Supporting Information

Expanding the Scope of Replicable Unnatural DNA: Stepwise Optimization of a Predominantly Hydrophobic Base Pair

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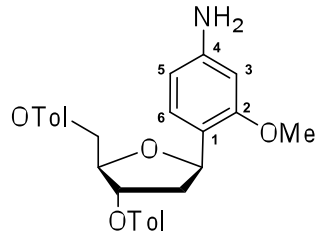
Synthetic Procedures and Characterizations

1,4-diiodo-2,5-dimethoxybenzene,[1] d**5FMTP**,[2] d**MMO2TP**,[3] d**NaMTP**,[2] d**DMOTP**,[4] d**PMO1TP**,[5] d**NMO1TP**[5] were synthesized as described previously.



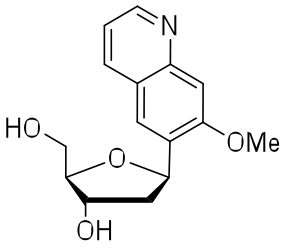
2

Compound 2. To a solution of **1** (1.5 g, 4.0 mmol, 1 equiv) in dry pyridine (10 mL) was added toluyl chloride (1.1 mL, 7.7 mmol, 2.6 equiv). The reaction mixture was stirred overnight at room temperature under nitrogen atmosphere, quenched with MeOH (2 mL), concentrated two-fold and diluted with ethyl acetate. The organic layer was washed with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of methanol (0 – 2%) in CH_2Cl_2. The eluted product was dissolved in ethyl acetate (35 mL) and Et_3N (170 μL, 1.2 mmol, 0.5 equiv) was added. The resulting solution was treated with 10% Pd/C (80 mg) under H_2 atmosphere and allowed to stir until the presence of the starting material could no longer be detected by TLC (~1 h). The reaction mixture was filtered through Celite and the filtrate was extracted with ethyl acetate and saturated aqueous $NaHCO_3$. The combined organic layers were dried (Na_2SO_4), filtered, and evaporated. The residue was subjected to silica gel column chromatography with a step gradient with EtOH (0 – 10%) in CH_2Cl_2 containing 1% triethylamine. The desired compound **2** was obtained as a white foam after evaporation of the solvent (1.05 mg, 2.21 mmol, 54% yield).

^{1}H NMR (600 MHz, CD_3CN) δ 7.95 (d, *J* = 7.9 Hz, 2H, Har), 7.91 (d, *J* = 7.9 Hz, 2H, Har), 7.32 (d, *J* = 7.8 Hz, 2H, Har), 7.28 (d, *J* = 7.9 Hz, 2H, Har), 7.10 (d, *J* = 8.1 Hz, 1H, H-6), 6.27 (s, 1H, H-3), 6.14 (d, *J* = 8.1 Hz, 1H, H-5), 5.52 (d, *J* = 5.9 Hz, 1H, H-3'), 5.34 (m, 1H, H-1'), 4.61 - 4.48 (m, 2H, H-5', H-5''), 4.37 (s, 1H, H-4'), 4.14 (s, 2H, NH_2), 3.73 (s, 3H, O*C*H_3), 2.41 (m, 7H, H-2', CH_3, CH_3), 2.11 (m, 1H, H-2'').

^{13}C NMR (151 MHz, CD_3CN) δ 166.89 (O*C*=O), 166.81 (O*C*=O), 158.59 ($C_{2\ (C\text{-}OMe)}$), 149.86 (C_4), 145.09 (Cq, Car), 144.9 (Cq, Car), 130.31 – 130.28 (CH, Ar), 130.08 (C_6) 128.27 – 127.21 (Cq, Car), 127.92 (C_1), 107.07 (C_5), 98.43 (C_3), 82.80 (C_4'), 78.24 (C_1'), 76.07 (C_3'), 65.48 (C_5'), 55.67 (O*C*H_3), 40.23 (C_2'), 21.55 (*C*H_3), 21.52 (*C*H_3).

HRMS-ESI: $[MH]^+$; calculated for $C_{28}H_{29}NO_6^+$: 476.2068 found: 476.2070.



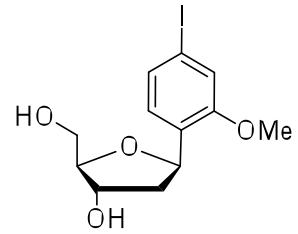
3

Compound 3. To a solution of **2** (100 mg, 0.21 mmol, 1 equiv) in a mixture of dry pyridine:CH_2Cl_2 1:1 (2 mL) was added tosyl chloride (50 mg, 0.25 mmol, 1.2 equiv). The reaction mixture was stirred 40 min at room temperature under a nitrogen atmosphere, quenched with MeOH (1 mL), concentrated two-fold, and diluted with ethyl acetate. The organic layer was washed with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered, and evaporated. The residue was dissolved in MeOH (1 mL) and Et_3N (8 μL, 0.08 mmol, 0.4 equiv) and acrolein (70 μL, 1.05 mmol, 5 equiv) were added. The resulting solution was allowed to stir at room temperature for 20 min. The reaction mixture was diluted with ethyl acetate and washed first with saturated aqueous $NaHCO_3$ and then with brine. The combined organic layers were dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of MeOH (0 – 7%) in CH_2Cl_2. The residue was dissolved in a mixture THF:HCl 3N 1:1 (2 mL) and stirred at 80 °C for 40 min under nitrogen atmosphere. The resulting mixture was diluted with ethyl acetate and quenched with saturated aqueous $NaHCO_3$ and washed with brine. The combined organic layers were dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of ethyl acetate (0 – 7.5%) in CH_2Cl_2. The eluted product was dissolved in a mixture of methanol:CH_2Cl_2 8:2 (3 mL) and cooled at 5 °C. A 30% solution of MeONa in MeOH (140 μL, 0.7 mmol, 5 equiv) was added. The mixture was stirred for 1 h at room temperature. The reaction mixture diluted with ethyl acetate, quenched with saturated aqueous NH_4Cl, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of methanol (0 – 8%) in CH_2Cl_2. The eluted product was dissolved in THF (3 mL) and potassium tert butoxide (78 mg, 0.7 mmol, 5 equiv) was added. The mixture was stirred for 3 h at 70 °C. The reaction mixture diluted with ethyl acetate, quenched with saturated aqueous NH_4Cl, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with CH_2Cl_2. The desired compound **3** was obtained as a colorless oil after evaporation of the solvent (28 mg, 0.1 mmol, 48% yield).

^{1}H NMR (600 MHz, MeOD) δ 8.70 (m, 1H, H-8), 8.25 (m, 1H, H-7), 8.08 (s, 1H, H-6), 7.36 (m, 1H, H-9), 7.34 (s, 1H, H-3), 5.47 (m, 1H, H-1'), 4.30 (m, 1H, H-3'), 3.99 (m, 4H, H-4', OC*H*$_3$), 3.73 (m, 2H, H-5', H-5''), 2.47 (ddd, *J* = 13.2, 5.8, 2.0 Hz, 1H, H-2'), 1.80 (m, 1H, H-2'').

^{13}C NMR (151 MHz, MeOD) δ 159.22 (C_2), 150.10 (C_8), 149.17 (C_4), 137.48 (C_{10}), 135.09 (C_1), 125.03 (C_6), 124.48 (C_5), 119.87 (C_9), 105.45 (C_3), 88.14 ($C_{4'}$), 76.02 ($C_{1'}$), 73.72 ($C_{3'}$), 63.49 ($C_{5'}$), 55.63 (O*C*H$_3$), 48.87 (s), 42.86 ($C_{2'}$).

HRMS-ESI: $[MH]^+$; calculated for $C_{15}H_{17}NO_4^+$: 276.1230 found: 276.1227.



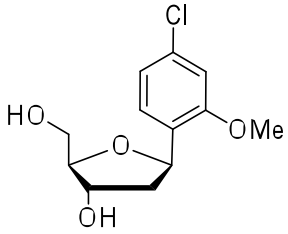
4

Compound 4. To a solution of **2** (300 mg, 0.63 mmol, 1 equiv) in THF (1.5 mL) cooled at 0 °C was added a solution of 6M aqueous chlorhydric acid (2.4 mL) dropwise over a period of 15 min. A solution of sodium nitrite (70 mg, 1.0 mmol, 1.6 equiv) in water (600 µL) was added dropwise over a period of 10 min. After an additional 30 min at 0 °C, potassium iodide (415 mg, 2.5 mmol, 4 equiv) in water (1.2 mL) was added dropwise over a period of 10 min. The reaction mixture was stirred at 0 °C for 30 min then at room temperature for an additional hour. The mixture was quenched with saturated aqueous $Na_2S_2O_3$ and saturated aqueous $NaHCO_3$, washed with brine, extracted with ethyl acetate, dried (Na_2SO_4), filtered and evaporated. The resulting residue was subjected to a silica gel column chromatography with a step gradient of CH_2Cl_2 (50 – 100%) in hexane. The eluted product was dissolved in a mixture of methanol:CH_2Cl_2 8:2 (5 mL) and cooled at 5 °C. A 30% solution of MeONa in MeOH (250 µL, 1.4 mmol, 4 equiv) was added. The mixture was stirred for 30 min at room temperature. The reaction mixture diluted with ethyl acetate, quenched with saturated aqueous NH_4Cl, washed with brine, dried (Na_2SO_4), filtered, and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of methanol (0 – 8%) in CH_2Cl_2. The desired compound **4** was obtained as white foam after evaporation of the solvent (105 mg, 0.3 mmol, 47 %).

^{1}H NMR (600 MHz, CD_3CN) δ 7.30 - 7.24 (m, 3H, H-3, H-5, H-6), 5.22 (m, 1H, H-1'), 4.20 (m, 1H, H-3'), 3.81 (m, 1H, H-4'), 3.79 (s, 3H, OC*H*$_3$), 3.57 (m, 2H, H-5', H-5''), 3.19 (s, 1H, OH-3'), 2.87 (s, 1H, OH-5'), 2.25 – 2.16 (m, 1H, H-2'), 1.68 (m, 1H, H-2'').

^{13}C NMR (151 MHz, CD_3CN) δ 157.73 (C_2), 132.18 (C_1), 130.43 (C_5), 128.51 (C_6), 120.43 (C_3), 92.74 (C_4), 88.16 ($C_{4'}$), 75.28 ($C_{1'}$), 73.88 ($C_{3'}$), 63.68 ($C_{5'}$), 56.37 (O*C*H$_3$), 42.87 ($C_{2'}$).

HRMS-ESI: $[MH]^+$; calculated for $C_{12}H_{15}IO_4^+$: 351.0088 found: 351.0085.



5

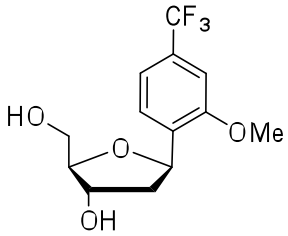
Compound 5. To a solution of **2** (100 mg, 0.21 mmol, 1 equiv) in THF (300 µL) cooled at 0 °C was added a solution of 6M aqueous chlorhydric acid (800 µL) dropwise over a period of 15 min. A solution of sodium nitrite (22 mg, 0.315 mmol, 1.5 equiv) in water (200 µL) was added dropwise over a period of 10 min. After an additional 30 min at 0 °C, copper chloride (42 mg, 0.42 mmol, 2 equiv) in water (200 µL) was added dropwise over a period of 10 min. The reaction mixture was stirred at 0 °C for 45 min then at room temperature for an additional

3 hours. The mixture was quenched with saturated aqueous $NaHCO_3$, washed with brine, extracted with ethyl acetate, dried (Na_2SO_4), filtered, and evaporated. The resulting residue was subjected to a long silica gel column and eluted with a step gradient of CH_2Cl_2 (50 – 100%) in hexane. The eluted product was dissolved in a mixture of methanol:CH_2Cl_2 8:2 (2 mL) and cooled at 5 °C. A 30% solution of MeONa in MeOH (50 μL, 0.25 mmol, 5 equiv) was added. The mixture was stirred for 1 h at room temperature. The reaction mixture was diluted with ethyl acetate, quenched with saturated aqueous NH_4Cl, washed with brine, dried (Na_2SO_4), filtered, and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of methanol (1 – 5%) in CH_2Cl_2. The desired compound **5** was obtained as white foam after evaporation of the solvent (12 mg, 0.05 mmol, 23 %).

^{1}H NMR (600 MHz, MeOD) δ 7.47 (d, *J* = 8.1 Hz, 1H, H-3), δ 6.94 (d, *J* = 1.9 Hz, 1H, H-6), 6.91 (dd, *J* = 8.2, 1.9 Hz, 1H, H-5), 5.33 (m, 1H, H-1'), 4.26 (m, 1H, H-3'), 3.90 (m, 1H, H-4'), 3.81 (s, 3H, OC*H*$_3$), 3.64 (m, 2H, H-5', H-5''), 2.30 (ddd, *J* = 13.2, 5.6, 1.8 Hz, 1, H-2'), 1.73 (m, 1H, H-2'').

^{13}C NMR (151 MHz, MeOD) δ 157.73 (C_2), 134.00 (C_4), 130.29 (C_6), 127.48 (C_1), 120.86 (C_5), 111.27 (C_3), 88.04 ($C_{4'}$), 75.43 ($C_{1'}$), 73.78 ($C_{3'}$), 63.45 ($C_{5'}$), 55.58 (O*C*H$_3$), 42.64 ($C_{2'}$).

HRMS-ESI: $[MH]^+$; calculated for $C_{12}H_{15}ClO_4^+$: 259.0732 found: 259.0741.



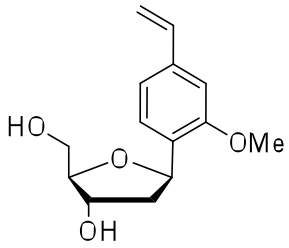
10

Compound 10. A mixture of compound **9** (70 mg, 0.12 mmol, 1 equiv), copper iodide (7 mg, 0.035 mmol, 0.3 equiv), 1,10-phenanthroline (7 mg, 0.035 mmol, 0.3 equiv) and potassium (trifluoromethyl)trimethoxyborate (100 mg, 0.47 mmol, 4 equiv) in neat DMSO (0.5 mL) was stirred at 70 °C for 16 h. The reaction was diluted in ethyl acetate and quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered, and evaporated. The resulting residue was subjected to silica gel column chromatography with a step gradient of CH_2Cl_2 (50 – 100%) in hexane. The eluted product was dissolved in a mixture of methanol and CH_2Cl_2 8:2 (1 mL) and cooled at 5 °C. A 30% solution of MeONa in MeOH (90 μL, 0.47 mmol, 5 equiv) was added. The mixture was stirred for 45 min at room temperature. The reaction mixture was diluted with ethyl acetate, quenched with saturated aqueous NH_4Cl, washed with brine, dried (Na_2SO_4), filtered, and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of methanol (0 – 6%) in CH_2Cl_2. The desired compound **10** was obtained as a colorless oil after evaporation of the solvent (41 mg, 0.07 mmol, 60 %).

^{1}H NMR (600 MHz, MeOD) δ 7.72 (d, *J* = 7.9 Hz, 1H, H-5), 7.24 (d, *J* = 7.8 Hz, 1H, H-6), 7.17 (s, 1H, H-3), 5.41 (m, 1H, H-1'), 4.28 (m, 1H, H-3'), 3.95 (m, 1H, H-4'), 3.89 (s, 3H, OC*H*$_3$), 3.68 (m, 2H, H-5', H-5''), 2.39 (dd, *J* = 13.1, 5.6 Hz, 1H, H-2'), 1.78 – 1.66 (m, 1H, H-2'').

^{13}C NMR (151 MHz, MeOD) δ 157.65 (C_2), 136.70-120.40 (CF3, C_4, C_1, C_6), 118.28 (q, J = 4.1 Hz, C_5), 107.63 (q, J = 3.7 Hz, C_3), 88.58 ($C_{4'}$), 76.05 ($C_{1'}$), 74.29 ($C_{3'}$), 63.95 ($C_{5'}$), 56.11 (O*C*H$_3$), 43.10 ($C_{2'}$).

HRMS-ESI: $[MH]^+$; calculated for $C_{13}H_{15}F_3O_4^+$: 293.0995 found: 293.0985.



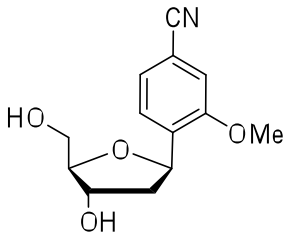
11

Compound 11. A mixture of compound **9** (140 mg, 0.23 mmol, 1 equiv), copper iodide (9 mg, 0.05 mmol, 0.2 equiv), tris(dibenzyllideneacetone)dipalladium (35 mg, 0.035 mmol, 0.15 equiv), vinyltributyltin (87 mg, 0.29 mmol, 1.2 equiv), and triphenylarsine (22 mg, 0.07 mmol, 0.3 equiv) in dioxane (1 mL) was stirred at 55 °C for 2 h. The reaction was diluted in ethyl acetate and quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The resulting residue was subjected to silica gel column chromatography with a step gradient of CH_2Cl_2 (55 – 100%) in hexane. The eluted product was dissolved in a mixture of methanol and CH_2Cl_2 8:2 (2 mL) and cooled at 5 °C. A 30% solution of MeONa in MeOH (115 μL, 0.6 mmol, 5 equiv) was added. The mixture was stirred for 45 min at room temperature. The reaction mixture diluted with ethyl acetate, quenched with saturated aqueous NH_4Cl, washed with brine, dried (Na_2SO_4), filtered, and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of methanol (0 – 5%) in CH_2Cl_2. The desired compound **11** was obtained as colorless oil after evaporation of the solvent (40 mg, 0.16 mmol, 65 %).

^{1}H NMR (600 MHz, MeOD) δ 7.46 – 7.42 (m, 1H, H-6), 7.01 – 6.95 (m, 2H, H-3, H-5), 6.70 (dd, J = 17.6, 10.9 Hz, 1H, H-7), 5.75 (dd, J = 17.6, 0.8 Hz, 1H, H-9), 5.39 (m, 1H, H-1'), 5.19 (dd, J = 10.9, 0.8 Hz, 1H, H-8), 4.26 (m, 1H, H-3'), 3.91 (m, 1H, H-4'), 3.82 (s, 3H, OC*H*$_3$), 3.65 (m, 2H, H-5', H-5''), 2.29 (ddd, J = 13.2, 5.6, 1.9 Hz, 1H, H-2'), 1.77 (ddd, J = 13.2, 10.2, 6.0 Hz, 1H, H-2'').

^{13}C NMR (151 MHz, MeOD) δ 157.28 (C_2), 138.77 (C_7), 137.60 (C_4), 130.94 (C_1), 126.51 (C_6), 119.25 (C_5), 113.25 (C_3), 108.17 (C_8), 88.00 ($C_{4'}$), 75.83 ($C_{1'}$), 73.88 ($C_{3'}$), 63.53 ($C_{5'}$), 55.22 (O*C*H$_3$), 42.65 ($C_{2'}$).

HRMS-ESI: $[MH]^+$; calculated for $C_{14}H_{18}O_4^+$: 251.1278 found: 251.1284.



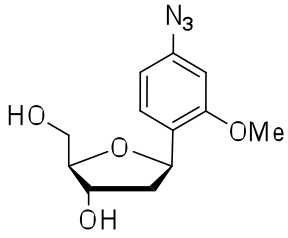
12

Compound 12. Compound **9** (120 mg, 0.2 mmol, 1 equiv), potassium hexacyanoferrate (II) (24 mg, 0.05 mmol, 0.25 equiv), potassium fluorine (12 mg, 0.2 mmol, 1 equiv), TBAB (66 mg, 0.2 mmol, 1 equiv), and palladium acetate (5 mg, 0.02 mmol, 0.1 equiv) in H_2O (2 mL) was heated to 150 °C for 15 min in a microwave synthesizer (Biotage AB, Sweden). The reaction was allowed to cool and the resulting mixture was concentrated, diluted with CH_2Cl_2, quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered, and evaporated. The resulting residue was subjected to silica gel column chromatography with a step gradient of ethyl acetate (0 – 20%) in CH_2Cl_2. The eluted product was dissolved in a mixture of methanol and CH_2Cl_2 8:2 (2 mL) and cooled at 5 °C. A 30% solution of MeONa in MeOH (60 μL, 0.34 mmol, 5 equiv) was added. The mixture was stirred for 1 h at room temperature. The reaction mixture was diluted with ethyl acetate, quenched with saturated aqueous NH_4Cl, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of methanol (0 – 6%) in CH_2Cl_2. The desired compound **12** was obtained as white foam after evaporation of the solvent (23 mg, 0.085 mmol, 44 %).

^{1}H NMR (600 MHz, MeOD) δ 7.71 (d, *J* = 7.8 Hz, 1H, H-6), 7.30 – 7.29 (m, 1H, H-5), 7.26 (s, 1H, H-3), 5.37 (m, 1H, H-1'), 4.26 (m, 1H, H-3'), 3.93 (m, 1H, H-4'), 3.87 (s, 3H, OC*H*$_3$), 3.65 (m, 2H, H-5', H-5''), 2.38 (ddd, *J* = 13.1, 5.7, 1.8 Hz, 1H, H-2'), 1.70 (m, 1H, H-2'').

^{13}C NMR (151 MHz, MeOD) δ 157.11 (C_2), 137.98 (C_1), 127.13 (C_5), 125.36 (C_6), 119.33 (CN), 113.63 (C_3), 111.92 (C_4), 88.22 ($C_{4'}$), 75.44 ($C_{1'}$), 73.68 ($C_{3'}$), 63.37 ($C_{5'}$), 55.77 (O*C*H$_3$), 42.52 ($C_{2'}$).

