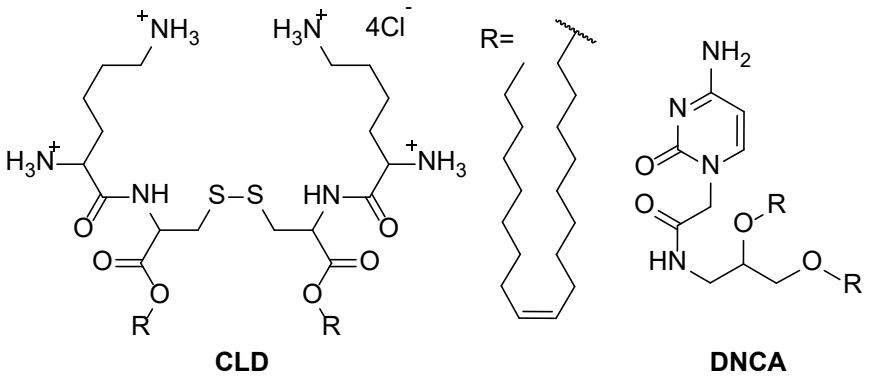

Figure S1. The structure of cationic lipid CLD and neutral lipid DNCA.

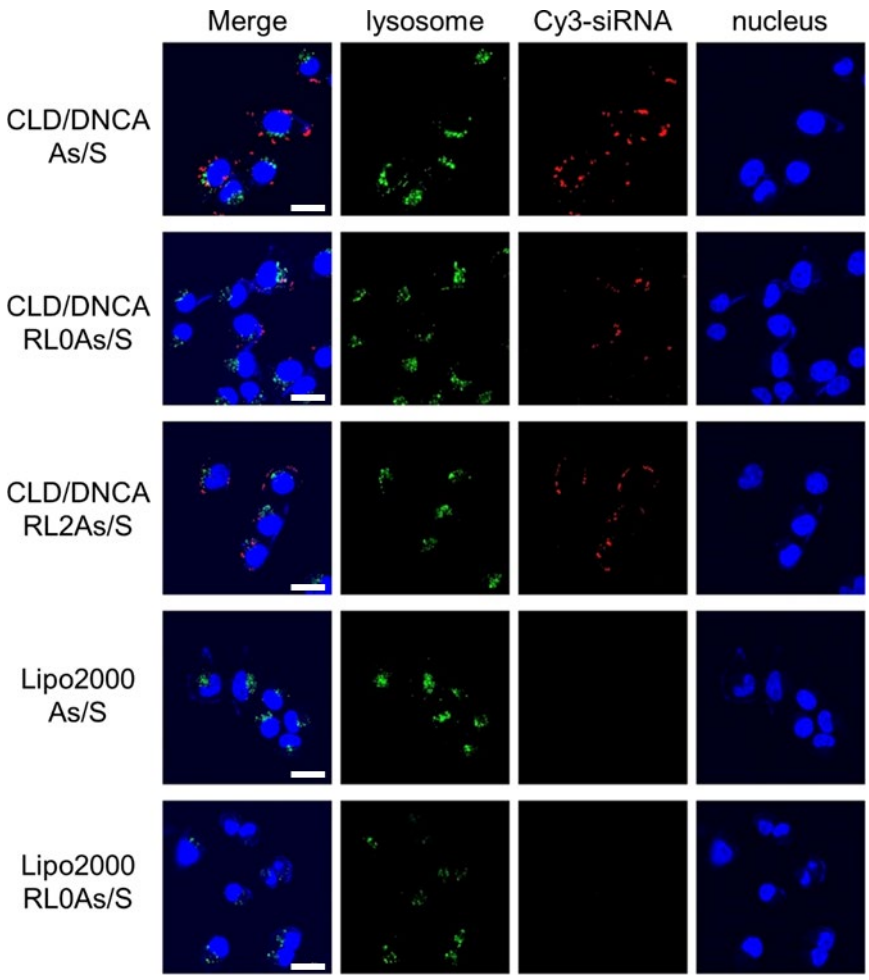

Figure S2. Confocal laser scanning microscopy (CLSM) images of A375 cells incubated with the Cy3-siRNA(50 nM) with different delivery system. Endosomes are stained by LysoBrite NIR and colored as green; nuclei were stained by Hoechst 33342 and colored as blue; Cy3-siRNA colored as red; merge of three layer. Lipo2000 represent Lipofectamine 2000. The white scale bar represents 25 μm.

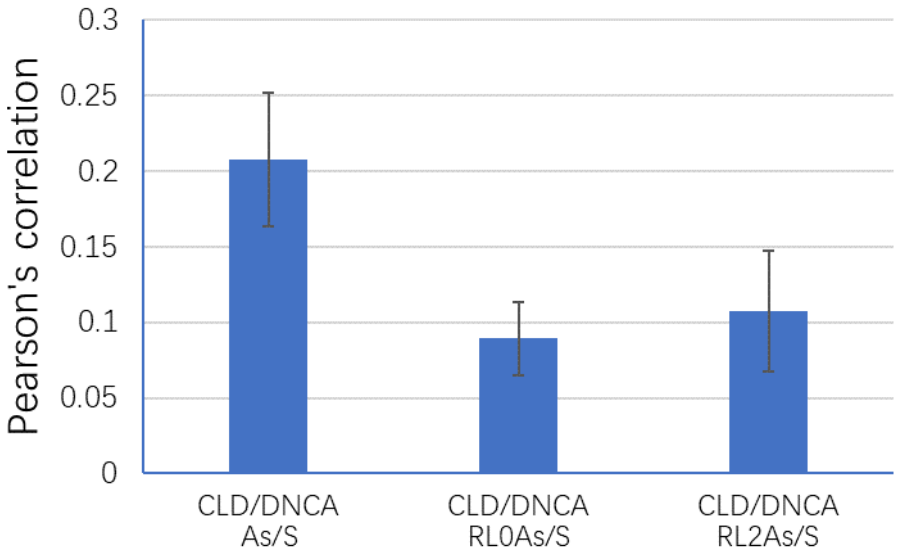

Figure S3. The quantitation of colocalization of cy3 labeled siMB3 and lysosome of Figure 3d.