HRMS-ESI: $[MH]^+$; calculated for $C_{13}H_{15}NO_4^+$: 250.1074 found: 250.1067.



13

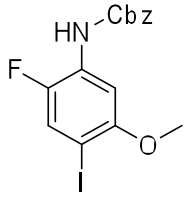
Compound 13. A mixture of compound **9** (80 mg, 0.14 mmol, 1 equiv), sodium azide (18 mg, 0.28 mmol, 2 equiv), copper iodide (3 mg, 0.014 mmol, 0.1 equiv), *N*,*N*'-dimethylethylenediamine (5 μL, 0.04 mmol, 0.3 equiv), and sodium ascorbate (1.5 mg, 0.007 mmol, 0.05 equiv) in EtOH/H_2O (1 mL) was stirred at 90 °C for 45 min. The reaction was diluted in ethyl acetate and quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The resulting residue was subjected to silica gel column chromatography with a step gradient of CH_2Cl_2 (55 – 100%) in hexane. The eluted

product was dissolved in a mixture of Methanol and CH_2Cl_2 8/2 (2 mL) and cooled at 5 °C. A 30% solution of MeONa in MeOH (100 μL, 0.5 mmol, 5 equiv) was added. The mixture was stirred for 50 min at room temperature. The reaction mixture diluted with ethyl acetate, quenched with saturated aqueous NH_4Cl, washed with brine, dried (Na_2SO_4), filtered, and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of methanol (0 – 6%) in CH_2Cl_2. The desired compound **13** was obtained as white foam after evaporation of the solvent (27 mg, 0.1 mmol, 73 %).

^{1}H NMR (600 MHz, CD_3CN) δ 7.52 (m, 1H, H-6), 6.66 (m, 1H, H-5), 6.58 (d, *J* = 2.1 Hz, 1H, H-3), 5.36 (m, 1H, H-1'), 4.27 (m, 1H, H-3'), 3.91 (m, 1H, H-4'), 3.82 (s, 3H, OC*H*$_3$), 3.66 (m, 2H, H-5', H-5''), 2.28 (m, 1H, H-2'), 1.76 (m, 1H, H-2'').

^{13}C NMR (151 MHz, CD_3CN) δ 158.22 (C_2), 140.84 (C_4), 128.31 (C_6), 127.71 (C_1), 111.03 (C_5), 102.14 (C_3), 88.00 ($C_{4'}$), 75.54 ($C_{1'}$), 73.83 ($C_{3'}$), 63.49 ($C_{5'}$), 55.42 (O*C*H$_3$), 42.66 ($C_{2'}$).

HRMS-ESI: $[MH]^+$; calculated for $C_{12}H_{15}N_3O_4^+$: 250.1135 found: 250.1127.



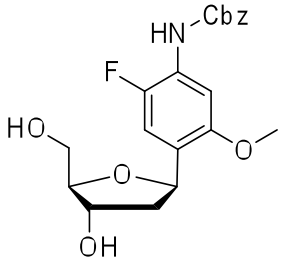
29

Compound 29. To a solution of 2-fluoro-5-methoxyaniline (2.4 g, 17.0 mmol, 1 equiv) in tetrahydrofuran (50 mL) at 0 °C was added $NaHCO_3$ (1.71 g, 20.41 mmol, 1.2 equiv). Benzyl chloroformate (2.9 mL, 20.41 mmol, 1.2 equiv) was then added dropwise under strong stirring. The mixture was allowed to warm to room temperature over 20 min, stirred for an additional hour, and then diluted with CH_2Cl_2. The organic layer was quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to a short silica gel column and eluted with a step gradient of CH_2Cl_2 (10 – 70%) in hexane affording pure NH-Cbz 2-fluoro-5-methoxyaniline. To a solution of NH-Cbz 2-fluoro-5-methoxyaniline in acetonitrile (140 mL) at -20 °C was added silver sulfate (5.76 g, 18.49 mmol) and iodine (4.69 g, 18.49 mmol). The mixture was stirred at -20 °C for 40 min, quenched with saturated aqueous $Na_2S_2O_3$ (14 mL) and filtered through a Celite pad. The filtrate was concentrated to 10% of its original volume and diluted with CH_2Cl_2 (200 mL). The organic layer was quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of CH_2Cl_2 (10 – 40%) in hexane. The desired compound **29** was obtained as white solid after evaporation of the solvent (5.52 g, 13.7 mmol, 81% yield).

^{1}H NMR (600 MHz, CD_3CN) δ 7.72 (br, 1H, NH), 7.68 (br, 1H, H-3), 7.54 (d, *J* = 10.1 Hz, 1H, H-6), 7.41 (m, 4H, H-ar (Cbz)), 7.35 (m, 1H, H-ar (Cbz)), 5.20 (s, 2H, OC*H*$_2$Ar (Cbz)), 3.80 (s, 3H, OC*H*$_3$).

^{13}C NMR (151 MHz, CD_3CN) δ 155.78 (d, *J* = 2.1 Hz, $C_{2\ (C\text{-}OMe)}$), 154.28 (NHC=O), δ 147.83 (d, *J* = 240.7 Hz, C_5), 137.44 (Cq, Car), 129.49-128.84 (CH, Ar), 128.41 (d, *J* = 12.1 Hz, C_4), 125.80 (d, *J* = 22.9 Hz, C_6), 104.66 (C_3), 76.37 (C_1), 67.76 (O*C*H$_2$Ph), 57.45 (O*C*H$_3$).

HRMS-ESI: $[MH]^+$; calculated for $C_{15}H_{14}FINO_3^+$: 401.9997 found: 402.0017.



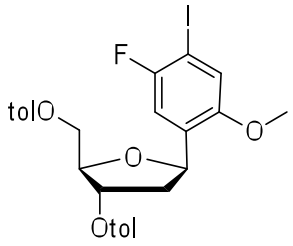
31

Compound 31. A mixture of palladium acetate (360 mg, 1.60 mmol, 0.15 equiv) and triphenylarsine (820 mg, 2.67, 0.25 equiv) in dry dimethylformamide (40 mL) was stirred under argon atmosphere at room temperature for 20 min. To this mixture was added **29** (6.43 g, 16.02 mmol, 1.5 equiv), **30** (3.68 g, 10.68 mmol, 1 equiv) and tri-n-butylamine (3.8 mL, 16.02 mmol, 1.5 equiv) in dimethylformamide (5 mL), and the resulting reaction mixture was stirred under nitrogen at 70 °C for 15 h. The reaction mixture was filtered through Celite and extracted with ethyl acetate and saturated aqueous $NaHCO_3$. The combined organic layers were dried (Na_2SO_4), filtered and evaporated. The residue was solubilized in THF (70 mL) and cooled in an ice bath. 1 M TBAF (25 mL, 23.5 mmol, 2.2 equiv) was added and the solution was stirred for 4 hours at room temperature. The reaction mixture was quenched with saturated aqueous NH_4Cl, extracted with ethyl acetate and saturated aqueous $NaHCO_3$. The combined organic layers were dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of ethyl acetate (0 – 20%) in CH_2Cl_2. The eluted product was dissolved in acetonitrile (15 mL), and the solution was cooled to -4 °C. Acetic acid (15 mL) then sodium triacetoxyborohydride (1.56 g, 5.65 mmol, 1.3 equiv) were added, and the mixture was stirred for 1 h. The reaction mixture was diluted with ethyl acetate, quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of ethyl acetate (0 – 100 %) in CH_2Cl_2. The desired compound **31** was obtained as white foam after evaporation of the solvent (2.05 g, 5.23 mmol, 49% yield).

^{1}H NMR (600 MHz, CD_3CN) δ 7.61 (br, 1H, NH), 7.54 (br, 1H, H-3), 7.41 (m, 4H, H-ar (Cbz)), 7.34 (m, 1H, H-ar (Cbz)), 7.28 (d, *J* = 11.9 Hz, 1H, H-6), 5.22 (m, 1H, H-1'), 5.19 (s, 2H, OC*H*$_2$Ar (Cbz)), 4.21 (m, 1H, H-3'), 3.81 (m, 1H, H-4'), 3.76 (s, 3H, OC*H*$_3$), 3.58 (m, 2H, H-5',H-5''), 3.19 (d, *J* = 4.0 Hz, 1H, OH-3'), 2.91 (t, *J* = 6.0 Hz, 1H, OH-5'), 2.21(m, 1H, H-2'), 1.68 (ddd, *J* = 13.0, 10.1, 5.9 Hz, 1H, H-2'').

^{13}C NMR (151 MHz, CD_3CN) δ 154.54 ($C_{2\ (C\text{-}OMe)}$), 153.09 (NH*C*=O), 148.37 (d, *J* = 235.6 Hz, C_5), 137.60 (Cq, Car), 129.46-128.80 (CH, Ar), 128.80 ($C_{1(c\text{-}sugar)}$), 125.99 (d, *J* = 12.5 Hz, C_4), 113.43 (d, *J* = 22.4 Hz, C_6), 105.42 (C_3), 88.24 (C_4'), 75.02 (C_1'), 73.92 (C_3'), 67.56 (O*C*H$_2$Ph), 63.70 (C_5'), 56.56 (O*C*H$_3$), 43.13 (C_2').

HRMS-ESI: $[MH]^+$; calculated for $C_{20}H_{23}FNO_6^+$: 392.1504 found: 392.1504.



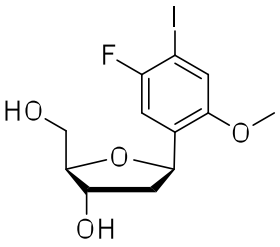
32

Compound 32. To a solution of **31** (577 mg, 1.47 mmol, 1 equiv) in dry pyridine (10 mL) was added toluyl chloride (519 μL, 3.83 mmol, 2.6 equiv). The reaction mixture was stirred for 3 h at room temperature under nitrogen atmosphere, quenched with MeOH (1.5 mL), concentrated two-fold and diluted with ethyl acetate. The organic layer was washed with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of methanol (0 – 4%) in CH_2Cl_2. The eluted product was dissolved in ethyl acetate (20 mL), and Et_3N (84 μL, 0.6 mmol, 0.5 equiv) was added. The resulting solution was treated with 10% Pd/C (38 mg) under H_2 atmosphere and allowed to stir until the presence of the starting material could no longer be detected by TLC (~1 h). The reaction mixture was filtered through Celite, and the filtrate was extracted with ethyl acetate and saturated aqueous $NaHCO_3$. The combined organic layers were dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient with CH_2Cl_2 (10 – 60%) in hexane containing 1% triethylamine. The eluted product was solubilized (450 mg, 1.0 mmol, 1 equiv) in THF (6 mL) cooled at 0 °C was added a solution of 6 M aqueous chlorhydric acid (4 mL) dropwise over a period of 15 min. A solution of sodium nitrite (101 mg, 1.46 mmol, 1.6 equiv) and potassium iodide (908 mg, 5.47 mmol, 6 equiv) in water (4 mL) was added dropwise over a period of 10 min. The reaction mixture was stirred at 0 °C for 45 min, then at room temperature for an additional hour. The mixture was quenched with saturated aqueous $Na_2S_2O_3$ and saturated aqueous $NaHCO_3$, washed with brine, extracted with ethyl acetate, dried (Na_2SO_4), filtered and evaporated. The resulting residue was subjected to a long silica gel column using 40 Å silica with a step gradient of CH_2Cl_2 (30 – 90%) in hexane. The desired compound **32** was obtained as a colorless oil after evaporation of the solvent (220 mg, 0.37 mmol, 40% yield).

^{1}H NMR (600 MHz, CD_3CN) δ 7.96 (d, *J* = 8.2 Hz, 2H, Har), 7.85 (d, *J* = 8.2 Hz, 2H, Har), 7.33 (d, *J* = 8.0 Hz, 2H, Har), 7.28 – 7.23(m, 4H, Har, H-3, H-6), 5.54 (d, *J* = 6.2 Hz, 1H, H-3'), 5.35 (m, 1H, H-1'), 4.68-4.51 (m, 2H, H-5', H-5''), 4.49 (m, 1H, H-4'), 3.78 (s, 3H, OC*H*$_3$), 2.66 (dd, *J* = 13.9, 5.5 Hz, 1H, H-2'), 2.41 (s, 3H, C*H*$_3$), 2.38 (s, 3H, C*H*$_3$), 2.01 – 1.95 (m, 1H, H-2'').

^{13}C NMR (151 MHz, CD_3CN) δ 166.96 (O*C*=O), 166.83 (O*C*=O), 157.28 (d, *J* = 235.2 Hz, C_5), 153.86 ($C_{2\ (C\text{-}OMe)}$), 145.28 (Cq, Car), 145.12 (Cq, Car), 134.05 (d, *J* = 6.3 Hz, C_1), 130.42-130.15 (CH, Ar), 128.21 (Cq, Car), 128.14 (Cq, Car), 121.59 (C_3), 113.16 (d, *J* = 27.8 Hz, C_6), 83.51 (C_4'), 78.96 (d, *J* = 27.5 Hz, C_4), 78.18 (C_1'), 76.15 (C_3'), 65.32 (C_5'), 56.99 (O*C*H$_3$), 40.32 (C_2'), 21.63 (*C*H$_3$), 21.59 (*C*H$_3$).

HRMS-ESI: $[MH]^+$; calculated for $C_{28}H_{27}FIO_6^+$: 605.0831 found: 605.0824



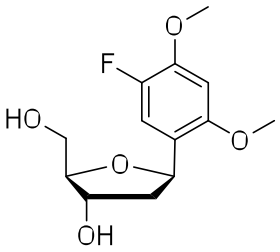
33

Compound 33. To a stirred solution of **32** (61 mg, 0.1 mmol, 1 equiv) in a mixture of Methanol and CH_2Cl_2 8/2 (2 mL) and cooled at 5 °C. A 30% solution of MeONa in MeOH (100 μL, 0.5 mmol, 5 equiv) was added. The mixture was stirred for 15 min at room temperature. The reaction mixture diluted with ethyl acetate, quenched with saturated aqueous NH_4Cl, washed with brine, dried (Na_2SO_4), filtered, and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of methanol (0 – 4%) in CH_2Cl_2. The desired compound **33** was obtained as white foam after evaporation of the solvent (34 mg, 0.09 mmol, 61 %).

^{1}H NMR (600 MHz, CD_3CN) δ 7.31 (d, *J* = 9.0 Hz, 1H, H-6), 7.26 (d, *J* = 5.1 Hz, 1H, H-3), 5.20 (m, 1H, H-1'), 4.21 (m, 1H, H-3'), 3.83 (m, 1H, H-4'), 3.78 (s, 3H, OC*H*$_3$), 3.58 (m, 2H, H-5', H-5''), 3.23 (s, 1H, OH-3'), 2.95 (s, 1H, OH-5'), 2.25 (ddd, *J* = 13.0, 5.7, 1.9 Hz, 1H, H-2'), 1.66 (ddd, *J* = 13.1, 10.1, 5.9 Hz, 1H, H-2'').

^{13}C NMR (151 MHz, CD_3CN) δ 157.31 (d, *J* = 234.9 Hz, C_5), 153.84 (C_2), 135.40 (d, *J* = 6.3 Hz, C_1), 121.40 (C_3), 113.44 (d, *J* = 6.3 Hz, C_6), 88.34 ($C_{4'}$), 78.39 (d, *J* = 27.6 Hz, C_4), 75.16 ($C_{1'}$), 73.80 ($C_{3'}$), 63.59 ($C_{5'}$), 56.95 (O*C*H$_3$), 42.91 ($C_{2'}$).

HRMS-ESI: $[MH]^+$; calculated for $C_{12}H_{15}FIO_4^+$: 368.9994 found: 368.9989.



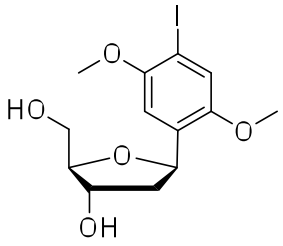
34

Compound 34. Compound **32** (120 mg, 0.20 mmol, 1 equiv), cesium carbonate (212 mg, 0.65 mmol, 2 equiv), copper iodide (13 mg, 0.07 mmol, 0.2 equiv), and 1,10-phenanthroline (24 mg, 0.1 mmol, 0.4 equiv) in methanol (0.8 mL) was heated to 110 °C for 6 h in a microwave synthesizer (Biotage AB, Sweden). The reaction was allowed to cool and the resulting mixture was concentrated, diluted with CH_2Cl_2, quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered, and evaporated. The resulting residue was subjected to silica gel column chromatography with a step gradient of methanol (0 – 4%) in CH_2Cl_2. The desired compound **34** was obtained as white foam after evaporation of the solvent (25 mg, 0.09 mmol, 46%).

^{1}H NMR (600 MHz, CD_3CN) δ 7.24 (d, *J* = 12.6 Hz, 1H, H-6), 6.67 (d, *J* = 7.2 Hz, 1H, H-3), 5.22 (m, 1H, H-1'), 4.21 (m, 1H, H-3'), 3.87 (s, 3H, OC*H*$_3$), 3.81 (s, 3H, OC*H*$_3$), 3.79 (m, 1H, H-4'), 3.58 (m, 2H, H-5', H-5''), 3.21 (s, 1H, OH-3'), 2.93 (s, 1H, OH-5'), 2.16 (dd, *J* = 12.9, 5.1 Hz, 1H, H-2'), 1.74 – 1.65 (m, 1H, H-2'').

^{13}C NMR (151 MHz, CD_3CN) δ 153.57 (C_2), 147.86 (d, J = 234.7 Hz, C_5), 147.55 (d, J = 11.9 Hz, C_4), 123.76 (d, J = 5.3 Hz, C_1), 114.21 (d, J = 21.0 Hz, C_6), 99.26 (C_3), 88.20 ($C_{4'}$), 74.87 ($C_{1'}$), 73.97 ($C_{3'}$), 63.72 ($C_{5'}$), 57.03 (O*C*H$_3$), 56.85 (O*C*H$_3$), 43.20 ($C_{2'}$).

HRMS-ESI: $[MH]^+$; calculated for $C_{13}H_{18}FO_5^+$: 273.1133 found: 273.1133.



38

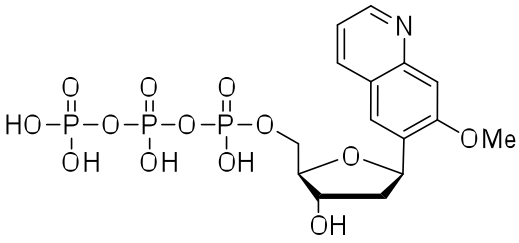
Compound 38. A mixture of palladium acetate (293 mg, 1.31 mmol, 0.15 equiv) and triphenylarsine (666 mg, 2.18, 0.25 equiv) in dry dimethylformamide (40 mL) was stirred under argon atmosphere at room temperature for 20 min. To this mixture was added 1,4-diiodo-2,5-dimethoxybenzene (6.79 g, 17.4 mmol, 2 equiv), **30** (3.0 g, 8.7 mmol, 1 equiv), and tri-n-butylamine (4.1 mL, 17.4 mmol, 2 equiv) in dimethylformamide (5 mL), and the resulting reaction mixture was stirred under nitrogen at 70 °C for 15 h. The reaction mixture was filtered through Celite and extracted with ethyl acetate and saturated aqueous $NaHCO_3$. The combined organic layers were dried (Na_2SO_4), filtered and evaporated. The residue was solubilized in THF (60 mL) and cooled in an ice bath. 1 M TBAF (24 mL, 22.6 mmol, 2.2 equiv) was added and the solution was stirred for 1 h 15 m at room temperature. The reaction mixture was quenched with saturated aqueous NH_4Cl extracted with ethyl acetate and saturated aqueous $NaHCO_3$. The combined organic layers were dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of ethyl acetate (0 – 30%) in CH_2Cl_2. The eluted product was dissolved in acetonitrile (7 mL) and the solution was cooled to -4 °C. Acetic acid (8 mL) then sodium triacetoxyborohydride (652 mg, 3.08 mmol, 1.3 equiv) were added, and the mixture was stirred for 45 min. The reaction mixture was diluted with ethyl acetate, quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient of ethyl acetate (0 – 60 %) in CH_2Cl_2. The desired compound **38** was obtained as white foam after evaporation of the solvent (794 mg, 2.1 mmol, 24% yield).

^{1}H NMR (600 MHz, CD_3CN) δ 7.30 (s, 1H, H-3), 7.15 (s, 1H, H-6), 5.23 (m, 1H, H-1'), 4.22 (m, 1H, H-3'), 3.84 (m, 1H, H-4'), 3.79 (s, 3H, OC*H*$_3$), 3.75 (s, 3H, OC*H*$_3$), 3.61 (m, 2H, H-5', H-5''), 3.16 (d, J = 4.0 Hz, 1H, OH-3'), 2.87 (t, J = 5.9 Hz, 1H, OH-5'), 2.23 (ddd, J = 13.0, 5.8, 2.1 Hz, 1H, H-2'), 1.73 (ddd, J = 13.1, 9.9, 5.9 Hz, 1H, H-2'').

^{13}C NMR (151 MHz, CD_3CN) δ 153.61 (C_5), 151.82 (C_2), 134.17 (C_1), 122.50 (C_3), 110.33 (C_6), 88.23 ($C_{4'}$), 83.43 ($C_{4(C\text{-}I)}$), 75.57 ($C_{1'}$), 73.81 ($C_{3'}$), 63.66 ($C_{5'}$), 57.47 (O*C*H$_3$), 56.89 (O*C*H$_3$), 42.99 ($C_{2'}$).

HRMS-ESI: $[MH]^+$; calculated for $C_{13}H_{18}IO_5^+$: 381.0193 found: 381.0186

General Procedure for Triphosphate Synthesis: Proton sponge (1.3 equiv) and the free nucleoside derivative (1.0 equiv, see below) were dissolved in dry trimethyl phosphate (40 equiv) and cooled to -15 °C under nitrogen atmosphere. Freshly distilled $POCl_3$ (1.3 equiv) was added dropwise and the resulting mixture was stirred at -10 °C for 2 h. Tributylamine (6.0 equiv) and a solution of tributylammonium pyrophosphate (5.0 eq.) in dimethylformamide (0.5 M) were added. Over 30 min, the reaction was allowed to warm slowly to 0 °C and then was quenched by addition of 0.5 M aqueous $Et_3NH_2CO_3$ (TEAB) pH 7.5 (2 vol-equiv). The mixture was diluted two-fold with H_2O and the product was subjected to anion exchange chromatography (DEAE Sephadex (GE Healthcare)) with an elution gradient of 0 to 1.2 M TEAB, then evaporated and co-distilled with H_2O (3×). Additional purification by reverse-phase (C18) HPLC (0 – 35% CH_3CN in 0.1 M TEAB, pH 7.5) was performed.



6

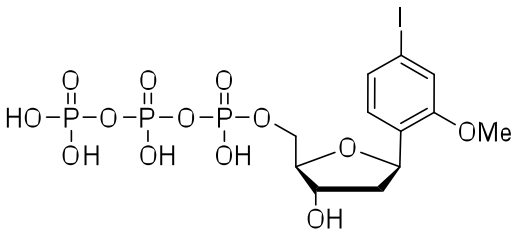
Compound 6. Compound **6** (7.0 mg, 13.6 μmol, 27%) was synthesized using the General Procedure described above starting from nucleoside **3 (**14 mg, 50.1 μmol).

^{31}P NMR (162 MHz, D_2O) δ -10.74 (d, *J* = 19.4 Hz, γ-P), -11.02 (d, *J* = 20.0 Hz, α-P), -23.11 (t, *J* = 19.8 Hz, β-P).

^{1}H NMR (400 MHz, D_2O) δ 8.94 (d, *J* = 8.3 Hz, 1H, H-8), 8.82 (m, 1H, H-7), 8.28 (m, 1H, H-6), 7.75 (m, 1H, H-9), 7.35 (s, 1H, H-3), 5.51 (m, 1H, H-1'), 4.56 (m, 1H, H-3'), 4.28 (m, 1H, H-4'), 4.20 (m, 2H, H-5'), 4.04 (s, 3H, OCH_3), 3.18 (q, *J* = 7.3 Hz, 24H, $N(CH_2CH_3)_3$), 2.51 (ddd, *J* = 13.6, 6.3, 2.5 Hz, 1H, H-2'), 2.01 (m, 1H, H-2''), 1.26 (t, *J* = 7.3 Hz, 36H, $N(CH_2CH_3)_3$).

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): $[M-H]^-$ calcd for $C_{15}H_{20}NO_{13}P_3$, 514.2; found, 514.8.

$\varepsilon_{(\lambda = 329\ nm)} = 4800\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 230\ nm)} = 26250\ M^{-1}\ cm^{-1}$



7

Compound 7. Compound **7** (20.2 mg, 34.2 μmol, 48%) was synthesized using the General Procedure described above starting from nucleoside **4** (25 mg, 71.4 μmol).

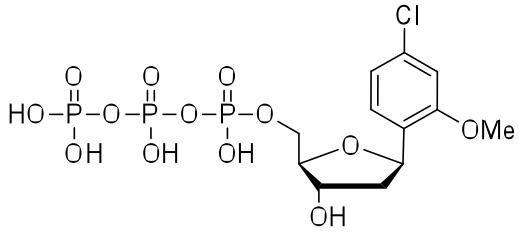
^{31}P NMR (162 MHz, D_2O) δ -10.36 (d, *J* = 19.9 Hz, γ-P), -10.85 (d, *J* = 20.3 Hz, α-P), -22.96 (t, *J* = 20.1 Hz, β-P).

^{1}H NMR (600 MHz, H_2O) δ 7.30 - 7.14 (m, 3H, H-3, H-5, H-6), 5.28 (dd, *J* = 10.3, 5.7 Hz, 1H, H-1'), 4.44 – 4.32 (m, 1H, H-3'), 4.05 – 4.00 (m, 1H, H-4'), 3.94 (m, 2H, H-5', H-5''), 3.73 (s, 3H, OC*H3*), 3.4 – 2.6 (m, 18H, (CH3C*H2*)$_3$N), 2.18 – 2.10 (m, 1H, H-2'), 2.01 – 1.87 (m, 1H, H-2''), 1.5 – 0.7 (m, 27H, (C*H3*CH2)$_3$N).

^{13}C NMR (151 MHz, D_2O) δ 157.75 (C_2), 131.02 (C_1), 129.70 (C_5), 128.91 (C_6), 121.24 (C_3), 94.01 (C_4), 85.97 (C_4'), 75.88 (C_1'), 73.62 (C_3'), 66.85 (C_5'), 56.69 (O*C*H3), 47.37 ((CH3*C*H2)$_3$N), 42.95 (C_2'), 11.32 – 8.16 ((*C*H3CH2)$_3$N).

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{12}H_{18}IO_{13}P_3$, 589.1 found, 589.7.

$\varepsilon_{(\lambda = 279\ nm)}$ = 3100 M^{-1} cm^{-1}; $\varepsilon_{(\lambda = 235\ nm)}$ = 12825 M^{-1} cm^{-1}



8

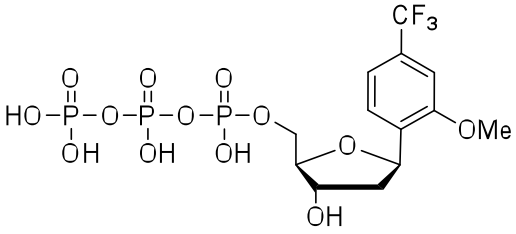
Compound 8. Compound **8** (9.9 mg, 19.8 μmol, 57%) was synthesized using the General Procedure described above starting from nucleoside **5 (**9 mg, 34.8 μmol).

^{31}P NMR (162 MHz, D_2O) δ -10.90 (d, *J* = 19.3 Hz, γ-P), -11.11 (d, *J* = 20.0 Hz, α-P), -23.35 (t, *J* = 19.7 Hz, β-P).

^{1}H NMR (400 MHz, D_2O) δ 7.50 (m, 1H, H-3), 7.11 (d, *J* = 1.9 Hz, 1H, H-6), 7.08 (dd, *J* = 8.2, 2.0 Hz, 1H, H-5), 5.45 (m, 1H, H-1'), 4.55 (m, 1H, H-3'), 4.17 (m, 1H, H-4'), 4.10 (m, 2H, H-5'), 3.85 (s, 3H, OCH_3), 3.19 (q, *J* = 7.3 Hz, 18H, N(CH_2CH$_3$)$_3$), 2.29 (m, 1H, H-2'), 2.12 (m, 1H, H-2''), 1.27 (t, *J* = 7.3 Hz, 27H, N(CH$_2$CH_3)$_3$).

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{12}H_{18}ClO_{13}P_3$, 497.6 found, 498.2.

$\varepsilon_{(\lambda = 278\ nm)} = 2050\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 228\ nm)} = 7650\ M^{-1}\ cm^{-1}$



14

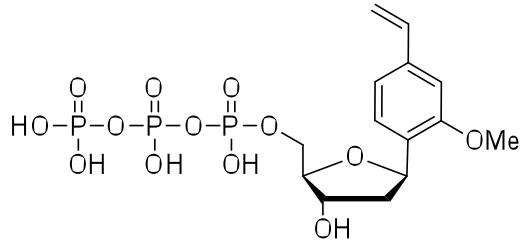
Compound 14. Compound **14 (**4.4 mg, 8.3 μmol, 20%) was synthesized using the General Procedure described above starting from nucleoside **10 (**12 mg, 41.1 μmol).

^{31}P NMR (162 MHz, D_2O) δ -10.11 – -11.63 (m, γ-P, α-P), -23.35 (m, β-P).

^{1}H NMR (400 MHz, D_2O) δ 7.70 (d, *J* = 7.9 Hz, 1H, H-5), 7.38 (d, *J* = 7.6 Hz, 1H, H-6), 7.33 (s, 1H, H-3), 5.52 (m, 1H, H-1'), 4.56 (m, 1H, H-3'), 4.22 (m, 1H, H-4'), 4.12 (m, 2H, H-5'), 3.91 (s, 3H, OC*H*$_3$), 3.19 (m, 24H, N(C*H*$_2$CH$_3$)$_3$), 2.38 (ddd, *J* = 13.5, 5.8, 2.0 Hz, 1H, H-2'), 2.07 (m 1H, H-2''), 1.27 (t, *J* = 7.3 Hz, 36H, N(CH$_2$C*H*$_3$)$_3$).

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): $[M-H]^-$ calcd for $C_{13}H_{18}F_3O_{13}P_3$, 531.2 found, 531.8.

$\varepsilon_{(\lambda = 278\ nm)} = 2450\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 223\ nm)} = 6900\ M^{-1}\ cm^{-1}$



15

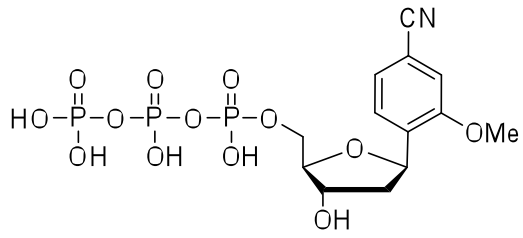
Compound 15. Compound **15** (6.3 mg, 12. 9 μmol, 36%) was synthesized using the General Procedure described above starting from nucleoside **11** (9 mg, 35.9 μmol).

^{31}P NMR (162 MHz, D_2O) δ -10.88 (d, *J* = 19.3 Hz, γ-P), -11.08 (d, *J* = 20.0 Hz, α-P), -23.32 (t, *J* = 19.6 Hz, β-P).

^{1}H NMR (400 MHz, D_2O) δ 7.52 (d, *J* = 7.8 Hz, 1H, H-6), 7.25 – 7.14 (m, 2H, H-3, H-5), 6.79 (m, 1H, H-7), 5.89 (d, *J* = 17.6 Hz, 1H, H-9), 5.49 (m, 1H, H-1'), 5.33 (d, *J* = 11.0 Hz, 1H, H-8), 4.56 (m, 1H, H-3'), 4.17 (m, 1H, H-4'), 4.11 (m, 2H, H-5'), 3.89 (s, 3H, OC*H*$_3$), 3.19 (q, *J* = 7.3 Hz, 20H, N(C*H*$_2$CH$_3$)$_3$), 2.30 (ddd, *J* = 13.6, 5.7, 1.7 Hz, 1H, H-2'), 2.13 (m, 1H, H-2''), 1.27 (t, *J* = 7.3 Hz, 30H N(CH$_2$C*H*$_3$)$_3$).

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{14}H_{21}O_{13}P_3$, 489.2 found, 489.9.

$\varepsilon_{(\lambda = 295\ nm)} = 3035\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 254\ nm)} = 11970\ M^{-1}\ cm^{-1}$



16

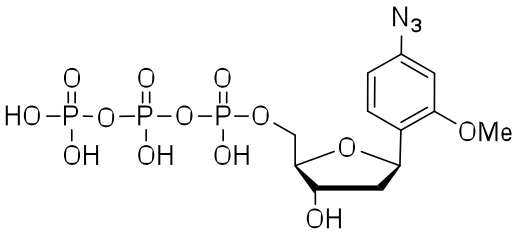
Compound 16. Compound **16** (8.2 mg, 16.8 μmol, 42%) was synthesized using the General Procedure described above starting from nucleoside **12 (**10 mg, 40.1 μmol).

^{31}P NMR (162 MHz, D_2O) δ -10.93 (d, *J* = 19.2 Hz, γ-P), -11.16 (d, *J* = 20.1 Hz, α-P), -23.39 (t, *J* = 19.6 Hz, β-P).

^{1}H NMR (400 MHz, D_2O) δ 7.69 (d, *J* = 7.9 Hz, 1H, H-6), 7.44 (dd, *J* = 7.9, 1.5 Hz, 1H, H-5), 7.36 (m, 1H, H-3), 5.50 (m, 1H, H-1'), 4.54 (m, 1H, H-3'), 4.21 (m, 1H, H-4'), 4.11 (m, 2H, H-5'), 3.88 (s, 3H, OC*H*$_3$), 3.19 (q, *J* = 7.3 Hz, 20H, N(C*H*$_2$CH$_3$)$_3$), 2.39 (m, 1H, H-2'), 2.03 (m, 1H, H-2''), 1.26 (t, *J* = 7.3 Hz, 30H).

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{13}H_{18}NO_{13}P_3$, 488.2 found, 488.7.

$\varepsilon_{(\lambda = 295\ nm)} = 4365\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 242\ nm)} = 13900\ M^{-1}\ cm^{-1}$



17

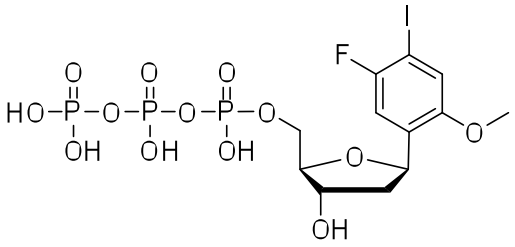
Compound 17. Compound **17** (4.8 mg, 9.5 μmol, 25%) was synthesized using the General Procedure described above starting from nucleoside **13** (10 mg, 37.7 μmol).

^{31}P NMR (162 MHz, D_2O) δ -10.92 (d, *J* = 19.2 Hz, γ-P), -11.11 (d, *J* = 20.1 Hz, α-P), -23.36 (t, *J* = 19.7 Hz, β-P).

^{1}H NMR (400 MHz, D_2O) δ 7.52 (d, *J* = 8.3 Hz, 1H, H-6), 6.79 (dd, *J* = 8.3, 2.2 Hz, 1H, H-5), 6.72 (d, *J* = 2.1 Hz, 1H, H-3), 5.45 (m, 1H, H-1'), 4.55 (m, 1H, H-3'), 4.15 (m, 1H, H-4'), 4.10 (m, 2H, H-5'), 3.85 (s, 3H, OC*H*$_3$), 3.19 (q, *J* = 7.3 Hz, 22H, N(C*H*$_2$CH$_3$)$_3$), 2.25 (m, 1H, H-2'), 2.15 (m, 1H, H-2''), 1.30 – 1.17 (t, *J* = 7.3 Hz, 33H, N(CH$_2$C*H*$_3$)$_3$).

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{12}H_{18}N_3O_{13}P_3$, 504.2 found, 504.6.

$\varepsilon_{(\lambda = 287\ nm)} = 3960\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 254\ nm)} = 10330\ M^{-1}\ cm^{-1}$



35

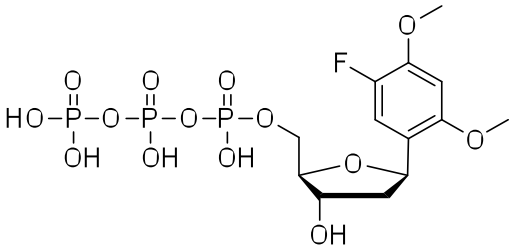
Compound 35. Compound **35** (3.9 mg, 6.5 μmol, 16%) was synthesized using the General Procedure described above starting from nucleoside **33 (**15 mg, 40.7 μmol).

^{31}P NMR (162 MHz, D_2O) δ -7.87 (d, *J* = 13.6 Hz, γ-P), -10.61 (d, *J* = 19.9 Hz, α-P), -22.32 (t, *J* = 20.1 Hz, β-P).

^{1}H NMR (400 MHz, D_2O) δ 7.42 (d, *J* = 5.1 Hz, 1H, H-3), 7.32 (d, *J* = 8.8 Hz, 1H, H-6), 5.40 (m, 1H, H-1'), 4.54 (m, 1H, H-3'), 4.18 (m, 1H, H-4'), 4.10 (m, 2H, H-5'), 3.82 (s, 3H, OC*H*$_3$), 3.19 (q, *J* = 7.3 Hz, 18H, N(C*H*$_2$CH$_3$)$_3$), 2.33 (ddd, *J* = 13.5, 5.8, 2.0 Hz, 1H, H-2'), 2.11 – 2.00 (m, 1H, H-2''), 1.27 (t, *J* = 7.3 Hz, 27H, N(CH$_2$C*H*$_3$)$_3$).

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{12}H_{17}FIO_{13}P_3$, 607.9 found, 607.4

$\varepsilon_{(\lambda = 232\ nm)} = 8570\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 288\ nm)} = 3605\ M^{-1}\ cm^{-1}$



36

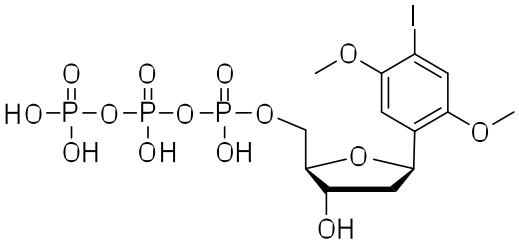
Compound 36. Compound **36** (4.5 mg, 8.8 μmol, 20%) was synthesized using the General Procedure described above starting from nucleoside **34** (12 mg, 44.1 μmol).

^{31}P NMR (162 MHz, D_2O) δ -7.95 (d, *J* = 12.4 Hz, γ-P), -10.59 (d, *J* = 20.0 Hz, α-P), -22.37 (t, *J* = 20.2 Hz, β-P).

^{1}H NMR (400 MHz, D_2O) δ 7.32 (d, *J* = 12.4 Hz, 1H, H-6), 6.82 (d, *J* = 7.3 Hz, 1H, H-3), 5.42 (m, 1H, H-1'), 4.56 (m, 1H, H-3'), 4.15 (m, 1H, H-4'), 4.07 (m, 2H, H-5'), 3.93 (s, 3H, OC*H*$_3$), 3.86 (s, 3H, OC*H*$_3$), 3.22 (q, *J* = 7.3 Hz, 18H, N(C*H*$_2$CH$_3$)$_3$), 2.23 (ddd, *J* = 13.4, 5.7, 1.8 Hz, 1H, H-2'), 2.13 (m, 1H, H-2''), 1.27 (t, *J* = 7.3 Hz, 27H, N(CH$_2$C*H*$_3$)$_3$).

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{13}H_{20}FO_{14}P_3$, 512.2; found, 512.5.

$\varepsilon_{(\lambda = 282\ nm)} = 6565\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 224\ nm)} = 13945\ M^{-1}\ cm^{-1}$



39

Compound 39. Compound **39** (6.1 mg, 9.9 μmol, 27%) was synthesized using the General Procedure described above starting from nucleoside **38** (14 mg, 36.8 μmol).

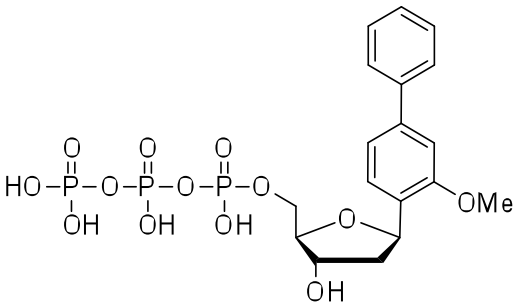
^{31}P NMR (162 MHz, D_2O) δ -6.42 (d, *J* = 20.3 Hz, γ-P), -10.66 (d, *J* = 19.9 Hz, α-P), -22.13 (t, *J* = 20.3 Hz, β-P).

^{1}H NMR (400 MHz, D_2O) δ 7.49 (s, 1H, H-3), 7.18 (s, 1H, H-6), 5.43 (m, 1H, H-1'), 4.58 (m, 1H, H-3'), 4.20 (s, 1H, H-4'), 4.14 (m, 2H, H-5'), 3.88 (s, 3H, OC*H*$_3$), 3.81 (s, 3H, OC*H*$_3$), 3.20 (q, *J* = 7.3 Hz, 18H, N(C*H*$_2$CH$_3$)$_3$), 2.31 (m, 1H, H-2'), 2.10 (m, 1H, H-2''), 1.26 (t, *J* = 7.3 Hz, 27H, N(CH$_2$C*H*$_3$)$_3$).

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{13}H_{20}IO_{14}P_3$, 619.9; found, 619.8

$\varepsilon_{(\lambda = 296\ nm)} = 3805\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 233\ nm)} = 8665\ M^{-1}\ cm^{-1}$

General Procedure for aqueous palladium cross-coupling on triphosphate: Palladium acetate (0.1 equiv) and TPPTS (0.5 equiv) were dissolved in a mixture of H_2O:ACN 2:1 and stirred under argon atmosphere at room temperature for 20 min. The resulting mixture was added to a solution of triphosphate derivative **7** (1 mg, 1.13 μmol, 1 equiv), boronic acid derivative (2 equiv) and cesium carbonate (6 equiv) in H_2O/ACN 2/1. The resulting reaction mixture was stirred under nitrogen at 70 °C for 30 min and filtered through a 0.45 micron filter. The product was isolated via anion exchange chromatography (DEAE Sephadex) with an elution gradient of 0 to 1.2 M TEAB, evaporated, co-distilled with H_2O (3×). Additional purification by reverse-phase (C18) HPLC (0 – 35% CH_3CN in 0.1 M TEAB, pH 7.5) was performed.

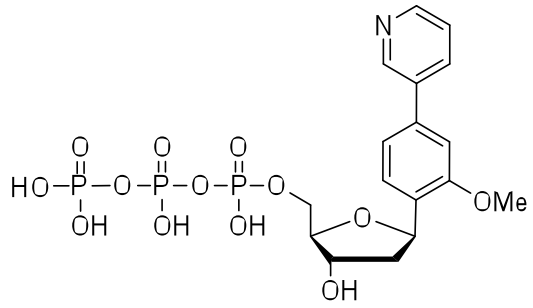


18

Compound 18. Compound **18** (528 μg, 0.96 μmol, 85%) was synthesized using the General Procedure described above using phenyl boronic acid.