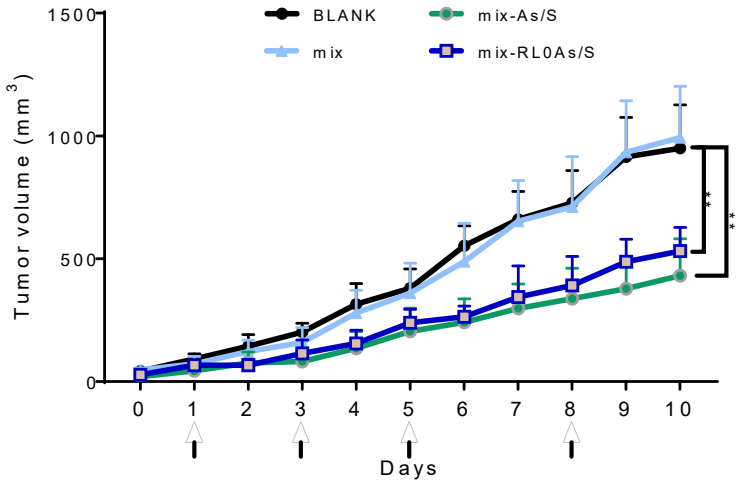

Figure S4. The tumor volume of A375 xenograft tumor-bearing nude mice treated with siMB3 or RL0As/s-siMB3 delivered by CLD/DNCA/PEG-DSPE(represented as mix). Different formulations were given by intravenous injection at days 1, 3, 5, 8 at siRNA dosage of 2 mg/kg.

2. Experimental Section

Synthesis of linker 0 (L0PA)

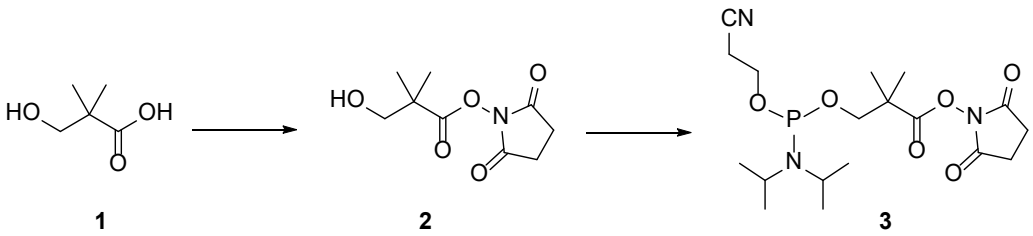

Figure S3. Synthesis of **linker 0 (L0PA)** for conjugation with cRGD

*Synthesis of compound **2***

As described in Figure S3, compound **1** (3.0 g, 25.4 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) (5.4 g, 30.2 mmol) were dissolved with 20 mL dry DCM (CH_2Cl_2). N-Hydroxysuccinimide (NHS) (3.3g, 28.6 mmol) was then added and the mixture was stirred overnight. The solvent was removed under reduced pressure, the crude reaction residue was purified by column chromatography [ethyl acetate (EA)/petroleum ether (PE), 1:1] to afford compound **2** (4.3g, 81%). ESI-MS m/z Calcd for compound **2**, 215.08, Found $[M+H]^+$ 216.21, $[M+Na]^+$ 238.18. ^1H NMR (400 MHz, Chloroform-*d*) δ 3.67 (d, 2H), 3.06 (s, 1H), 2.82 (s, 4H), 1.32 (s, 6H).

*Synthesis of compound **3***

2-Cyanoethyl N,N,N',N'-tetraisopropyl phosphorodiamidite (1.5 g, 5.0 mmol in 2 mL DCM) was added to a mixture of compound **2** and tetrazole (0.44 g, 6.28 mmol) in dry DCM (2 mL) under N_2. The reaction mixture was stirred at room temperature for 2 h. The desired product was purified by column chromatography (EA/PE, 1:10) to afford compound **3**. ESI-MS m/z Calcd for compound **3**, 415.19, Found 416.22, $[M+Na]^+$ 438.16. 1H NMR (400 MHz, Chloroform-*d*) δ 3.91 – 3.86 (m, 2H), 3.70 – 3.60 (m, 2H), 3.60 – 3.52 (m, 2H), 2.84 (s, 4H), 2.64 (t, 2H), 1.61 (s, 6H), 1.24 – 1.19 (m, 12H). ^{31}P NMR (162 MHz, $CDCl_3$) δ 147.94.

Synthesis of linker 1 (L1PA)

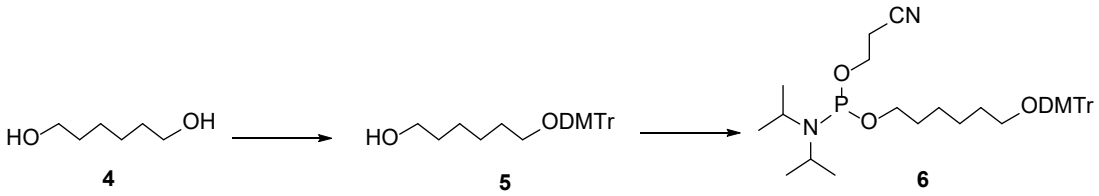

Figure S4. Synthesis of **linker 1 (L1PA)** for cRGD modified siRNA

*Synthesis of compound **5***

As described in Figure S4, compound **4** (0.5 g, 4.2 mmol) and triethylamine (0.43 g, 4.2 mmol) were dissolved with 10 mL dry DCM. DMTrCl (0.64 g, 1.9 mmol) dissolved in DCM was then added slowly to the above mixture. The reaction mixture was stirred at room temperature for 4 h. Solvent was removed at reduced pressure, the crude reaction residue was purified by column chromatography (EA/PE, 1/5) to afford compound **5** (0.67 g, 38%). ESI-MS m/z Calcd for compound **5**, 420.23, Found $[M+Na]^+$ 443.20, [DMTr] 303.27. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 7.44 – 7.36 (m, 2H), 7.33 – 7.18 (m, 7H), 6.91 – 6.83 (m, 4H), 3.72 (s, 6H), 3.44 – 3.37 (m, 2H), 3.02 – 2.92 (m, 2H), 1.59 – 1.49 (m, 2H), 1.46 – 1.38 (m, 2H), 1.33 – 1.23 (m, 4H).

Synthesis of compound **6**

2-Cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite (0.64 , 2.1 mmol) in 2 mL DCM was added to a mixture of compound **5** (0.45 g, 1.1 mmol) and tetrazole (0.18 g, 2.6 mmol) in dry DCM (2 mL) under N_2. The reaction mixture was stirred at room temperature for 2 h. The desired product was purified by column chromatography (EA/PE, 1:10) to afford compound **6** (0.48 g, 72.7%). ESI-MS m/z Calcd for compound **6**, 620.34; Found $[M+H]^+$ 621.34, $[M+Na]^+$ 643.26, [DMTr] 303.17. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.39 – 7.19 (m, 9H), 6.88

(d, 4H), 3.73 (s, 6H), 3.72 – 3.64 (m, 2H), 3.64 – 3.46 (m, 4H), 2.95 (t, 2H), 2.74 (t, 2H), 1.60 – 1.46 (m, 4H), 1.37 – 1.24 (m, 4H), 1.12 (dd, 12H). ^{31}P NMR (162 MHz, DMSO-*d*6) δ 146.34.

Synthesis and purification of naked siRNAs and their corresponding cRGD conjugates

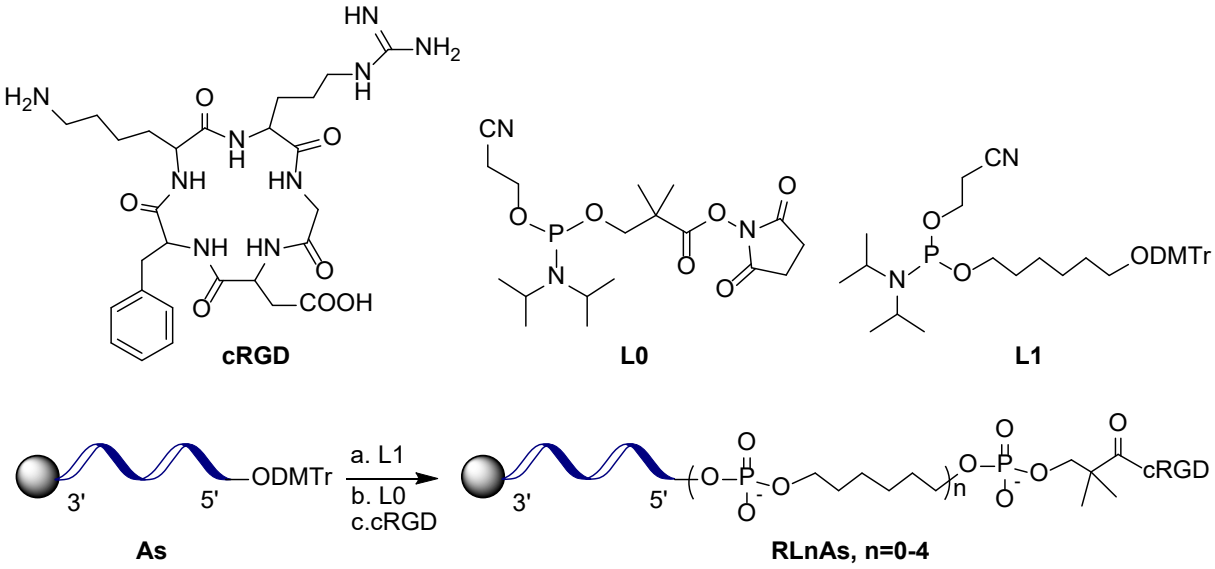

Figure S5. The synthetic route for the CPG of cRGD modified single stranded RNA. Step a, Step b used solid synthesis by phosphoroamidite chemistry. Step c 1.5 eq DIPEA in DMF.

The CPG for the cRGD modified single strand RNA was synthesized by two different alkyl phosphoroamidites inserted at 5′-terminal of RNA by solid synthesizer, respectively. Then it was conjugated with cRGD by amide reaction in DMF for 24 h. The desired CPG was obtained, washed three times by 1 mL EtOH, and washed once by aether, and allow to air dry.

AMA solution of aqueous ammonium hydroxide (28%) and aqueous methylamine (40%) (V/V=1/1) was used to cleave RNA from CPG (60℃, 90 min). Then the solution was collected and concentrated by vacuum evaporation. The residue was resolved completely in 50 μL DMSO, and 50 μL triethylamine trihydrofluoride (TEA·3HF) was added to remove the silyl protecting groups (60℃, 90 min). Butanol (250 μL) and 10 μL NaOAc (3M) was added to precipitate the desired compounds, then it was stored in -30 ℃ refrigerator for 30 min to get the precipitate. Then precipitate was collected for further purification.

All the native single strand RNAs were purified using Waters 1525 HPLC under the reversed-phase conditions: A, 0.1 M triethylammonium bicarbonate buffer (TEAB, pH 8.5); B, acetonitrile. (A: 98-75% in 25 min. HPLC column: XBridge^{TM} OST C18 2.5 μm 4.6×50 mm Column). The target fraction solution was collected and concentrated by vacuum evaporation.

The residue was desalted by HiTrap^{TM} 5mL Desalting column to achieve high purity. The target product fractions were collected and characterized by electrospray ionization mass (ESI-MS).