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{18}H_{23}O_{13}P_3$, 539.3 found, 539.2.

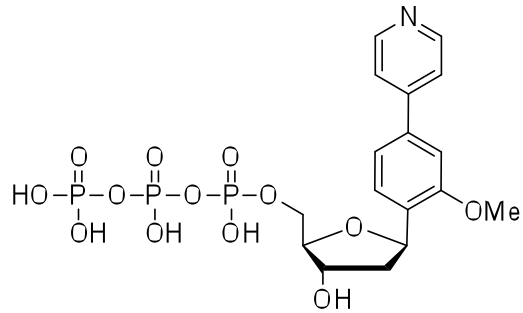
^{31}P NMR (162 MHz, D_2O) δ -9.82 (d, *J* = 20.2 Hz, γ-P), -10.73 (d, *J* = 20.1 Hz, α-P), -22.74 (t, *J* = 20.0 Hz, β-P).



19

Compound 19. Compound **19** (448 μg, 0.81 μmol, 72%) was synthesized using the General Procedure described above using pyridine-3-boronic acid.

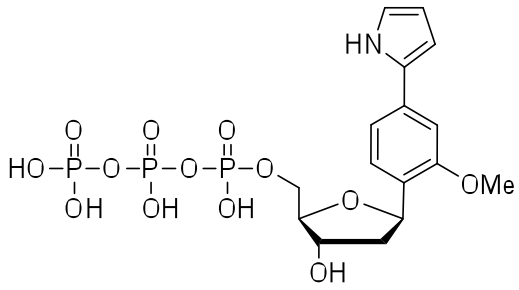
MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{17}H_{22}NO_{13}P_3$, 540.3 found, 540.9.



20

Compound 20. Compound **20** (465 μg, 0.86 μmol, 76%) was synthesized using the General Procedure described above using pyridine-4-boronic acid.

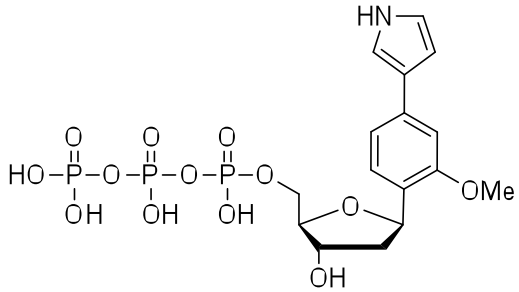
MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{17}H_{22}NO_{13}P_3$, 540.3 found, 540.7



21

Compound 21. Compound **21** (418 μg, 0.79 μmol, 70%) was synthesized using the General Procedure described above using *N*-Boc-pyrrole-2-boronic acid.

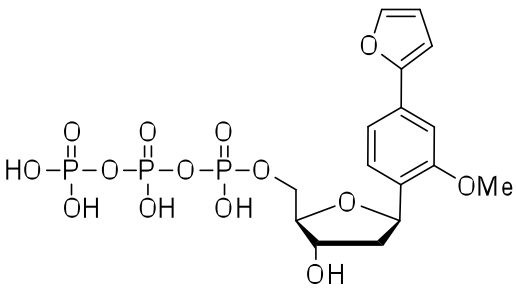
MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{16}H_{22}NO_{13}P_3$, 528.3 found, 528.8.



22

Compound 22. Compound **22** (424 μg, 0.80 μmol, 71%) was synthesized using the General Procedure described above using 1-(triisopropylsilyl)-1H-pyrrole-3-boronic acid.

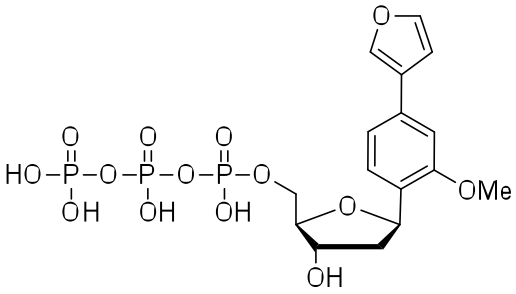
MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{16}H_{22}NO_{13}P_3$, 528.3 found, 528.9.



23

Compound 23. Compound **23** (515 μg, 0.97 μmol, 86%) was synthesized using the General Procedure described above using 2-furanboronic acid.

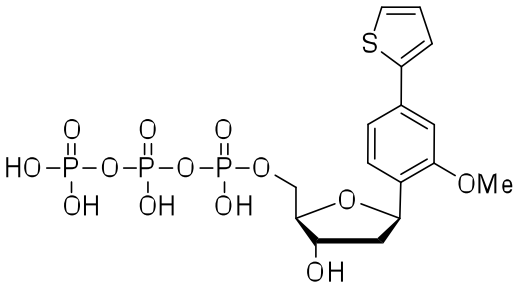
MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{16}H_{21}O_{14}P_3$, 529.3 found, 529.7.



24

Compound 24. Compound **24** (473 μg, 0.89 μmol, 79%) was synthesized using the General Procedure described above using 3-furanboronic acid.

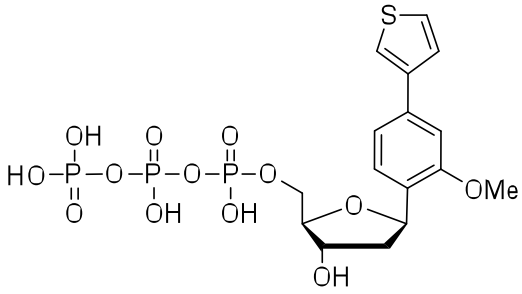
MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{16}H_{21}O_{14}P_3$, 529.3 found, 529.9.



25

Compound 25. Compound **25** (482 μg, 0.88 μmol, 78%) was synthesized using the General Procedure described above using 2-thiopheneboronic acid.

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{16}H_{21}O_{13}P_3S$, 545.3 found, 545.8.

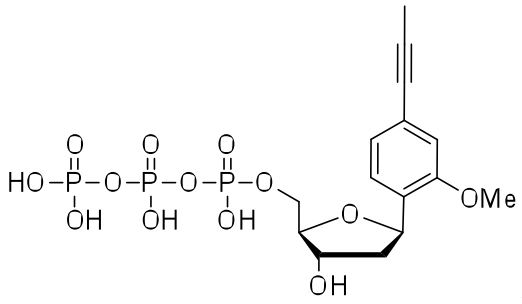


26

Compound 26. Compound **26** (475 μg, 0.87 μmol, 77%) was synthesized using the General Procedure described above using 3-thiopheneboronic acid.

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{16}H_{21}O_{13}P_3S$, 545.3 found, 545.9.

General Procedure for aqueous copper catalyzed Sonogashira coupling on triphosphate: Palladium acetate (0.1 equiv) and TPPTS (0.5 equiv) were dissolved in a mixture of H_2O:ACN 2:1 and stirred under argon atmosphere at room temperature for 20 min. The resulting mixture was added to a solution of triphosphate derivative **7** (1.5 mg, 1.69 μmol, 1 equiv), copper iodide (0.1 equiv), alkyne derivative (10 to 50 equiv), and triethylamine (8 equiv) in H_2O:ACN 2:1. The resulting reaction mixture was stirred under nitrogen at 55–70 °C for 30 m and filtered through a 0.45 micron filter. The product was isolated via anion exchange chromatography (DEAE Sephadex) with an elution gradient of 0 to 1.2 M TEAB, then evaporated and co-distilled with H_2O (3×). Additional purification by reverse-phase (C18) HPLC (0 – 35% CH_3CN in 0.1 M TEAB, pH 7.5) was performed.



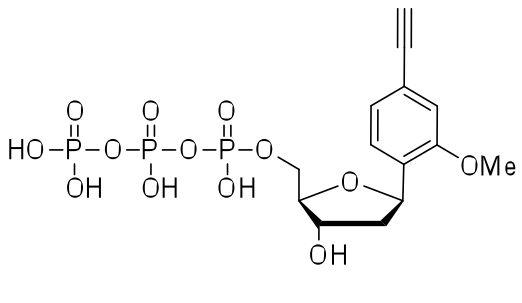
27

Compound 27. Compound **27 (**593 μg, 1.18 μmol, 70%) was synthesized using the General Procedure described above using propyne gas.

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{15}H_{21}O_{13}P_3$, 501.2 found, 501.6.

^{31}P NMR (162 MHz, D_2O) δ -9.91 (d, *J* = 20.0 Hz, γ-P), -11.01 (d, *J* = 20.0 Hz, α-P), -22.80 (t, *J* = 20.0 Hz, β-P).

$\varepsilon_{(\lambda = 254\ nm)} = 12100\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 288\ nm)} = 2460\ M^{-1}\ cm^{-1}$

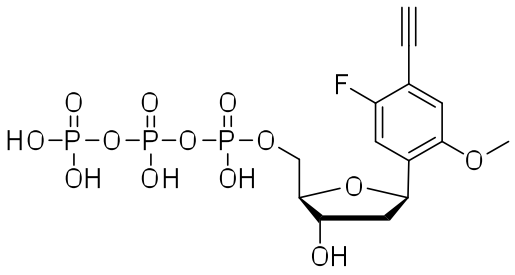


28

Compound 28. Compound **28** (495 μg, 1.01 μmol, 60 %) was synthesized using the General Procedure described above using triethylsilylacetylene. The triethylsilyl-protected triphosphate derivative was treated with 30% NH_4OH and stirred at room temperature for 1 h. The desired acetylene derivative was obtained after evaporation of the solvents.

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{14}H_{19}O_{13}P_3$, 487.2 found, 487.2.

$\varepsilon_{(\lambda = 252\ nm)} = 12990\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 295\ nm)} = 2650\ M^{-1}\ cm^{-1}$

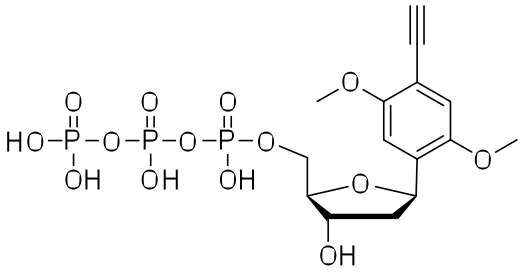


37

Compound 37. Compound **37** (622 μg, 1.23 μmol, 50%) was synthesized using the General Procedure described above starting from triphosphate derivative **35** (1.5 mg, 2.46 μmol).

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{14}H_{18}FO_{13}P_3$, 506.0; found, 506.6.

$\varepsilon_{(\lambda = 300\ nm)} = 3720\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 248\ nm)} = 11900\ M^{-1}\ cm^{-1}$



40

Compound 40. Compound **40** (508 μg, 0.98 μmol, 40 %) was synthesized using the General Procedure described above starting from triphosphate derivative **39** (1.5 mg, 2.42 μmol).

MS (MALDI-TOF$^-$, matrix: 9-aminoacridine) (*m/z*): [M-H]$^-$ calcd for $C_{15}H_{21}O_{14}P_3$, 518.0; found, 518.6.

$\varepsilon_{(\lambda = 282\ nm)} = 6565\ M^{-1}\ cm^{-1}$; $\varepsilon_{(\lambda = 224\ nm)} = 13945\ M^{-1}\ cm^{-1}$