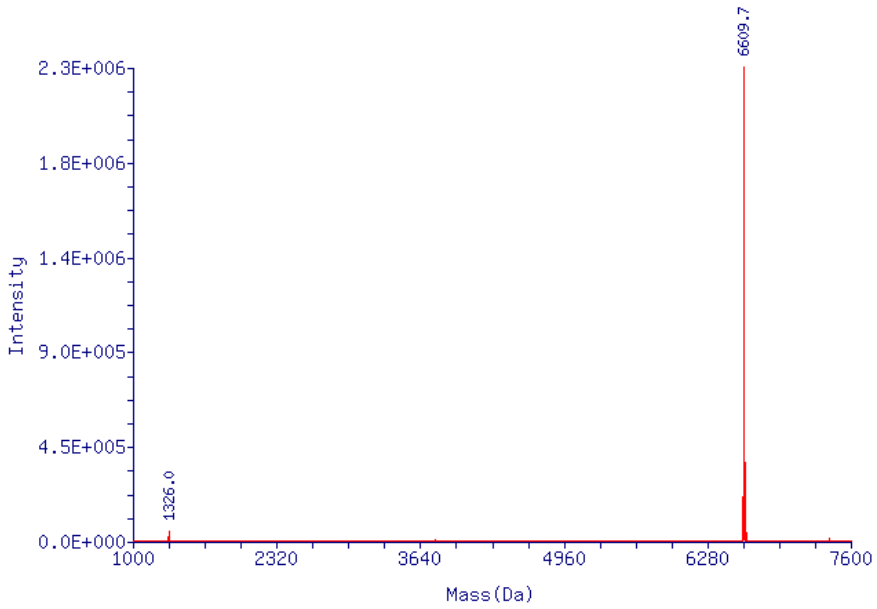

Figure S6. The ESI-MS of As strand

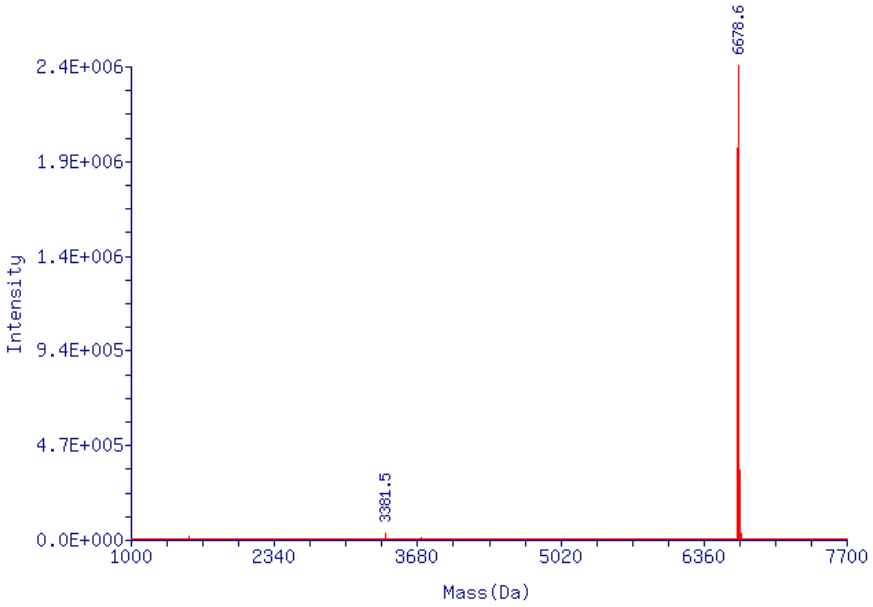

Figure S7. The ESI-MS of S strand

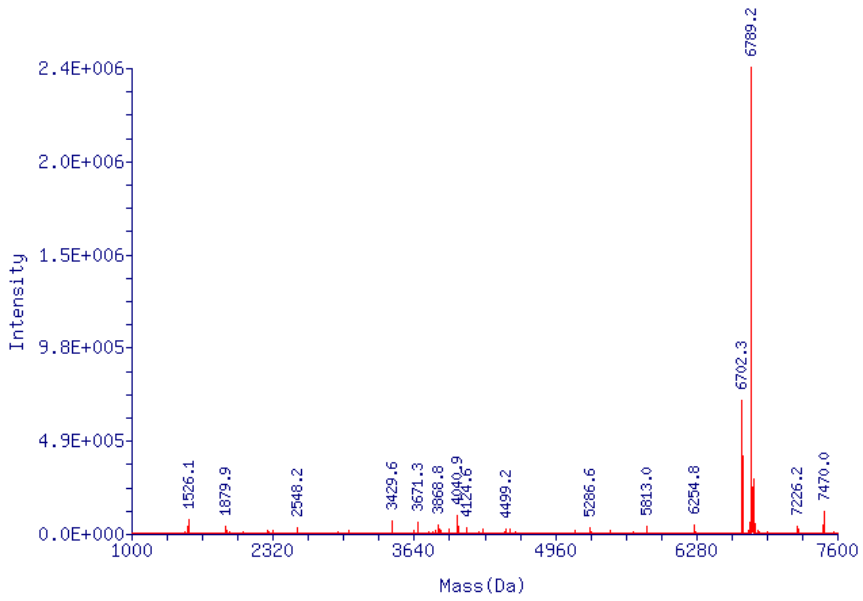

Figure S8. The ESI-MS of L0As strand