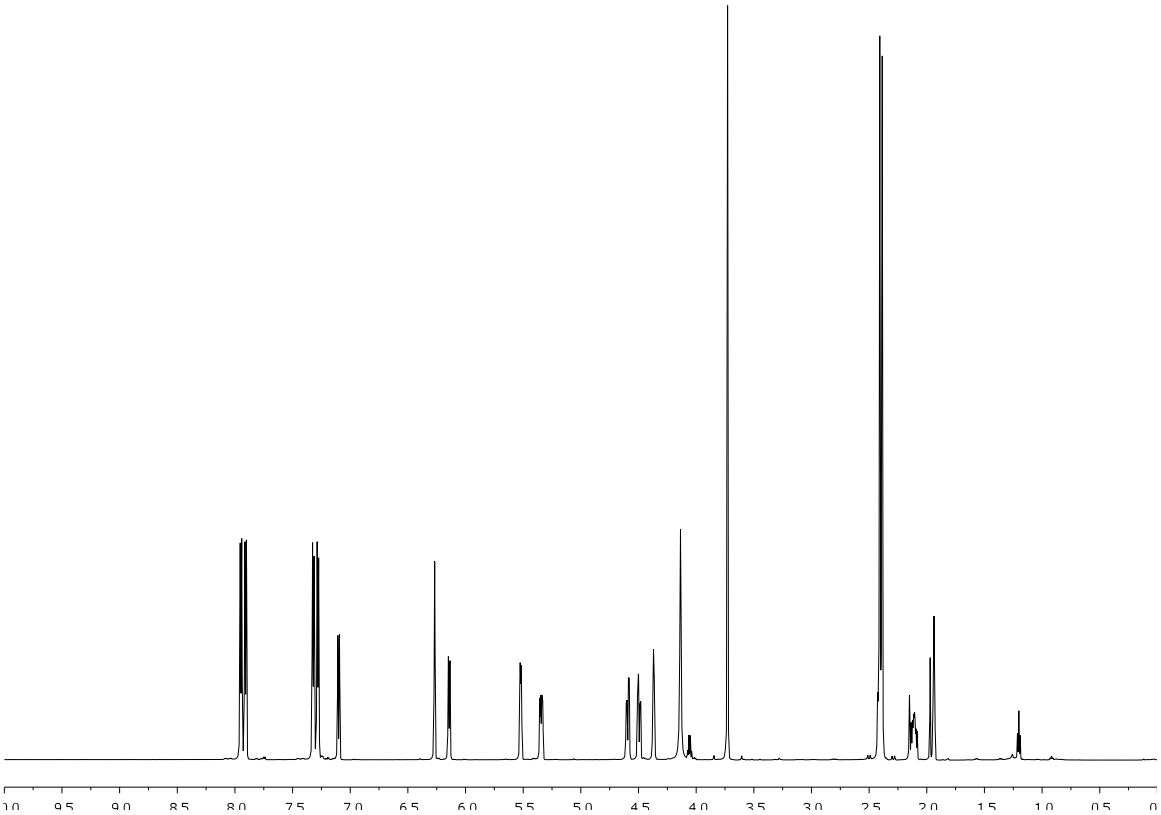


Figure S1: 600 MHz ^{1}H NMR spectra (CD_3CN) of **2**

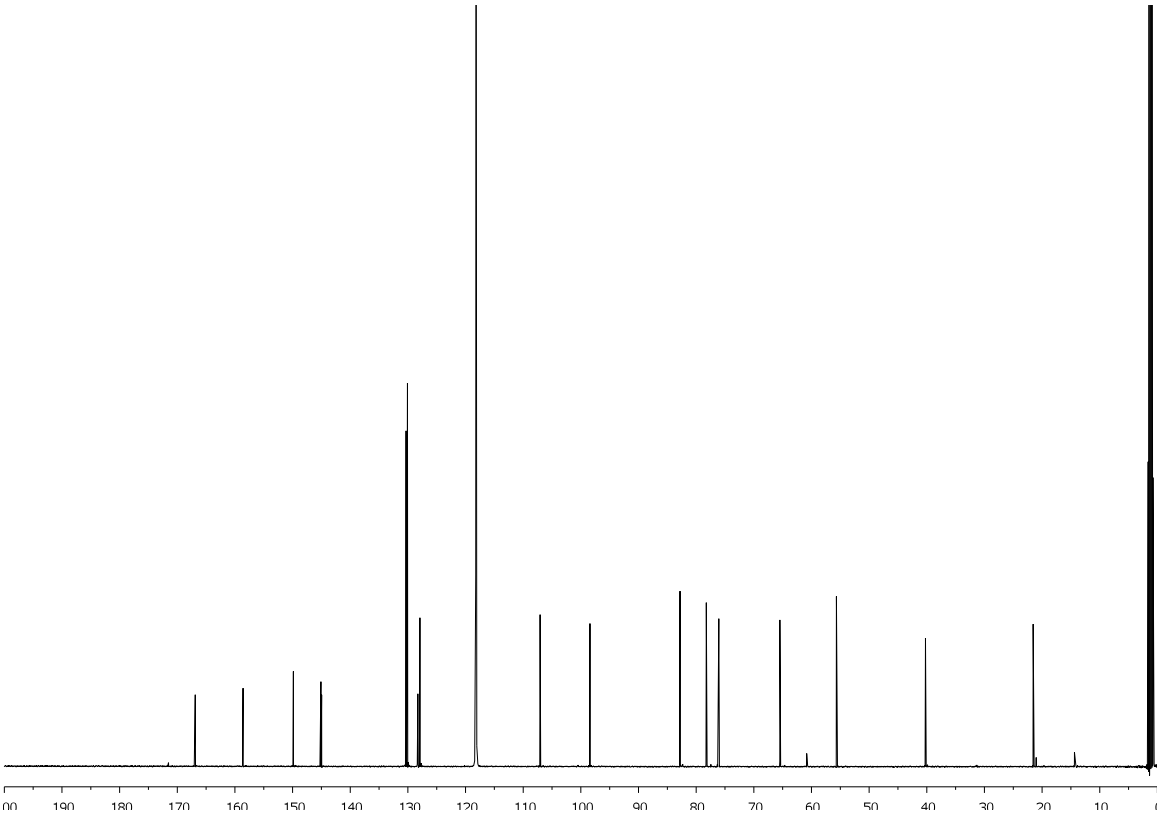


Figure S2: 151 MHz ^{13}C NMR spectrum (CD_3CN) of **2**

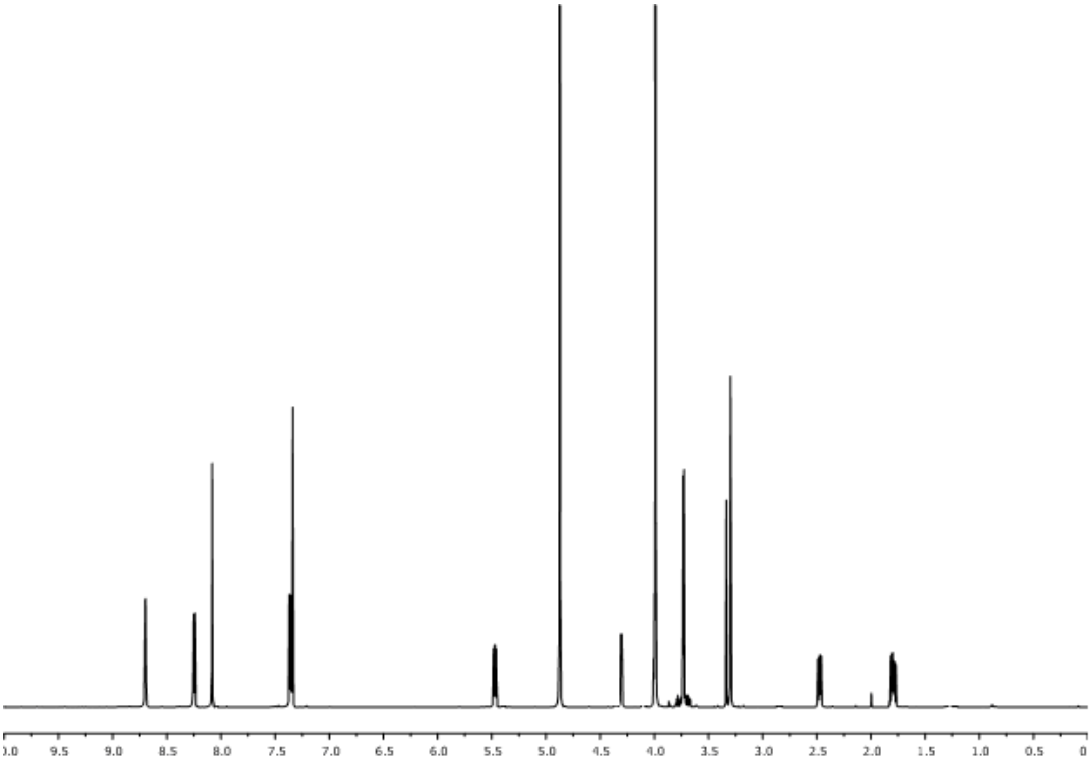


Figure S3: 600 MHz ^{1}H NMR spectra (MeOD) of **3**

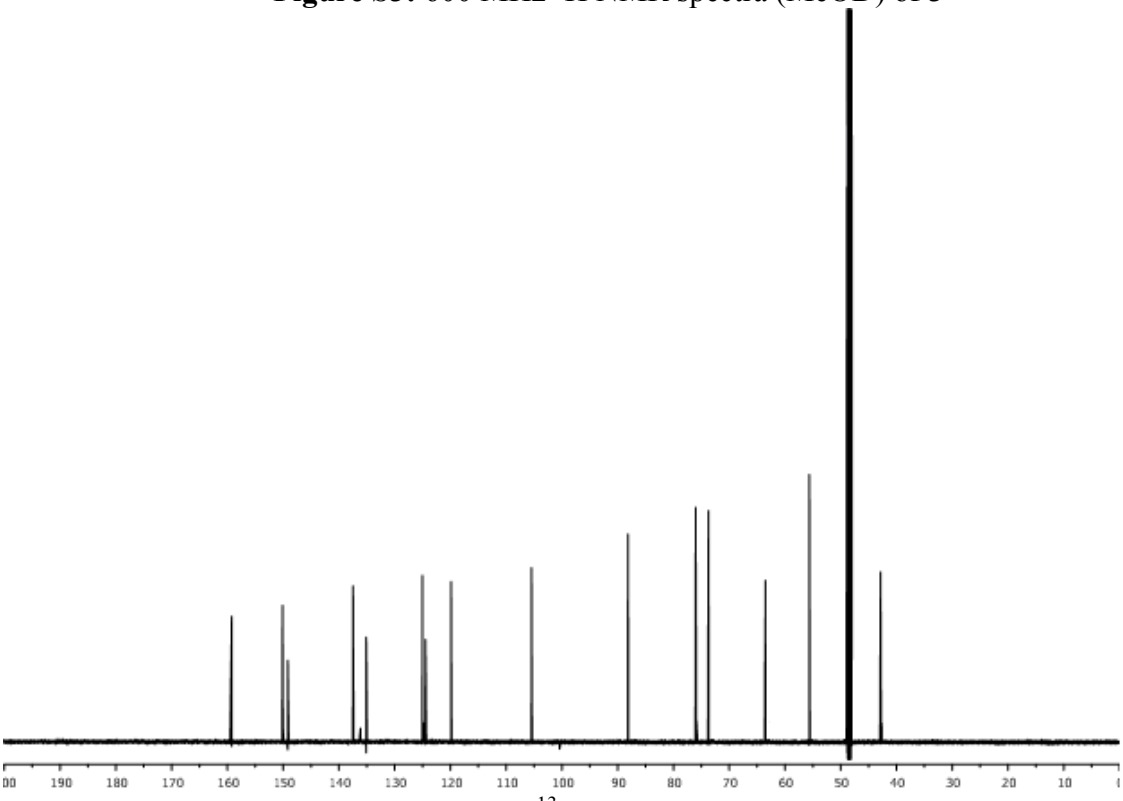


Figure S4: 151 MHz ^{13}C NMR spectrum (MeOD) of **3**

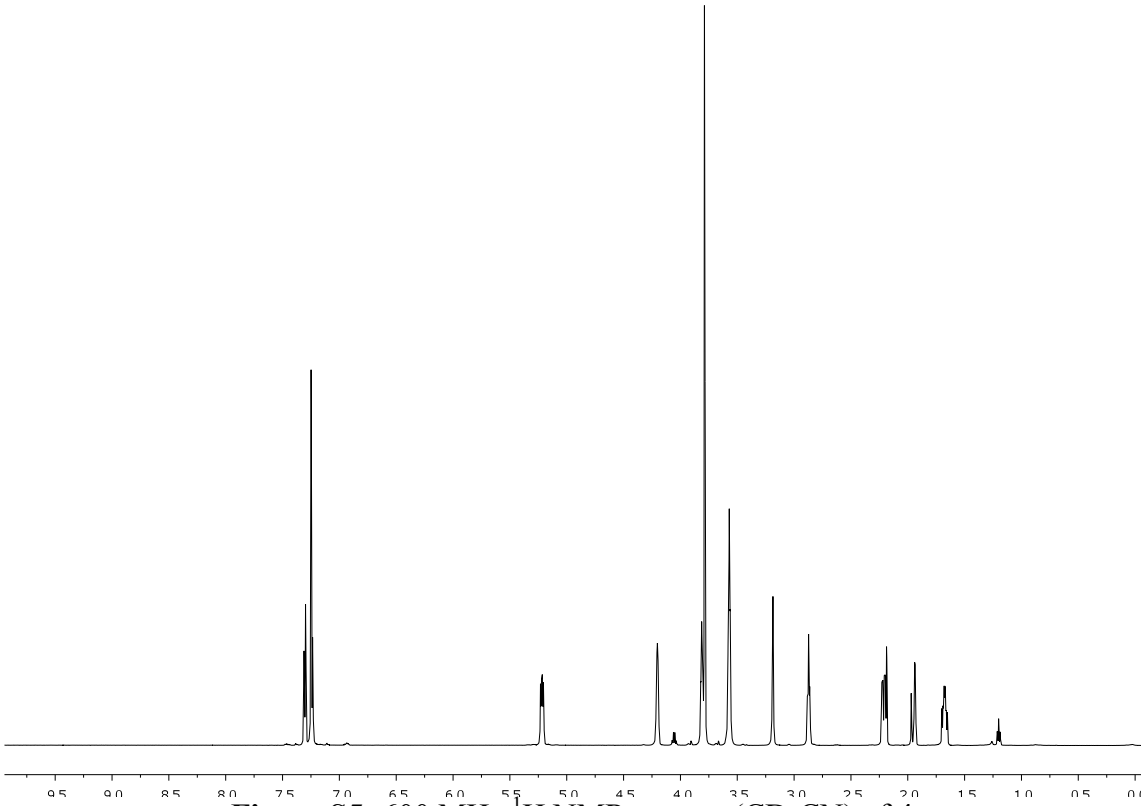


Figure S5: 600 MHz ^{1}H NMR spectra (CD_3CN) of **4**

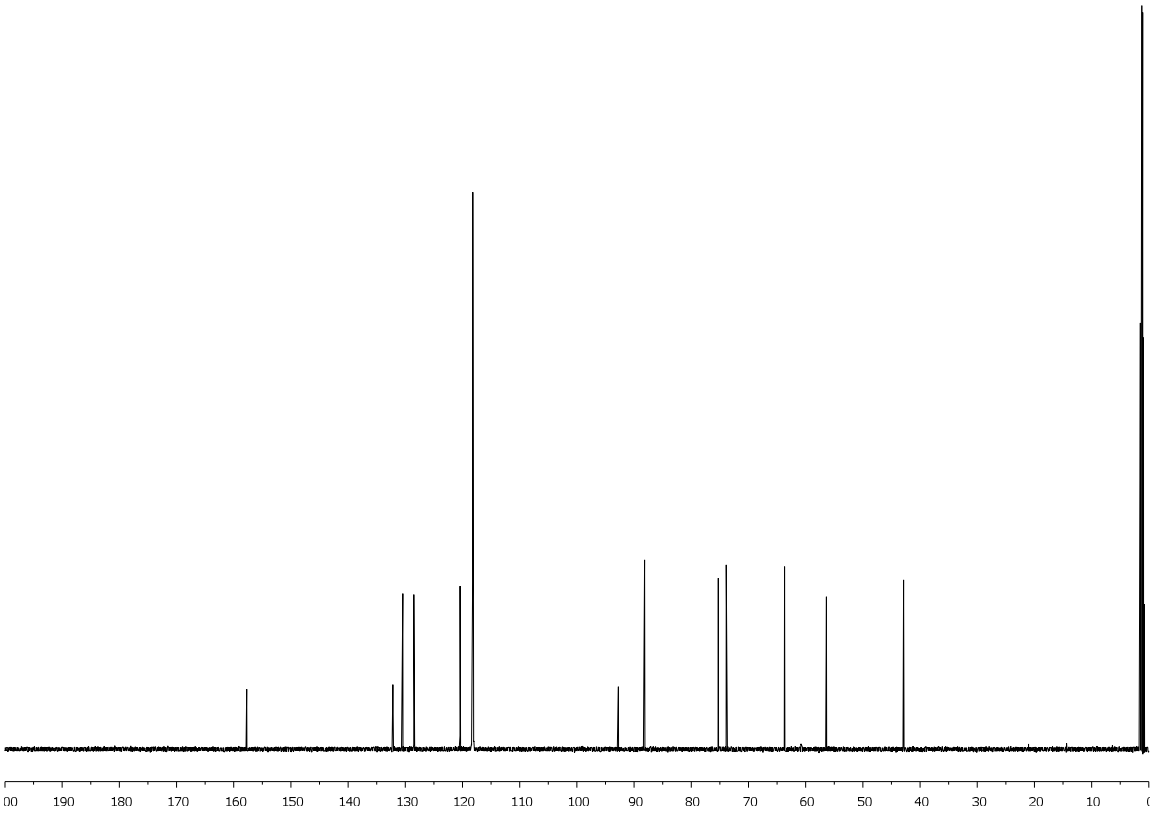


Figure S6: 151 MHz ^{13}C NMR spectrum (CD_3CN) of **4**

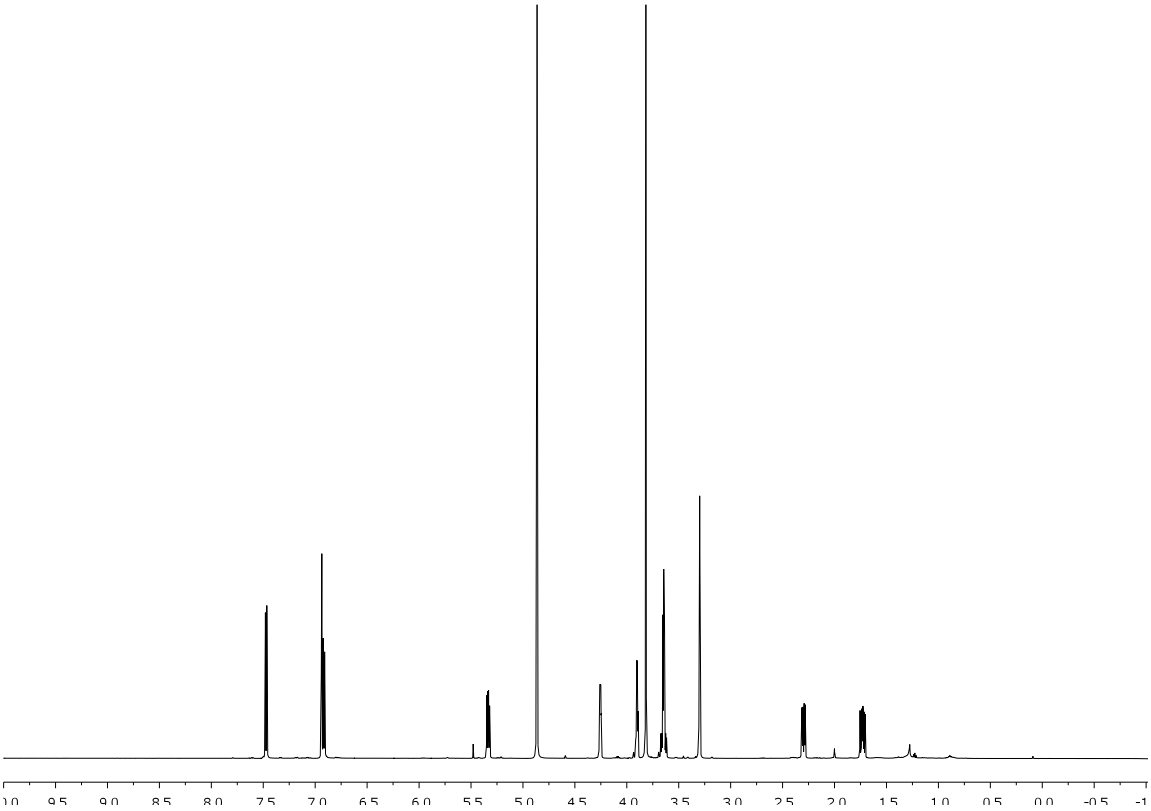


Figure S7: 600 MHz ^{1}H NMR spectra (MeOD) of **5**

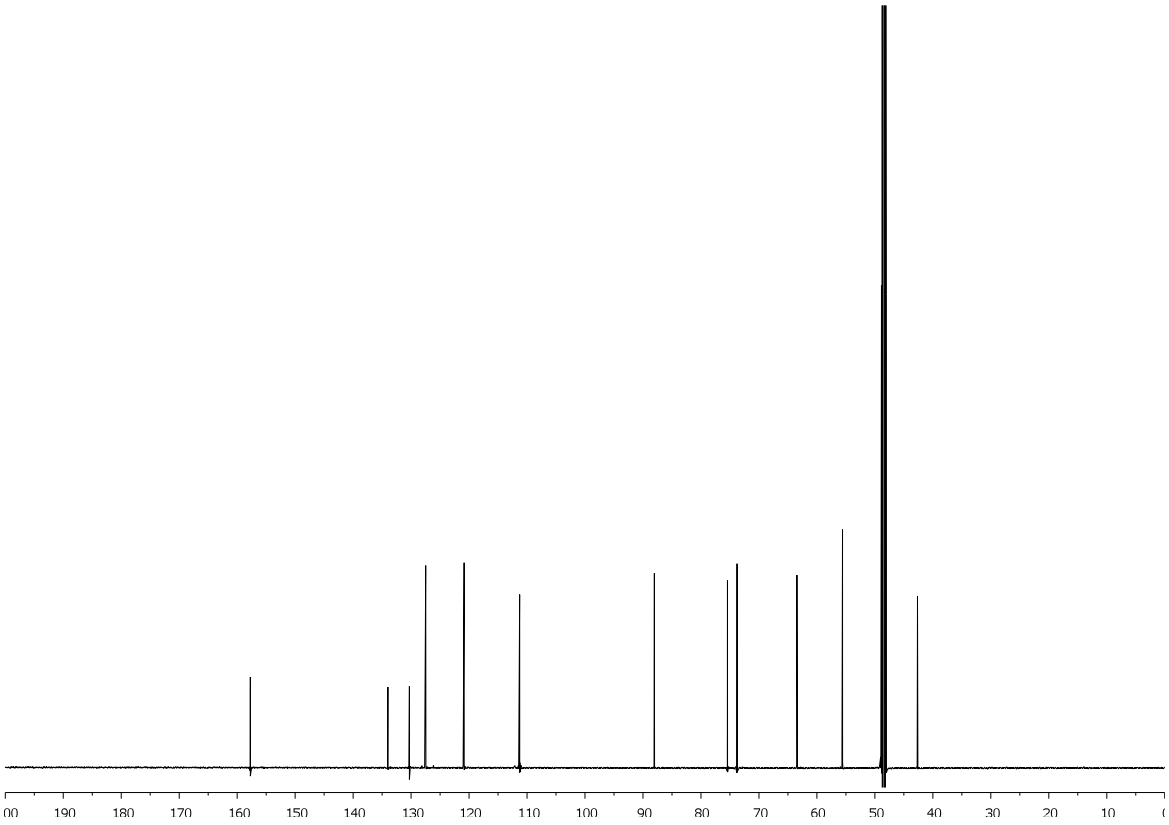


Figure S8: 151 MHz ^{13}C NMR spectrum (MeOD) of **5**

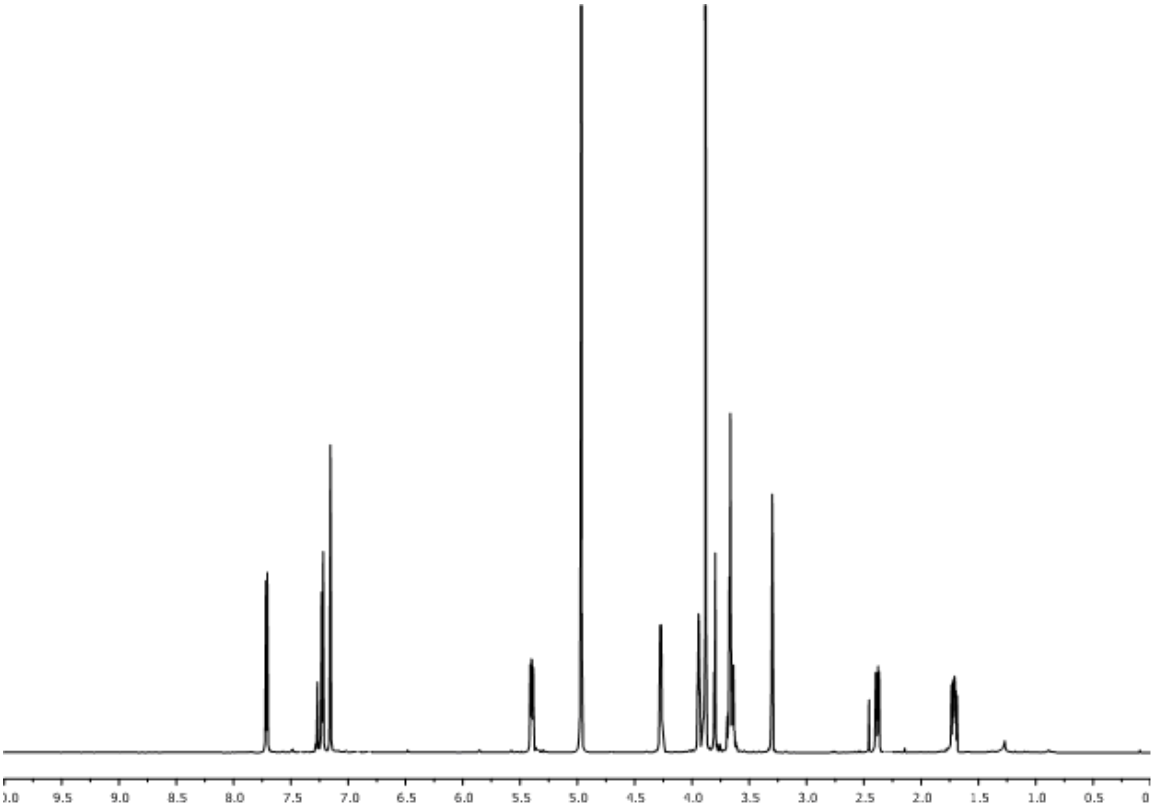


Figure S9: 600 MHz ^{1}H NMR spectra (MeOD) of **10**

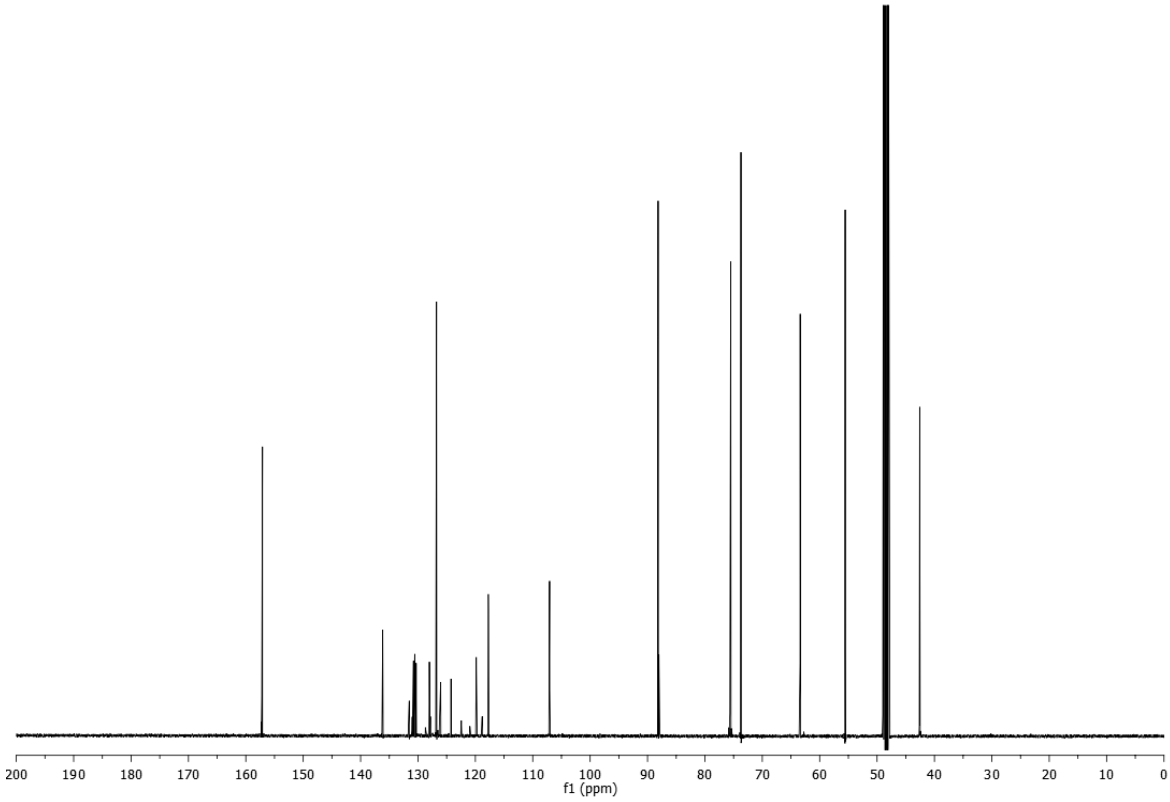


Figure S10: 151 MHz ^{13}C NMR spectrum (MeOD) of **10**

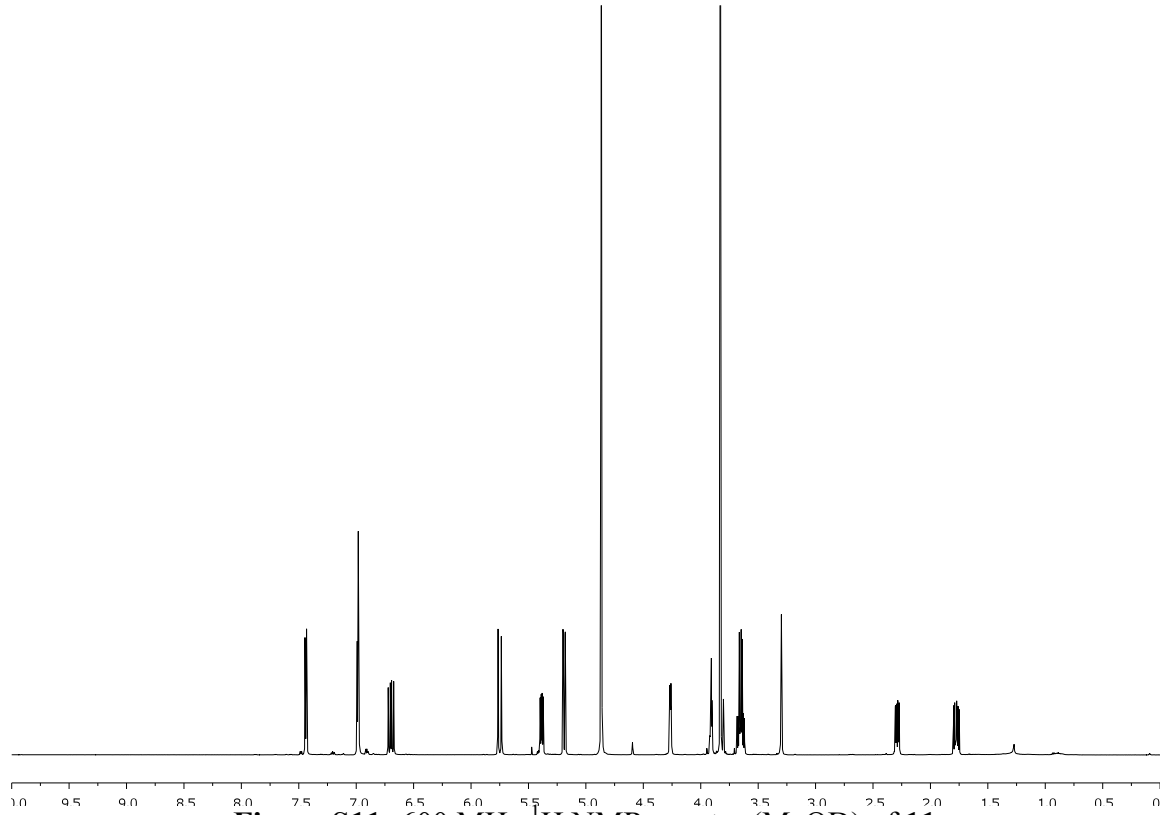


Figure S11: 600 MHz ^{1}H NMR spectra (MeOD) of **11**

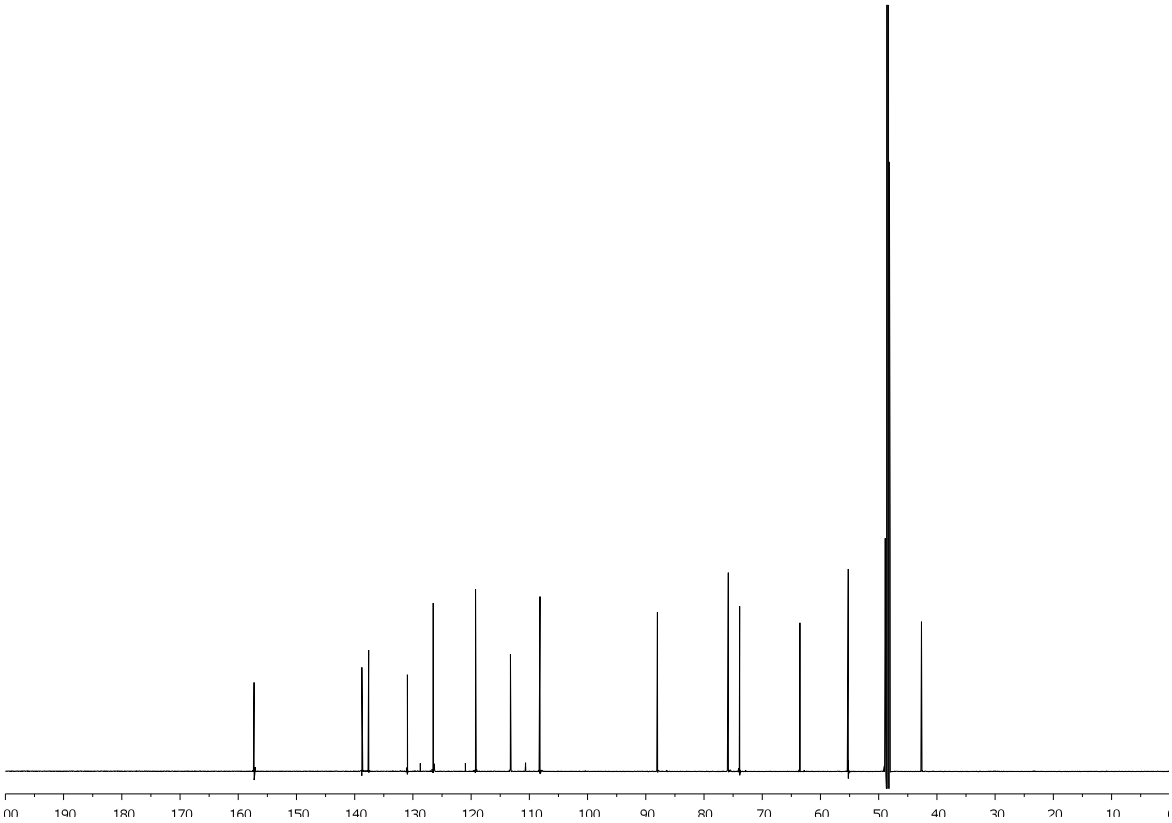


Figure S12: 151 MHz ^{13}C NMR spectrum (MeOD) of **11**

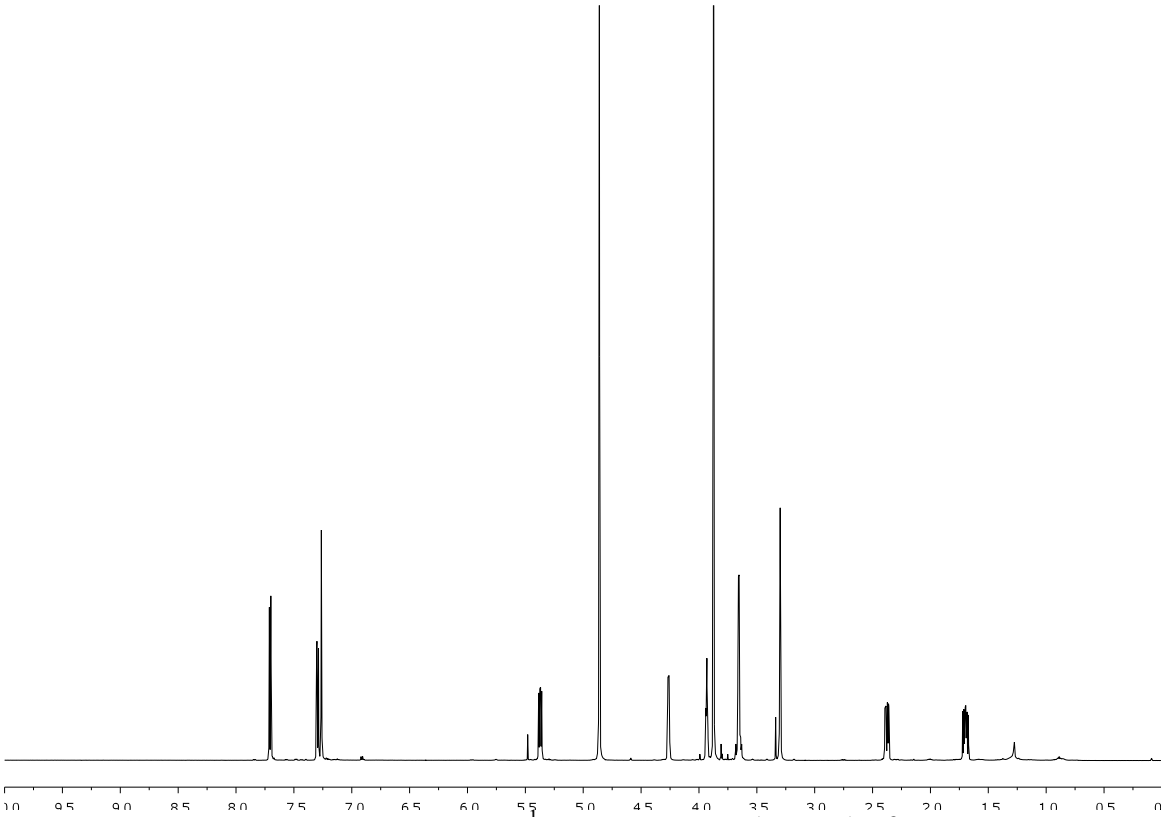


Figure S13: 600 MHz ^{1}H NMR spectra (MeOD) of **12**

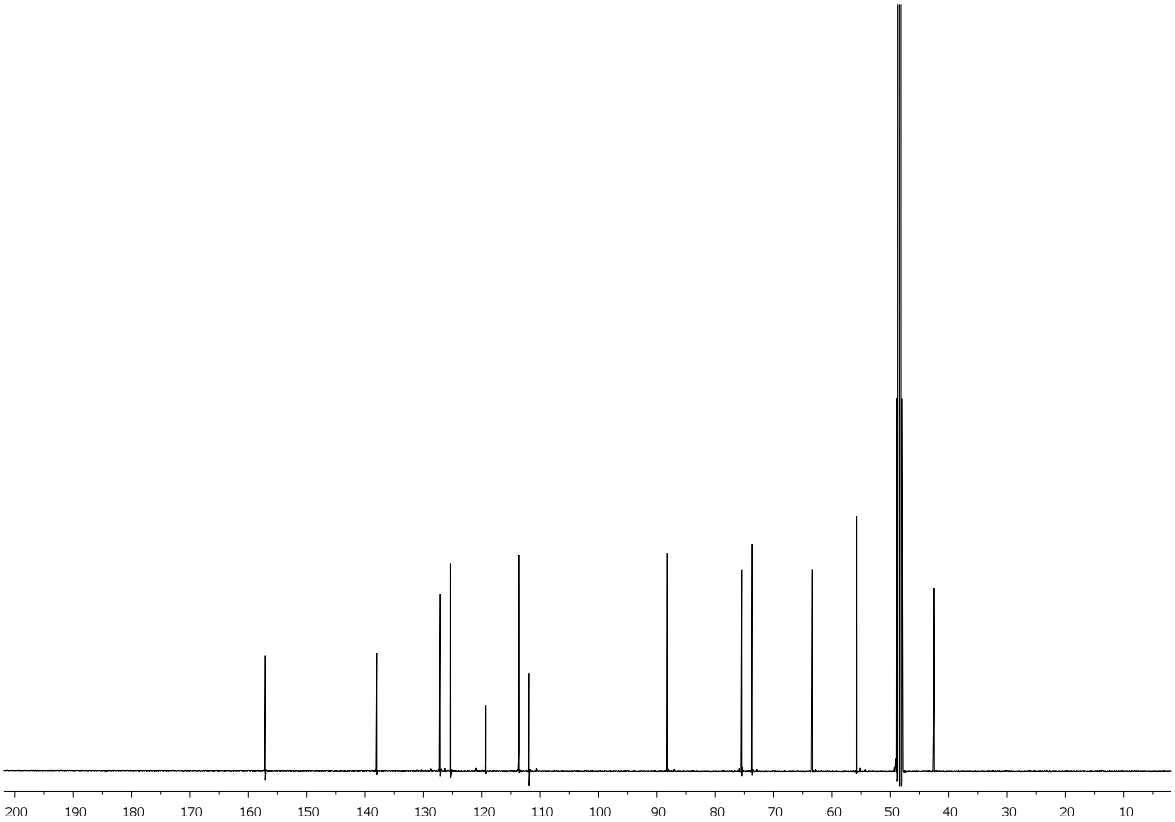


Figure S14: 151 MHz ^{13}C NMR spectrum (MeOD) of **12**

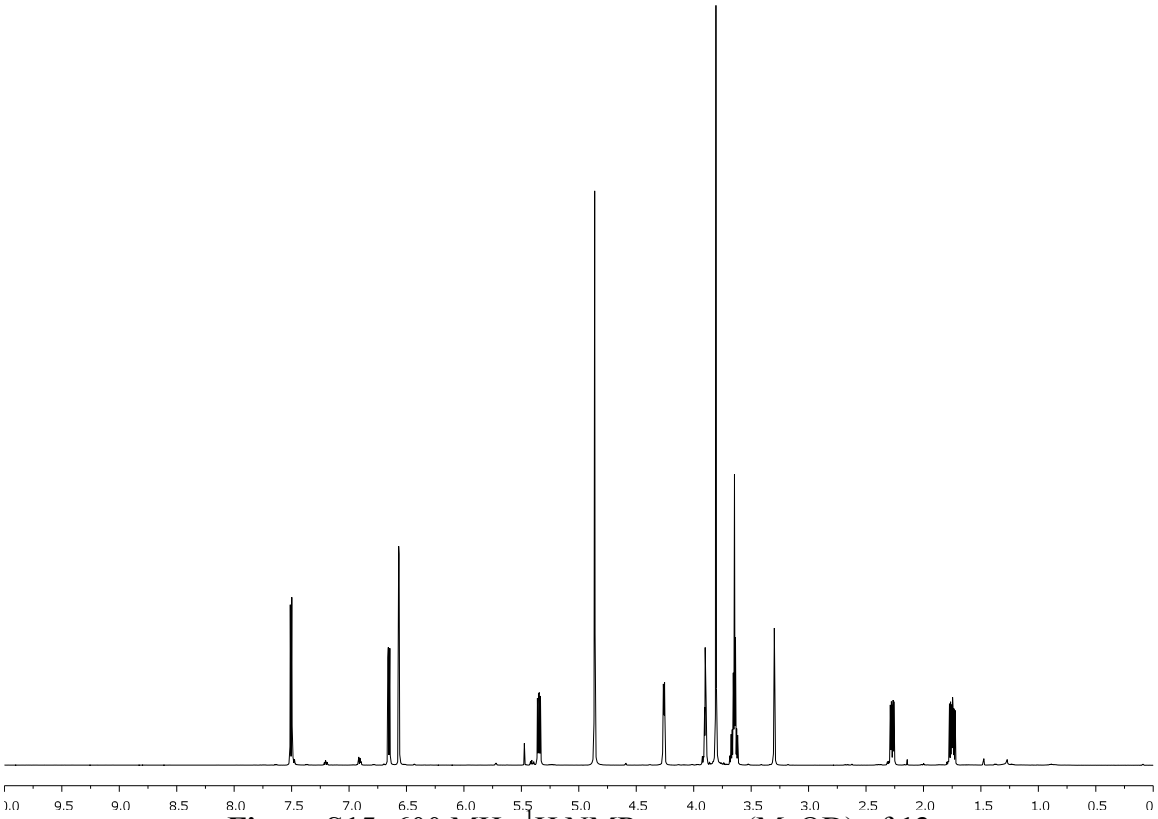


Figure S15: 600 MHz ^{1}H NMR spectra (MeOD) of **13**

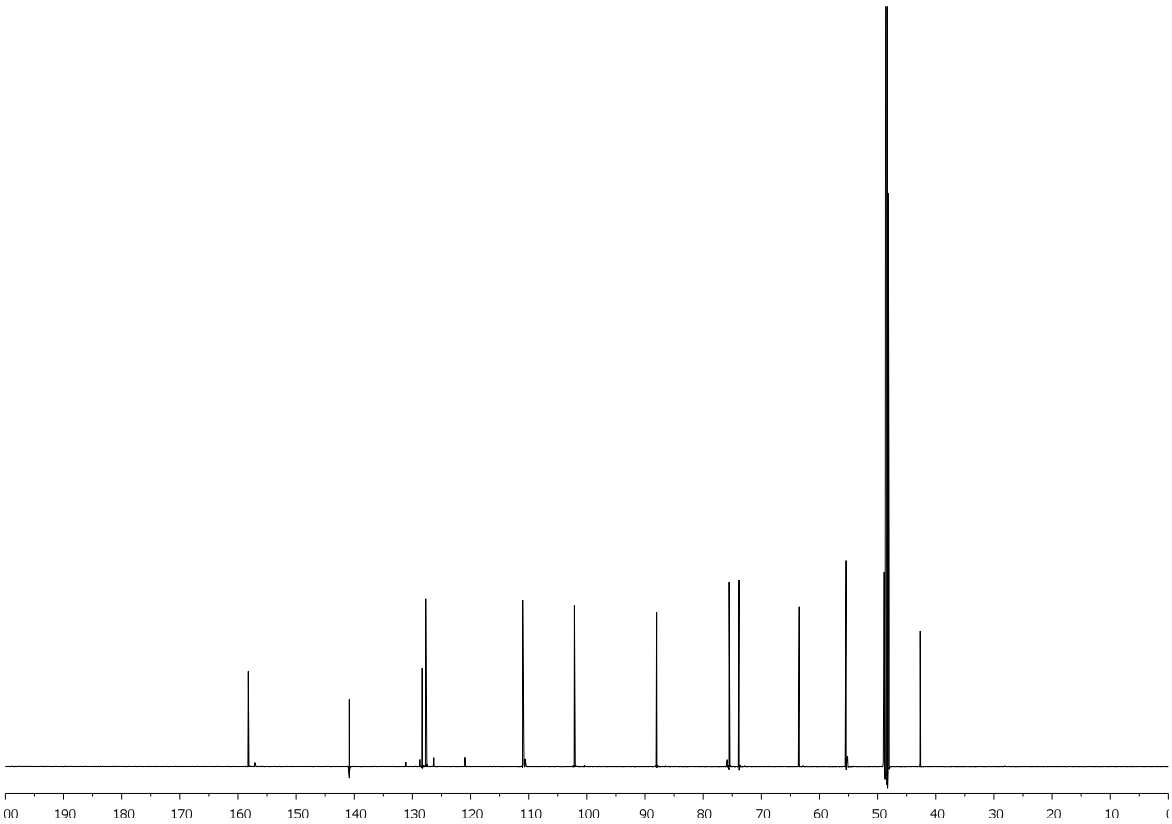


Figure S16: 151 MHz ^{13}C NMR spectrum (MeOD) of **13**

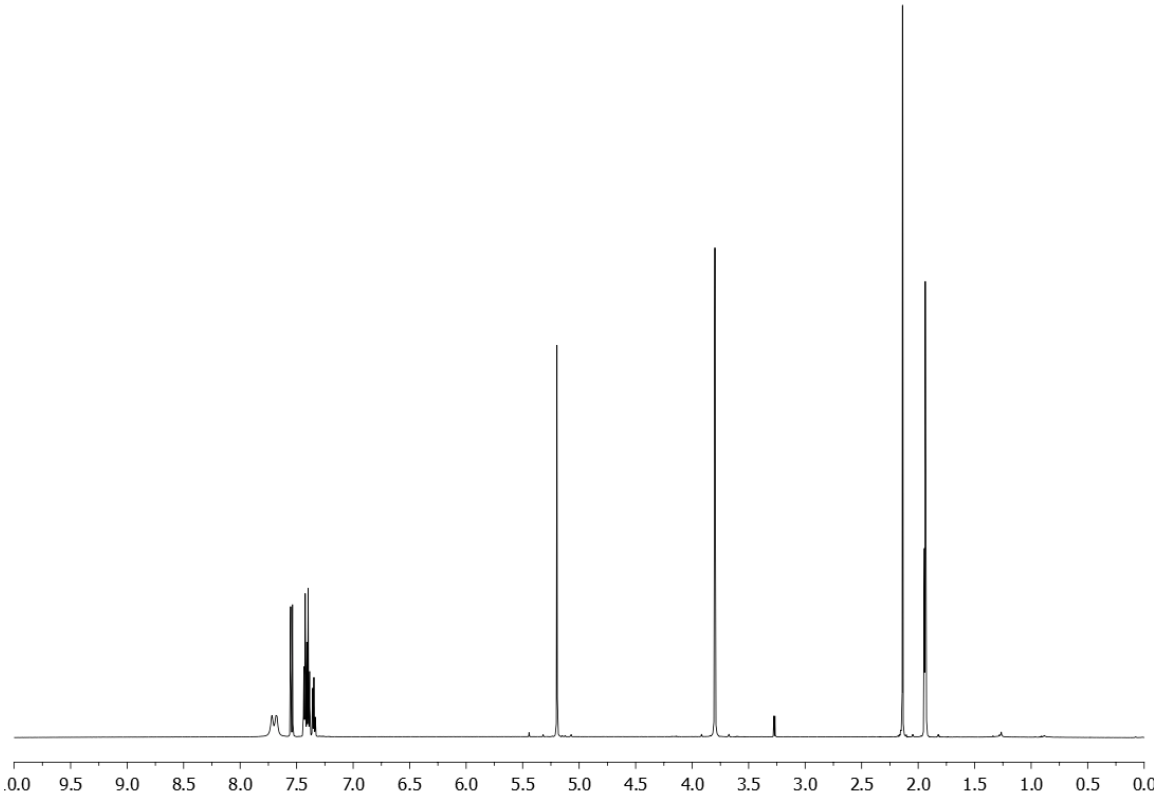