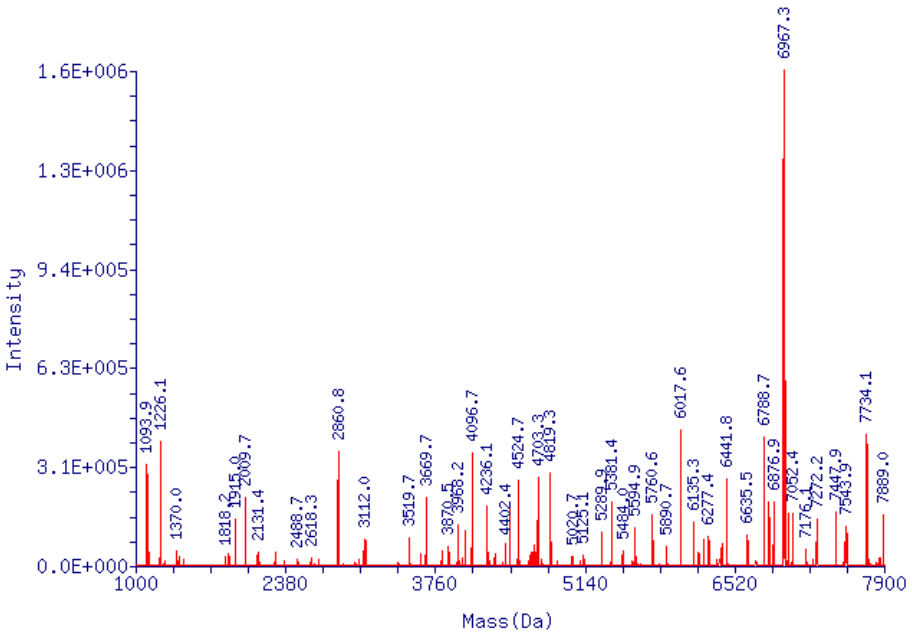

Figure S9. The ESI-MS of L1As strand

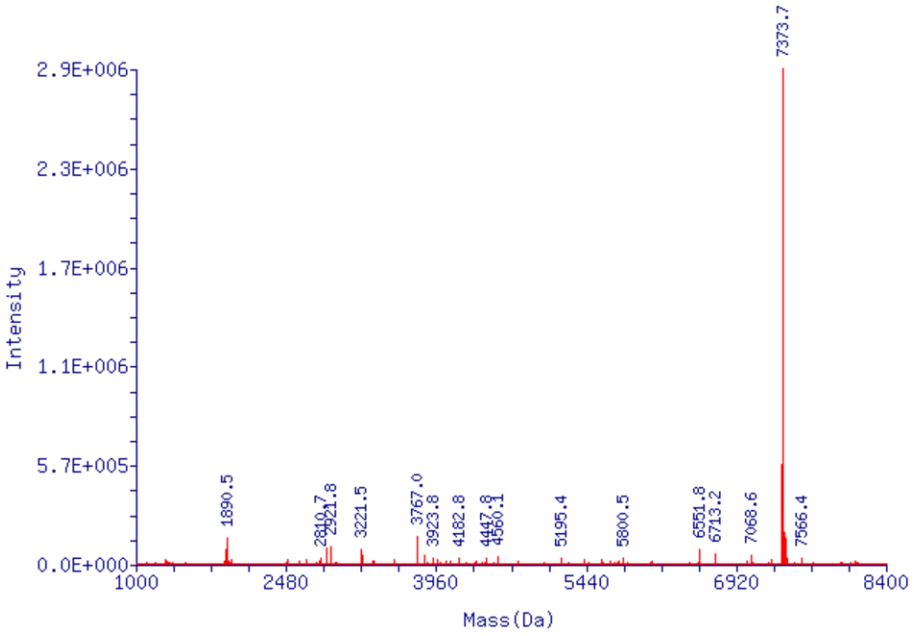

Figure S10. The ESI-MS of RL0As strand

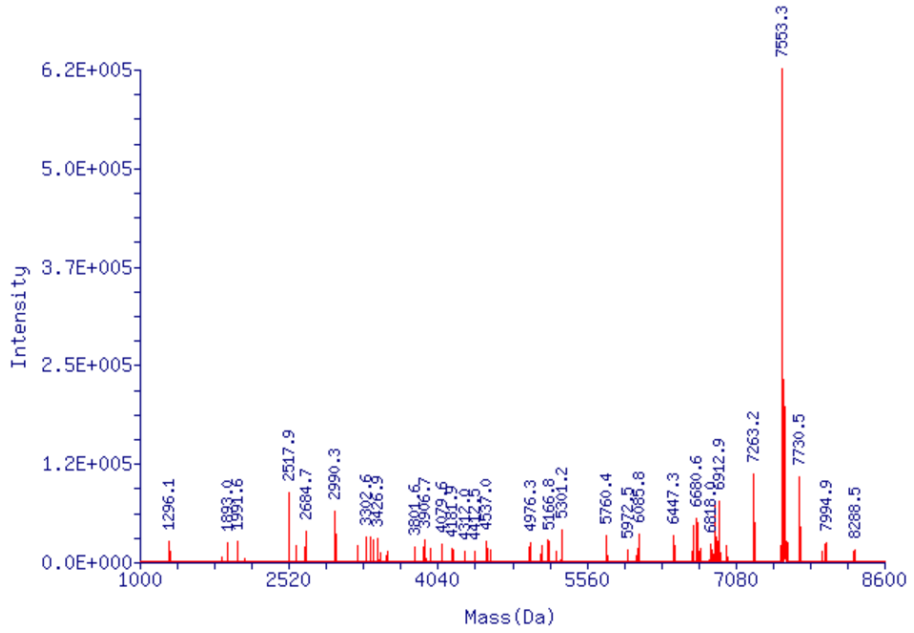

Figure S11. The ESI-MS of RL1As strand

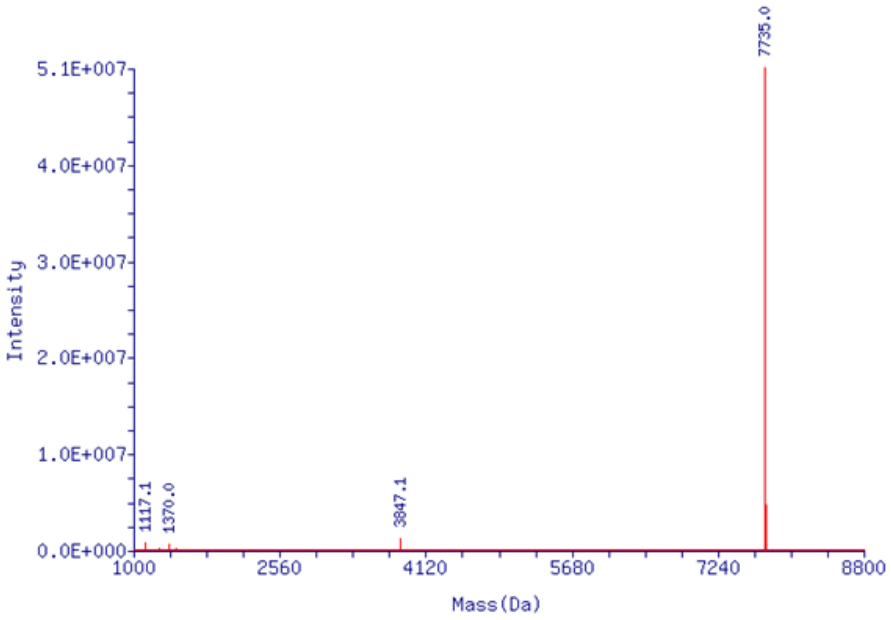

Figure S12. The ESI-MS of RL2As strand

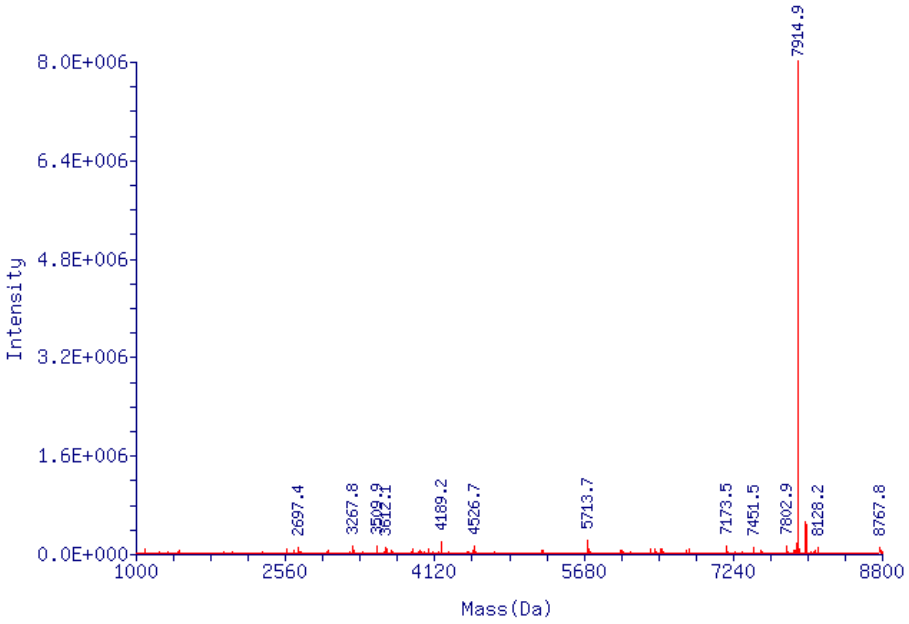

Figure S13. The ESI-MS of RL3As strand

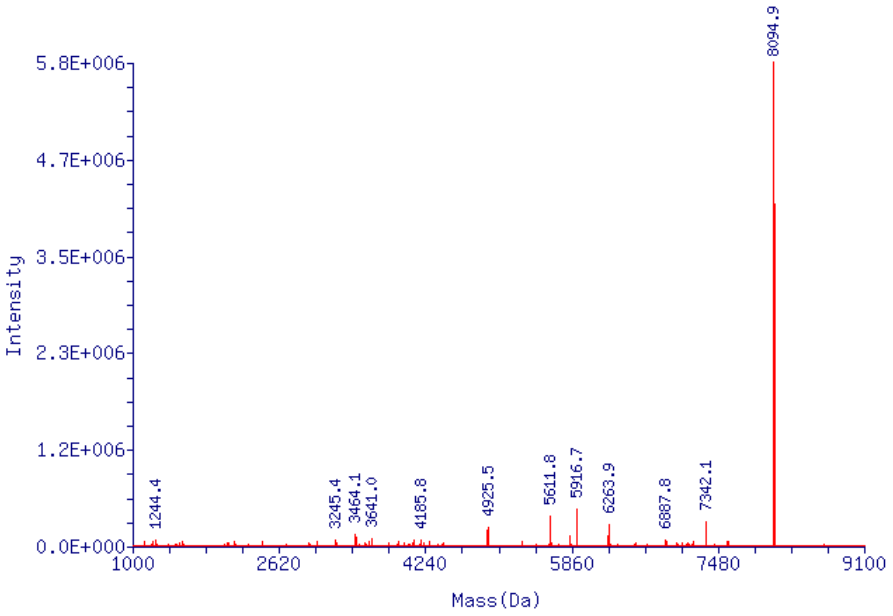

Figure S14. The ESI-MS of RL4As strand