Figure S17: 600 MHz ^{1}H NMR spectra (CD_3CN) of **29**

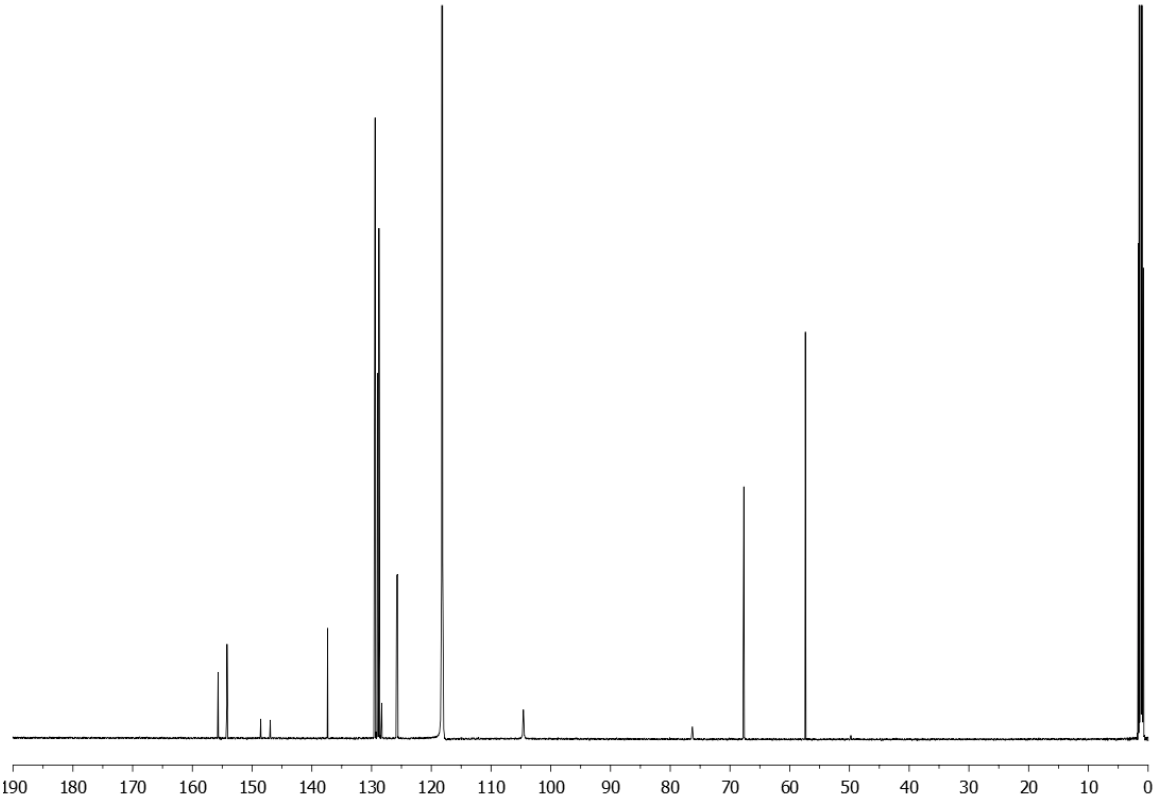


Figure S18: 151 MHz ^{13}C NMR spectrum (CD_3CN) of **29**

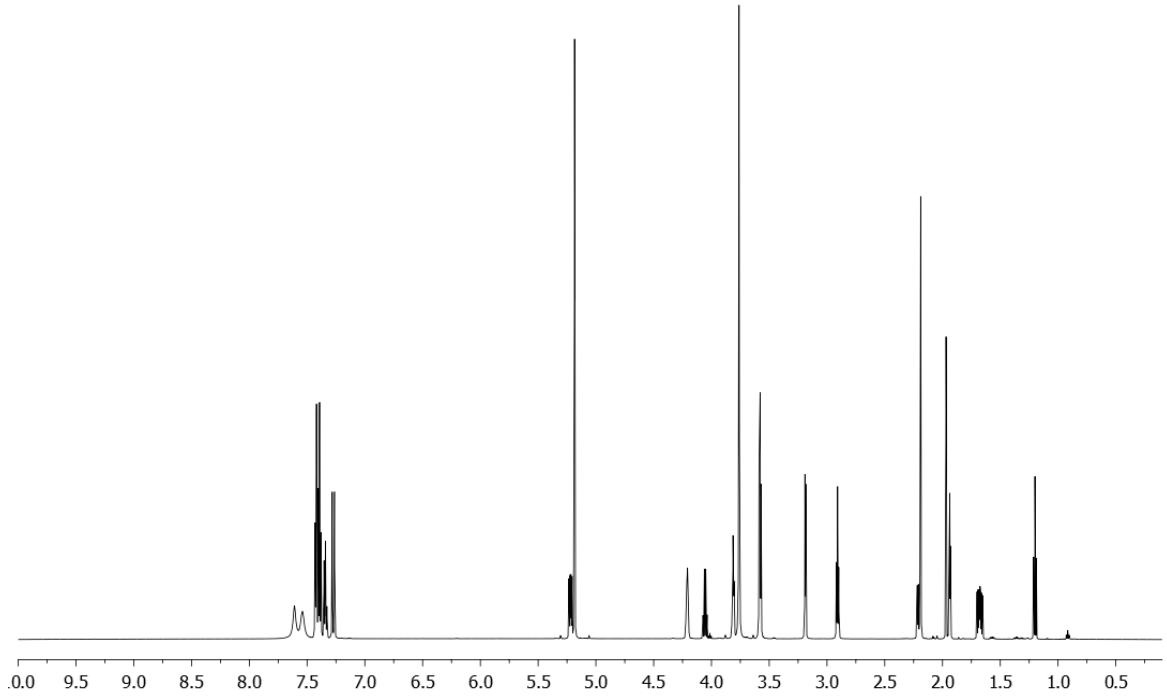


Figure S19: 600 MHz ^{1}H NMR spectra (CD_3CN) of **31**

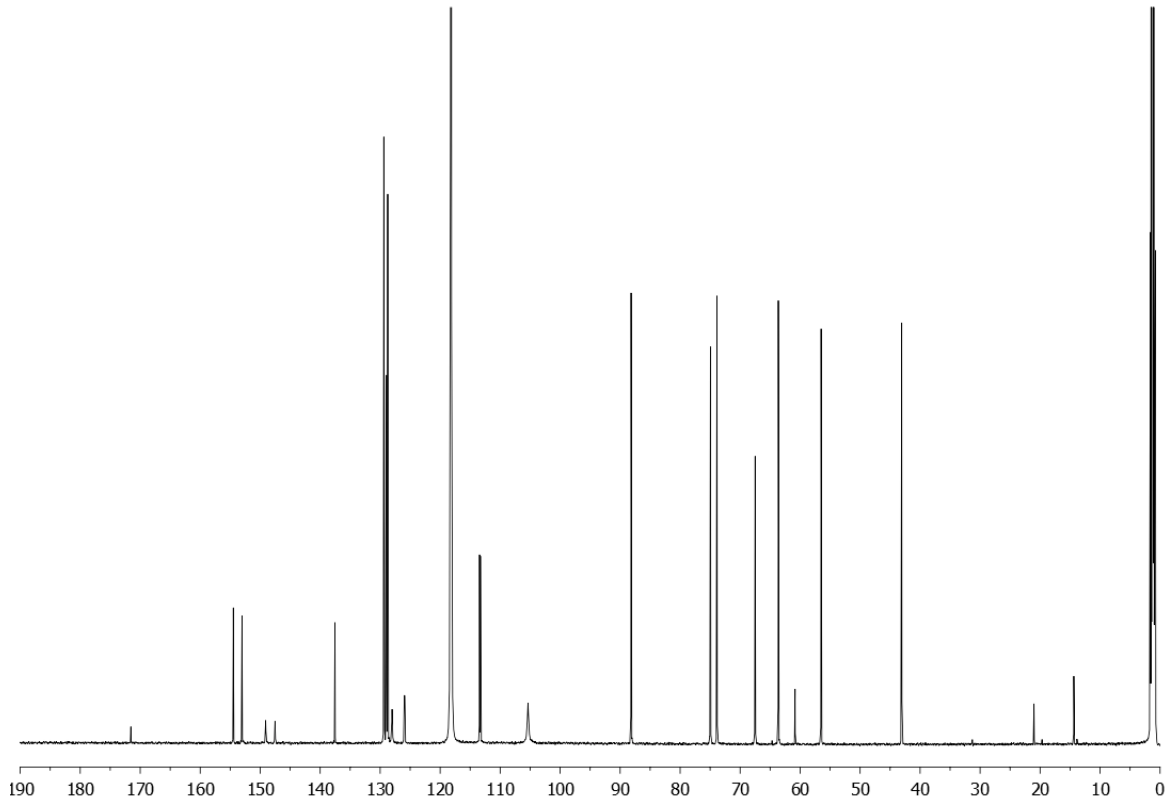


Figure S20: 151 MHz ^{13}C NMR spectrum (CD_3CN) of **31**

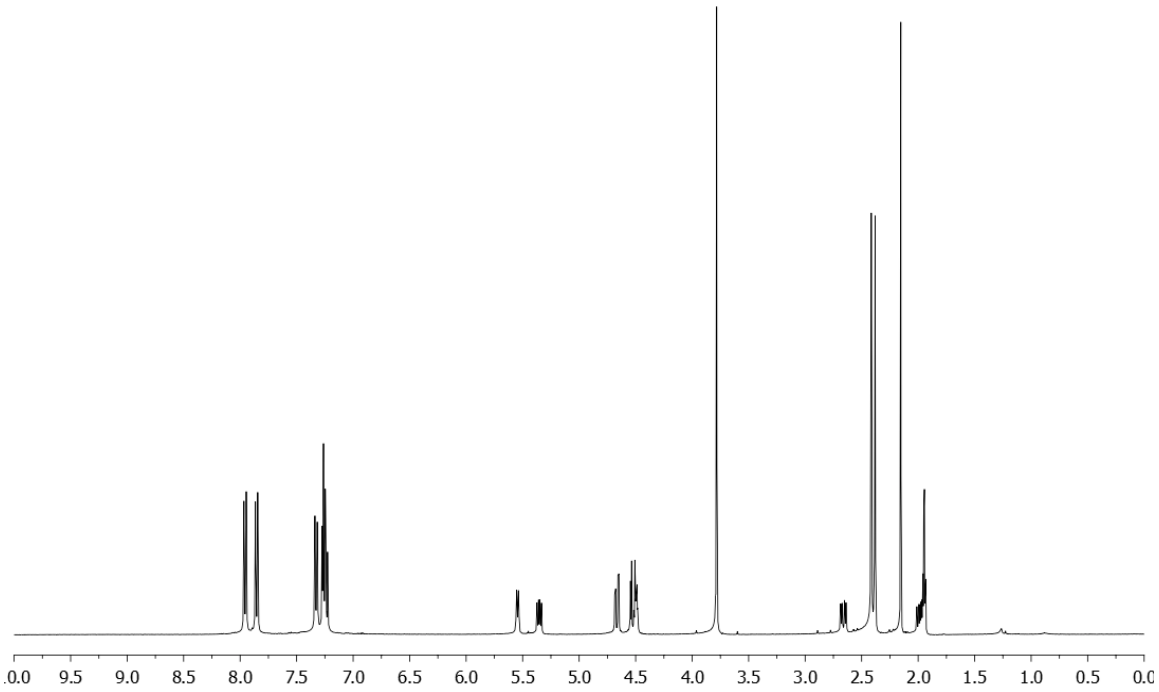


Figure S21: 600 MHz ^{1}H NMR spectra (CD_3CN) of **32**

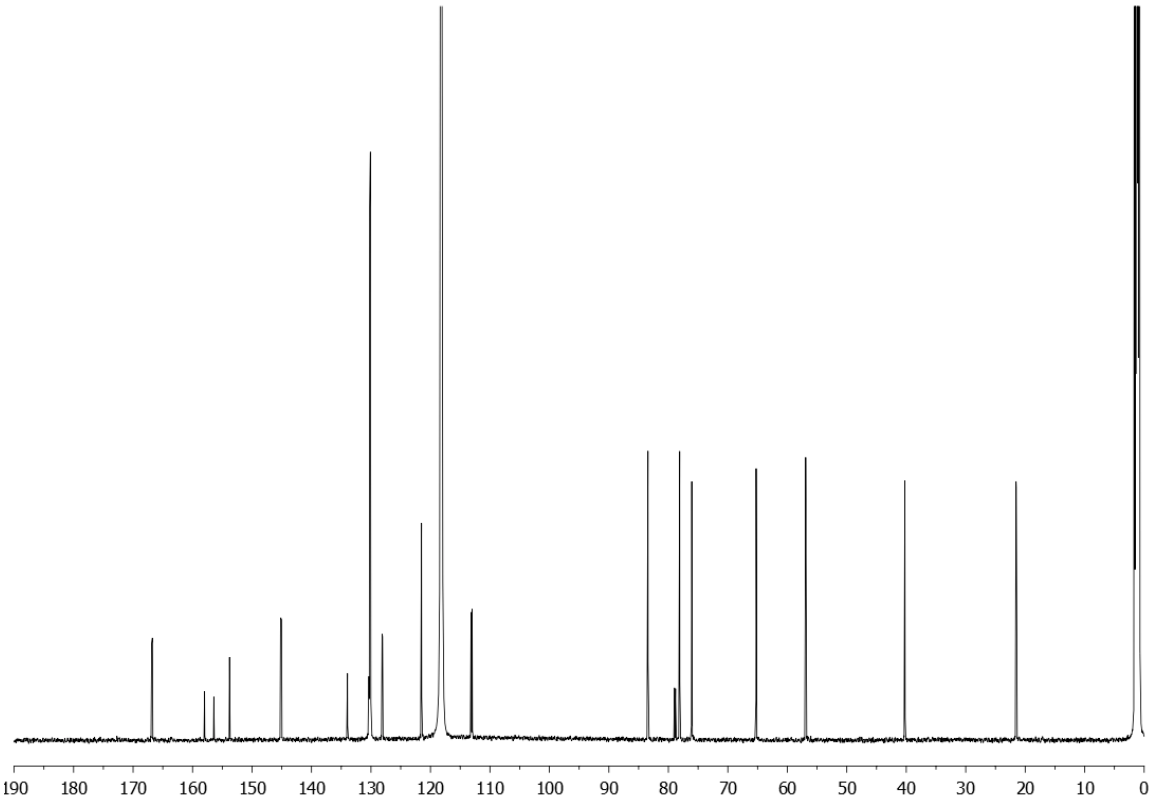


Figure S22: 151 MHz ^{13}C NMR spectrum (CD_3CN) of **32**

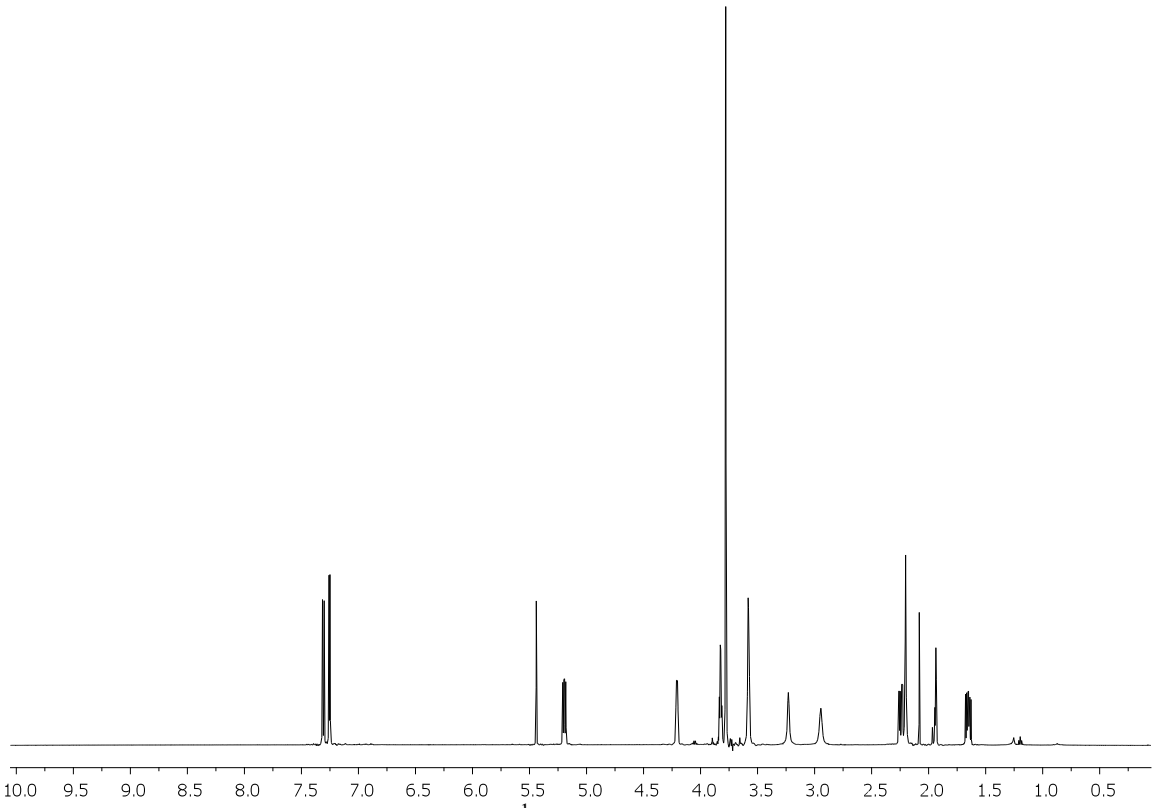


Figure S23: 600 MHz ^{1}H NMR spectra (CD_3CN) of **33**

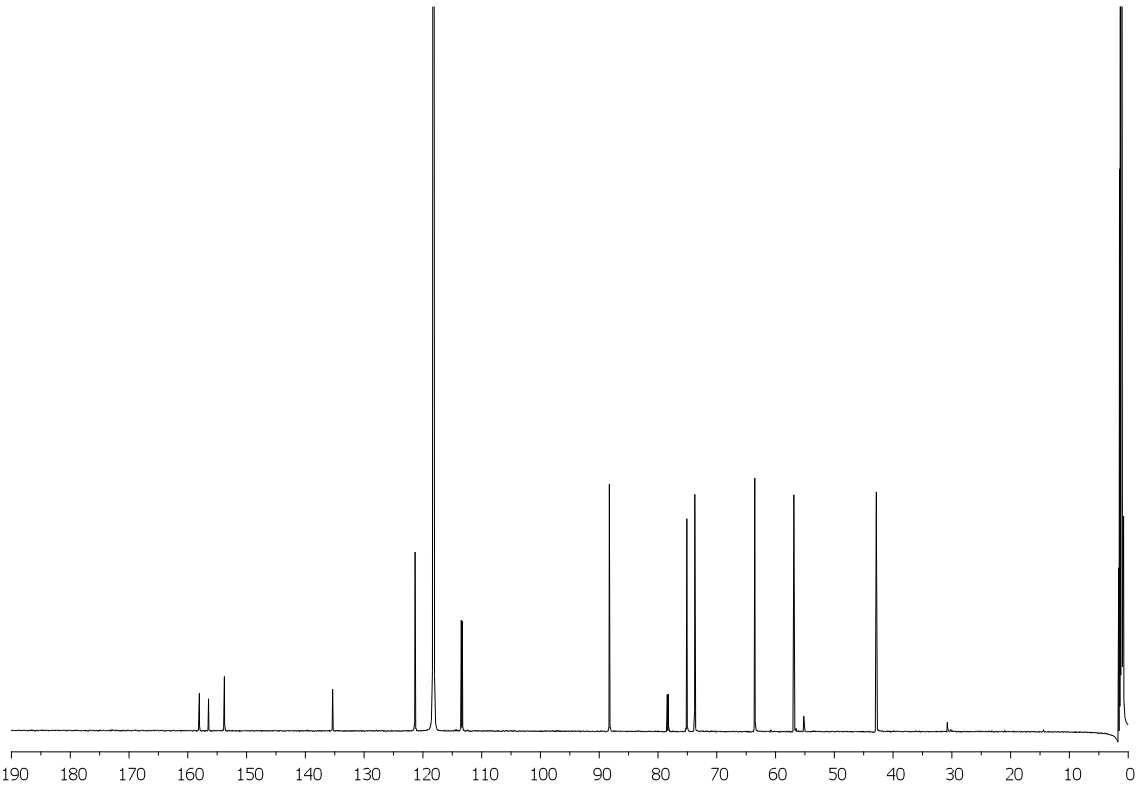


Figure S24: 151 MHz ^{13}C NMR spectrum (CD_3CN) of **33**

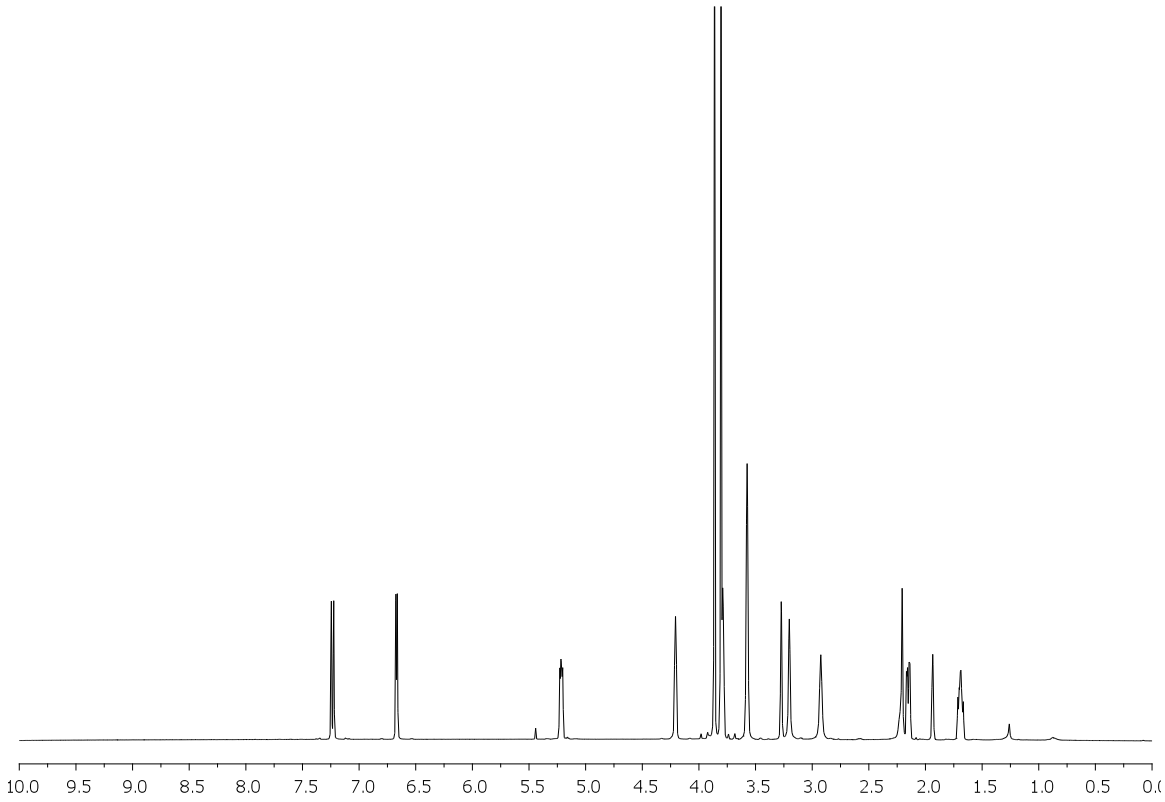