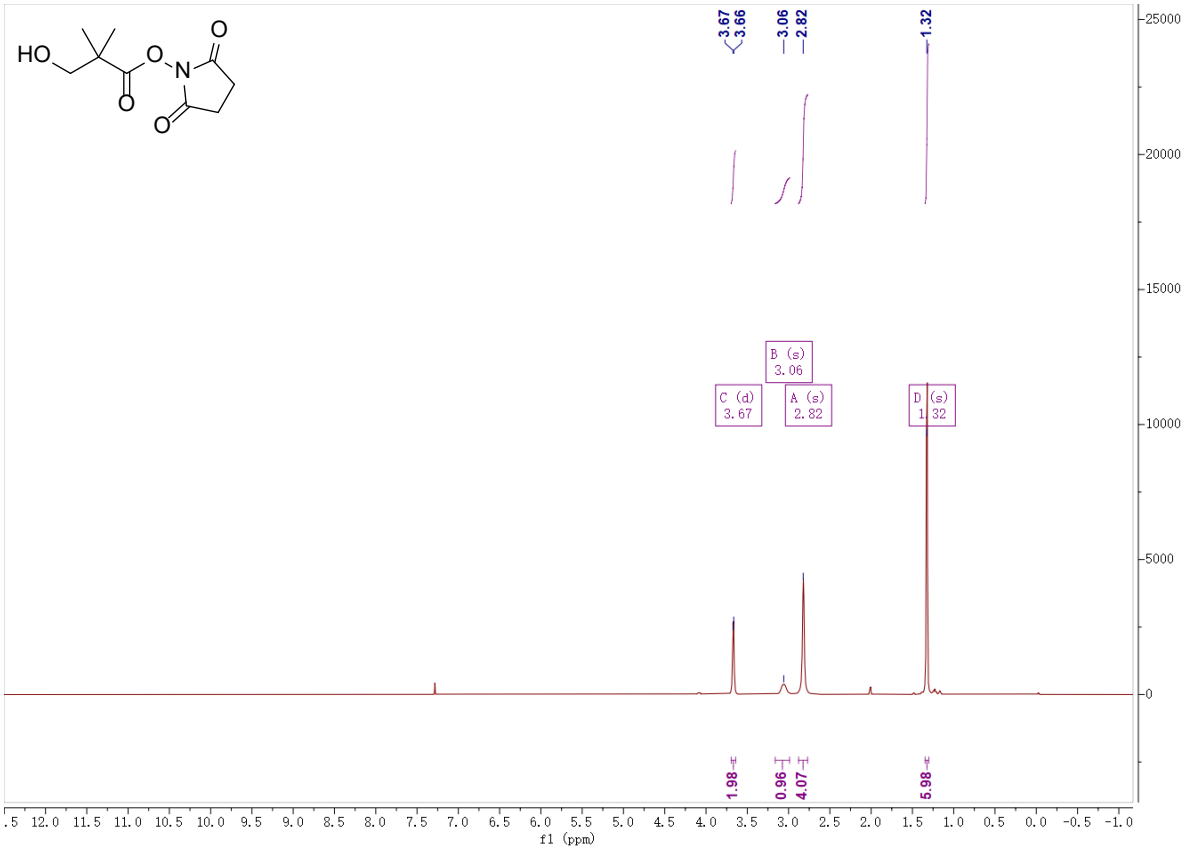

Figure S15. ^{1}H NMR of compound **2**

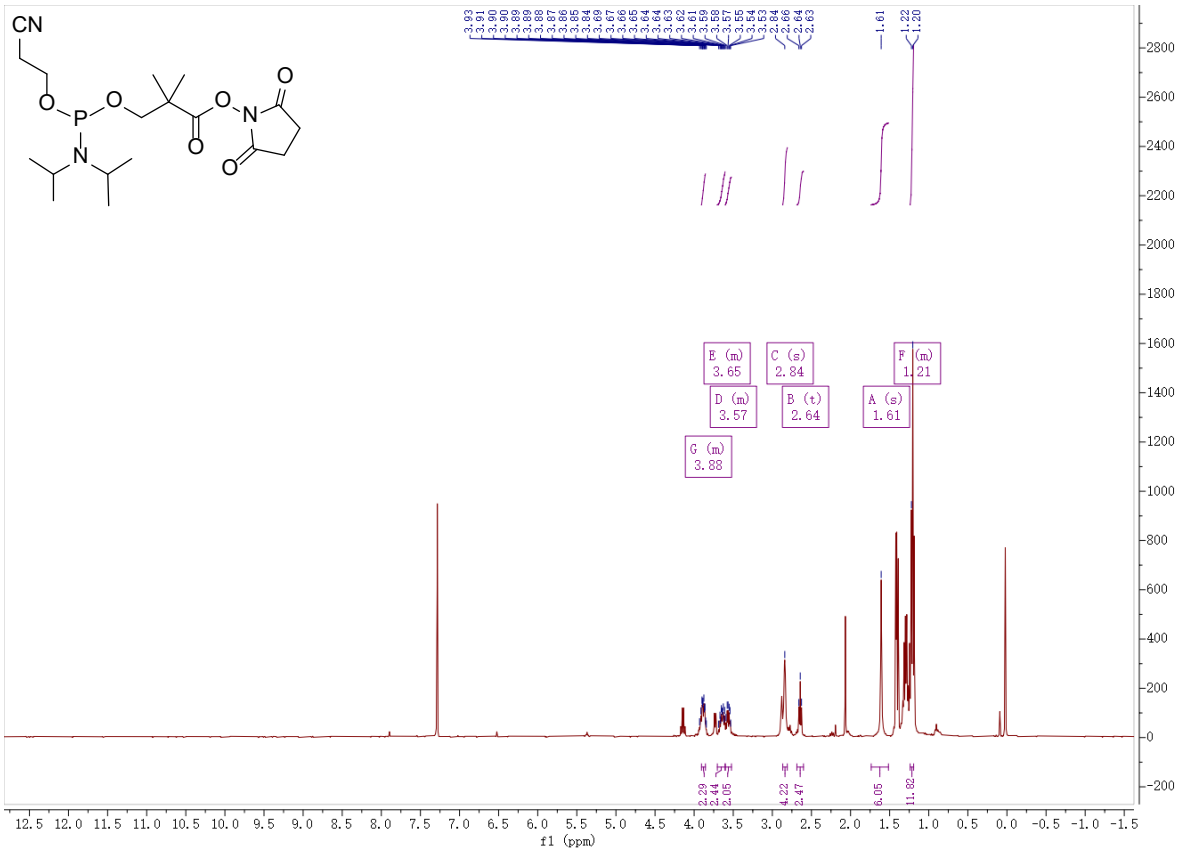

Figure S16. ^{1}H NMR of compound **3**

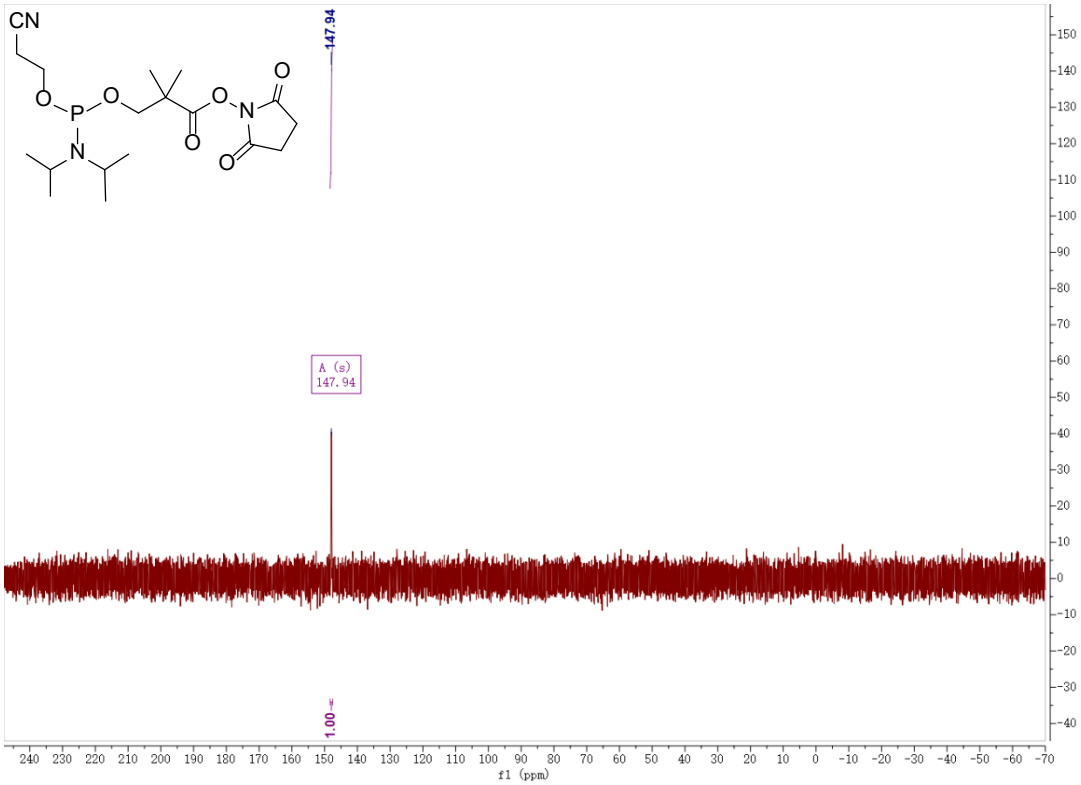

Figure S17. ^{31}P NMR of compound **3**

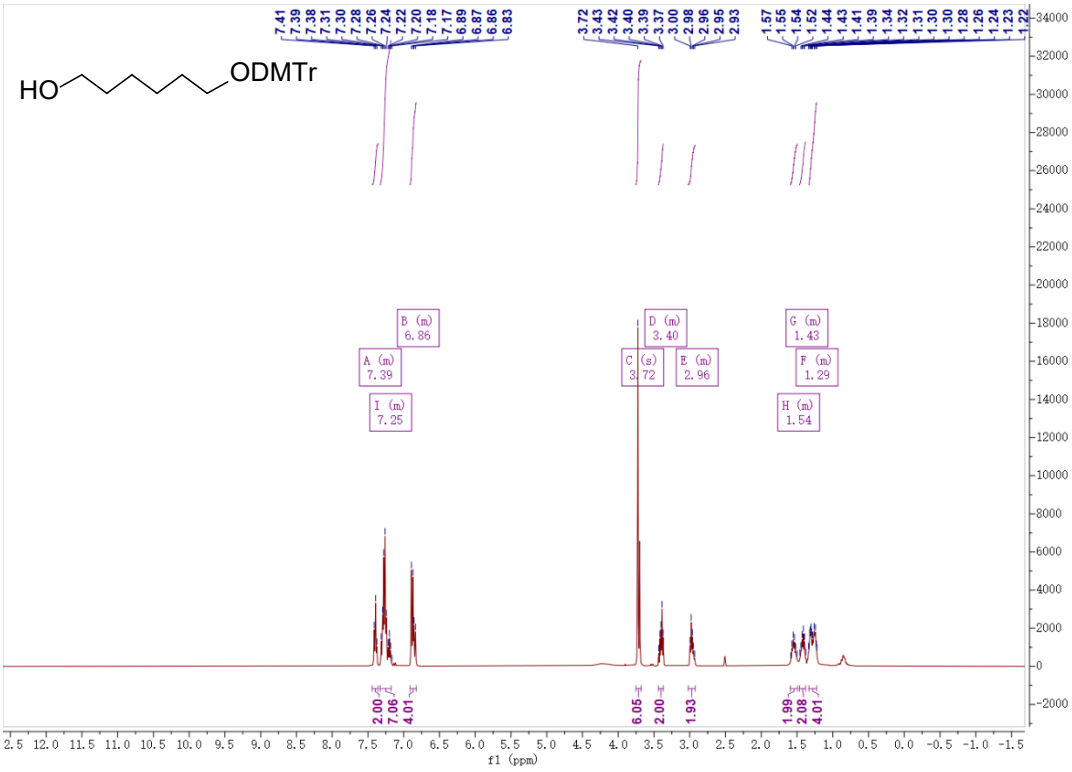

Figure S18. ^{1}H NMR of compound **5**

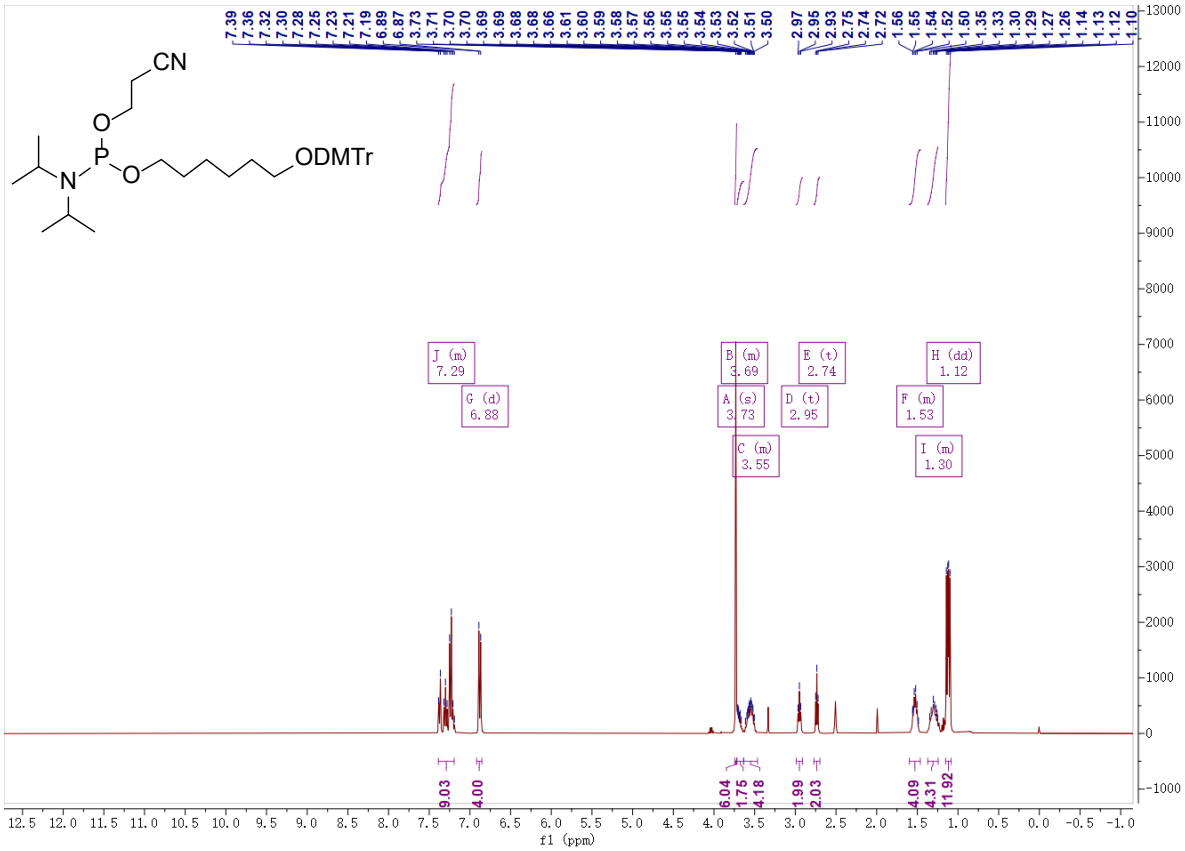

Figure S19. ^{1}H NMR of compound **6**

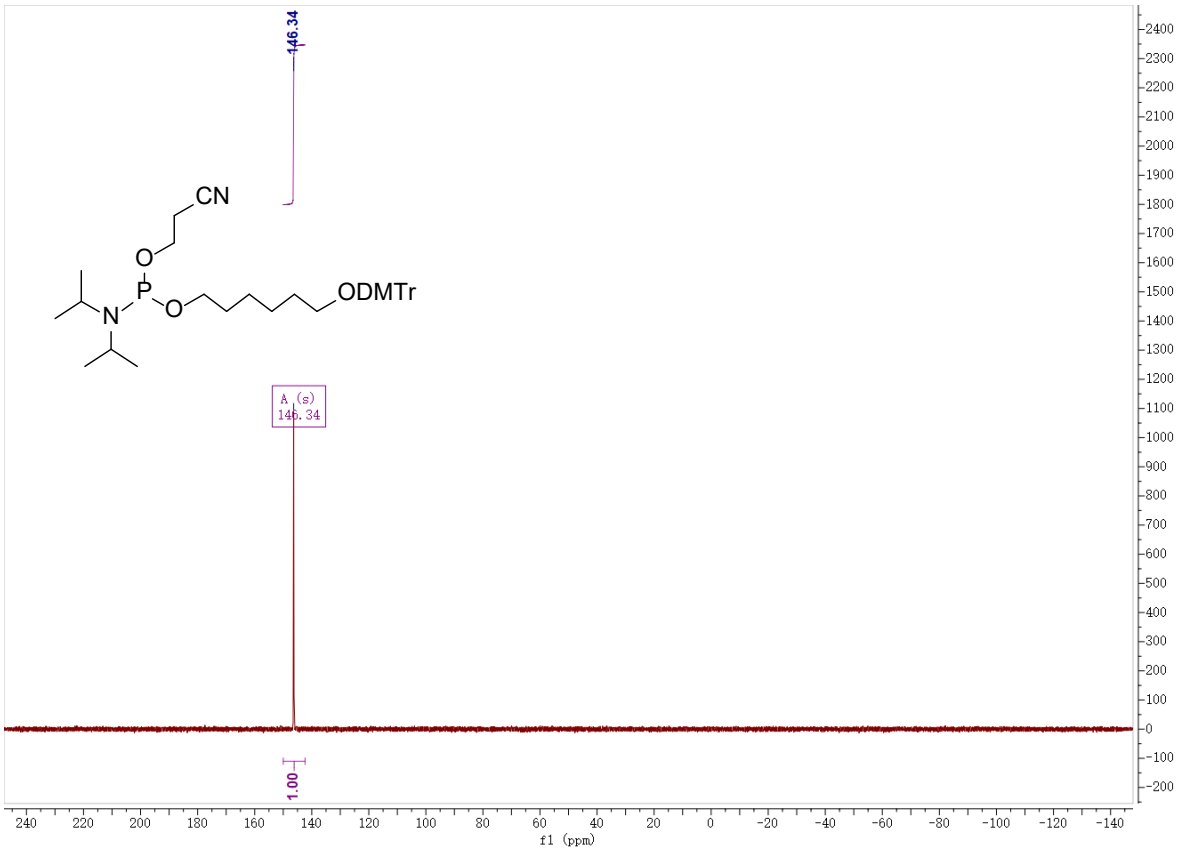

Figure S20. ^{31}P NMR of compound **6**