Figure S25: 600 MHz ^{1}H NMR spectra (CD_3CN) of **34**

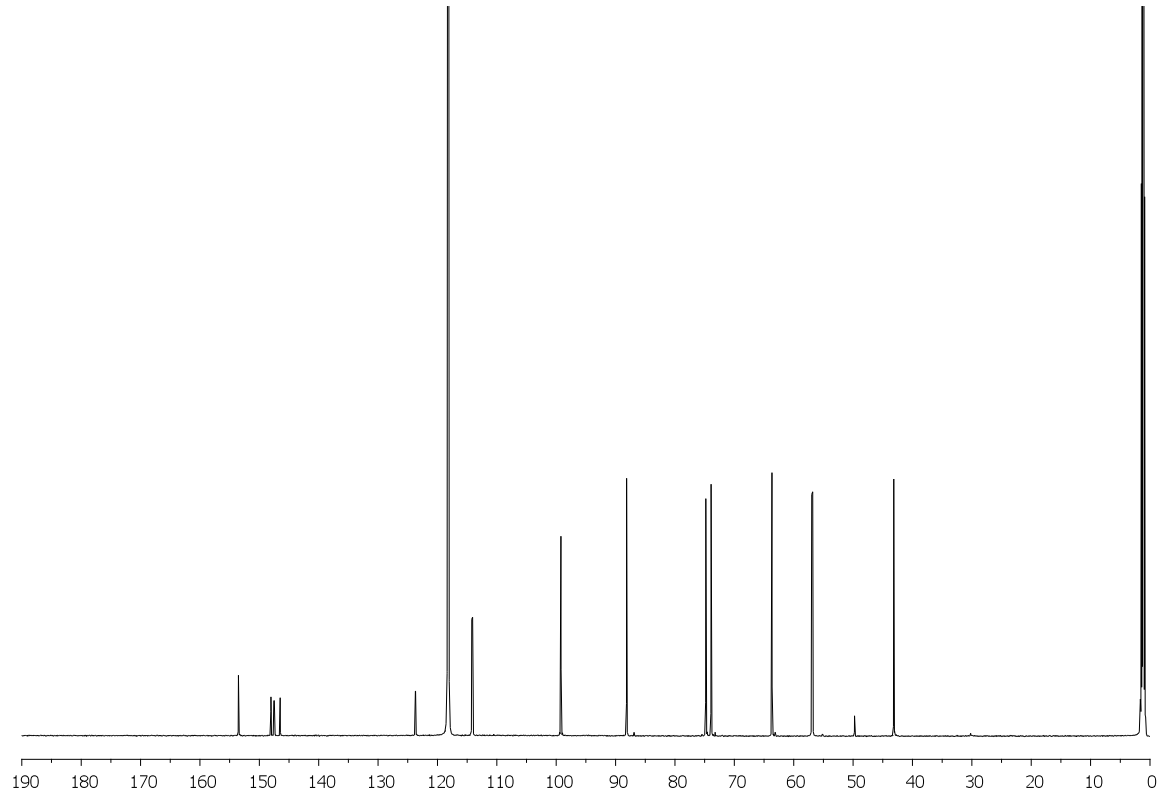


Figure S26: 151 MHz ^{13}C NMR spectrum (CD_3CN) of **34**

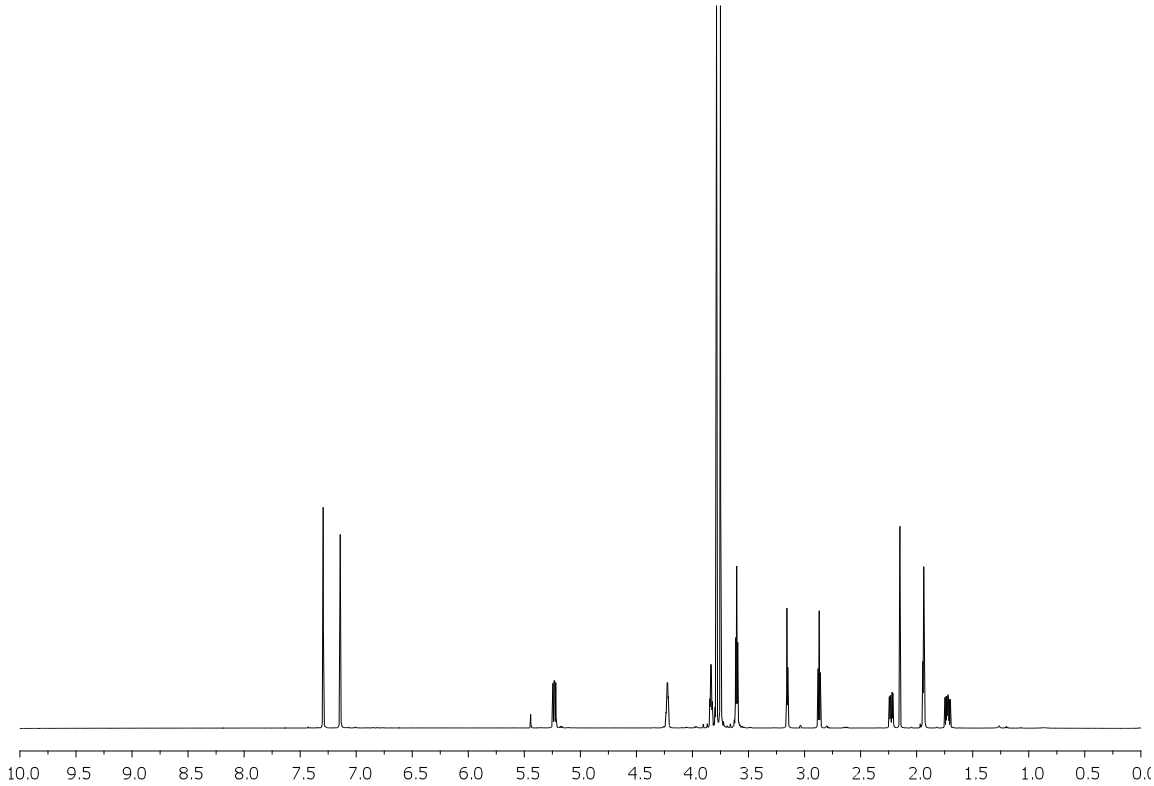


Figure S27: 600 MHz ^{1}H NMR spectra (CD_3CN) of **38**

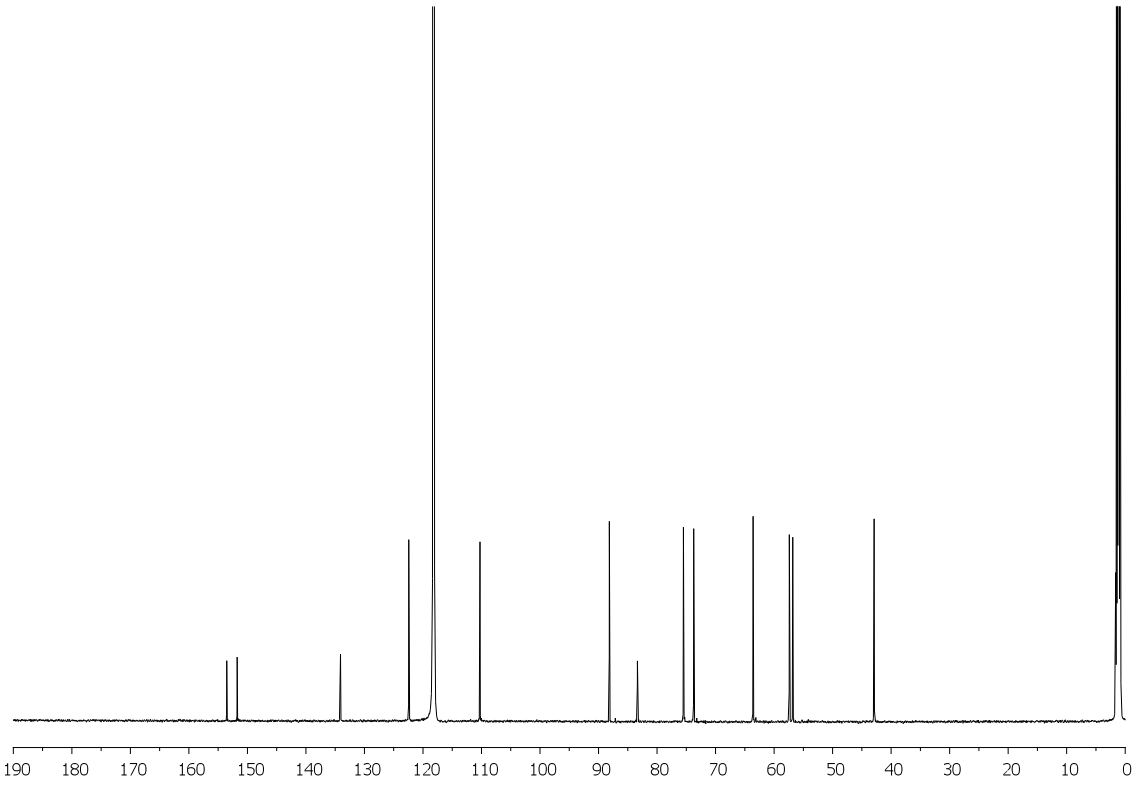


Figure S28: 151 MHz ^{13}C NMR spectrum (CD_3CN) of **38**

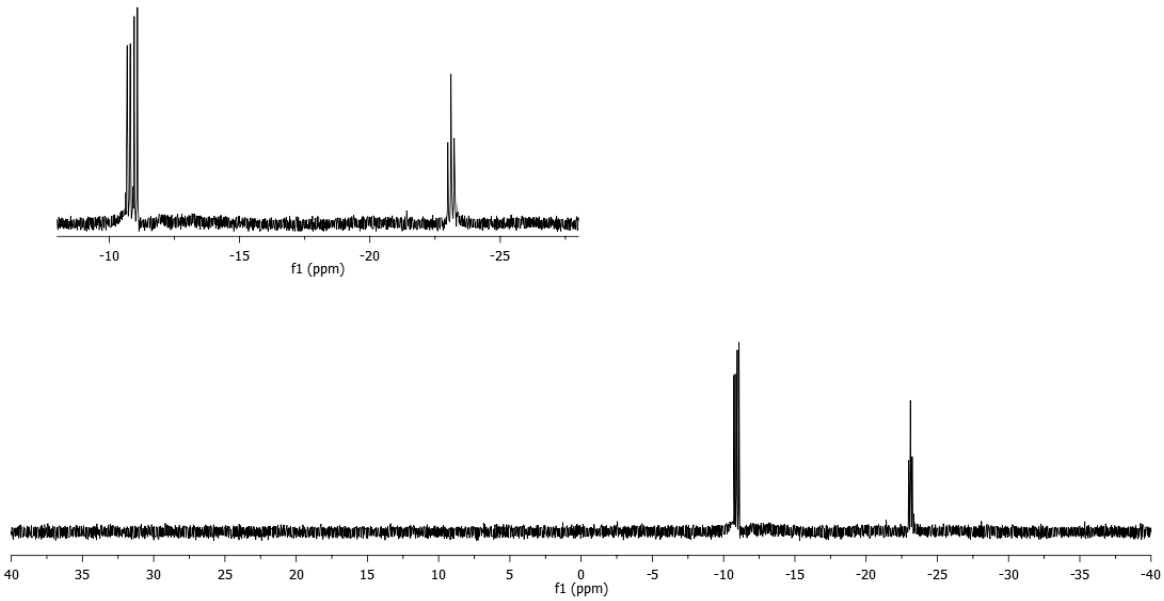


Figure S29: 162 MHz ^{31}P NMR spectra (D_2O) of **6**

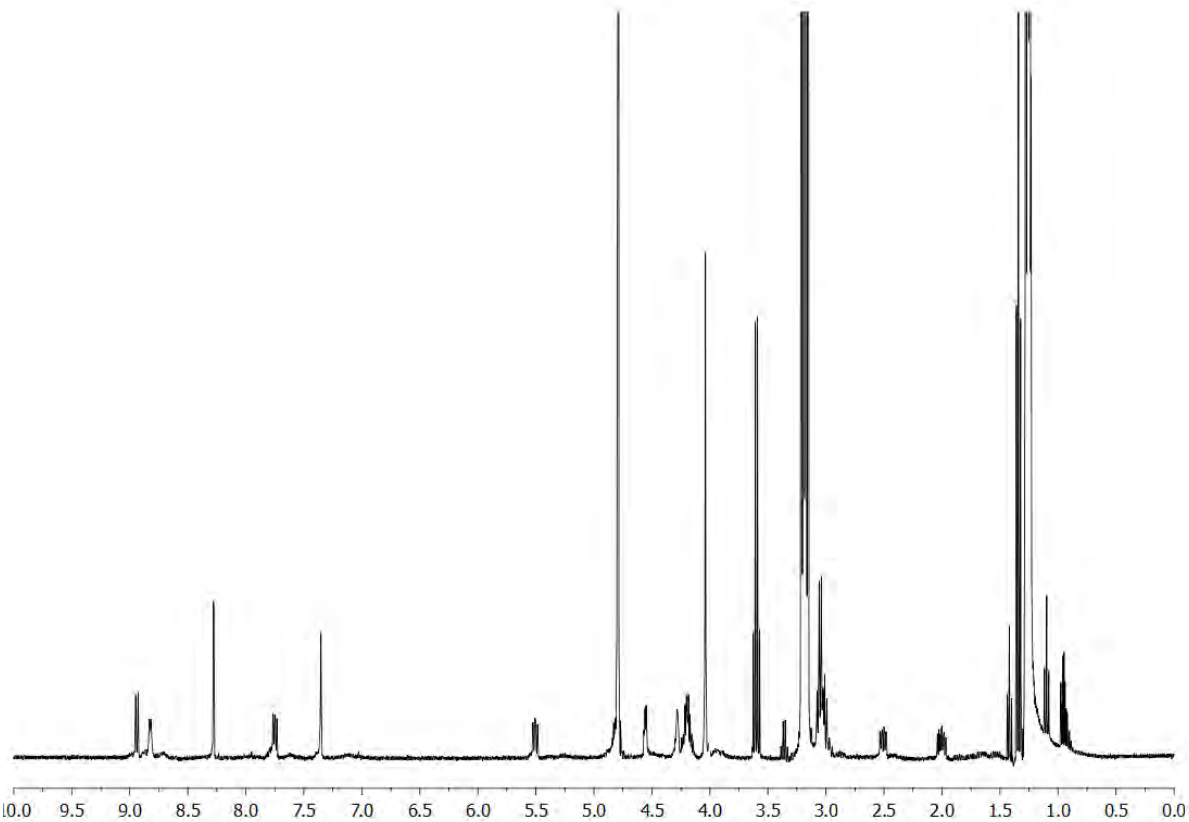


Figure S30: 400 MHz ^{1}H NMR spectra (D_2O) of **6**

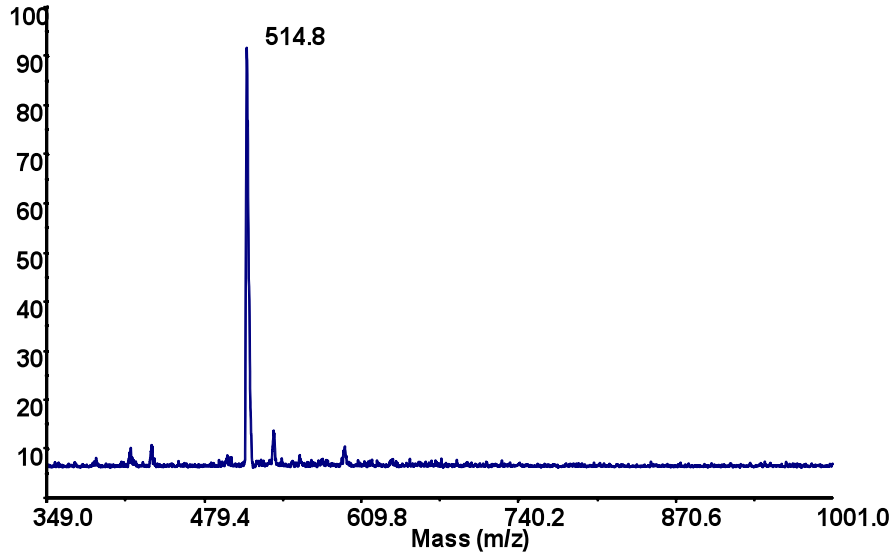


Figure S31: MALDI-TOF MS analysis of **6**

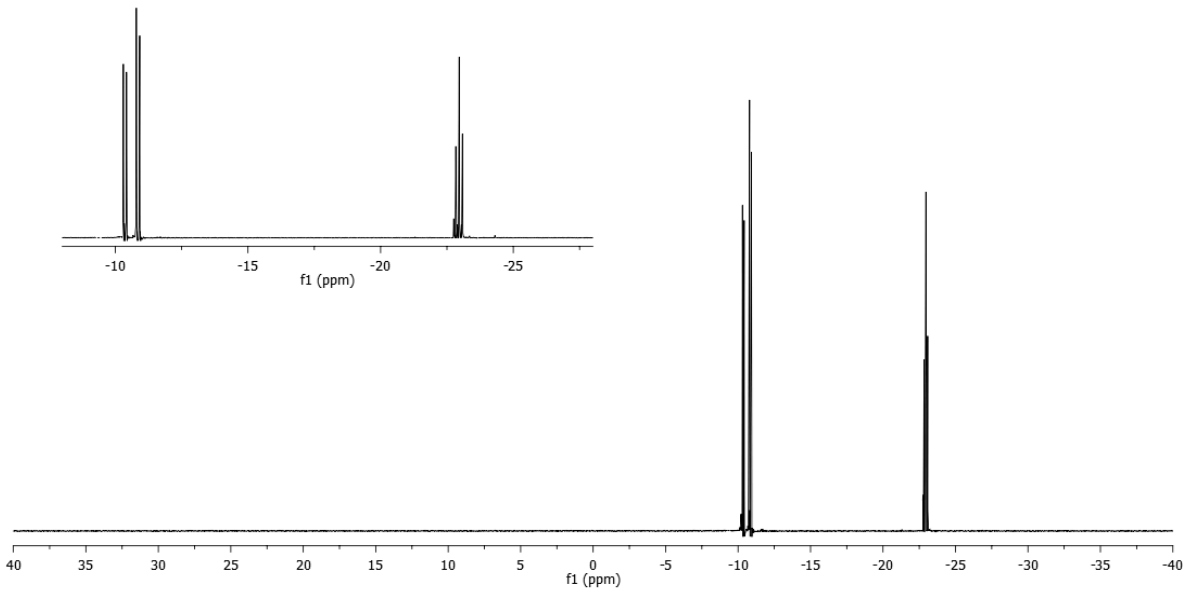


Figure S32: 162 MHz ^{31}P NMR spectra (D_2O) of **7**

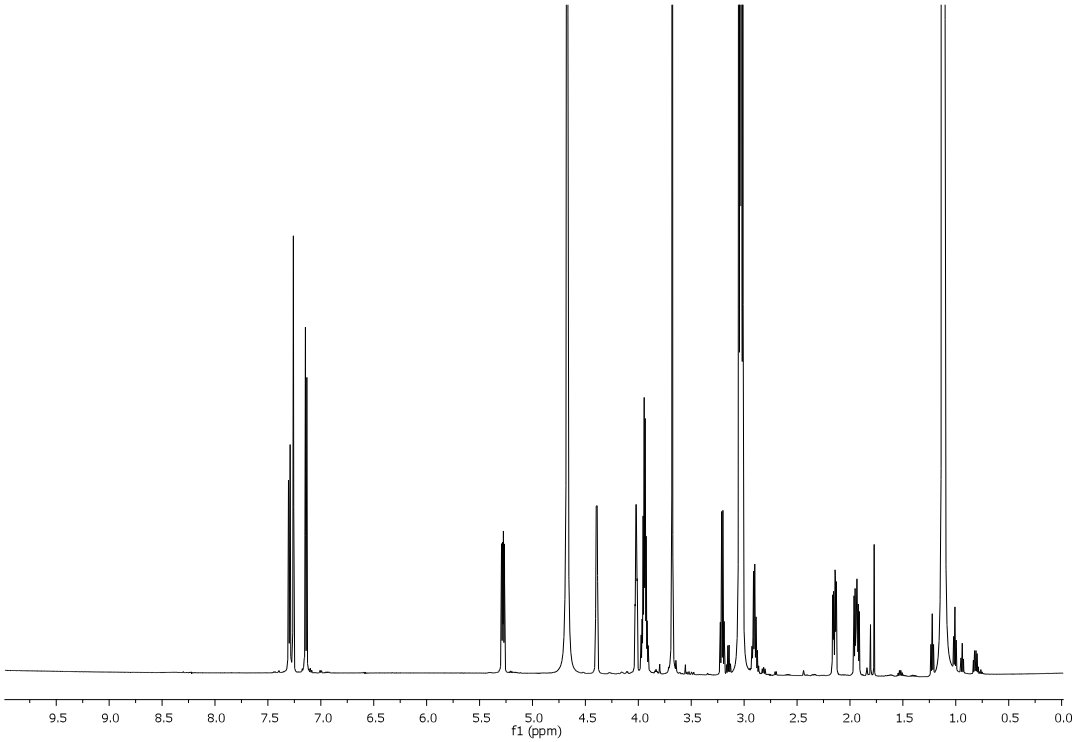


Figure S33: 600 MHz ^{1}H NMR spectra (D_2O) of **7**

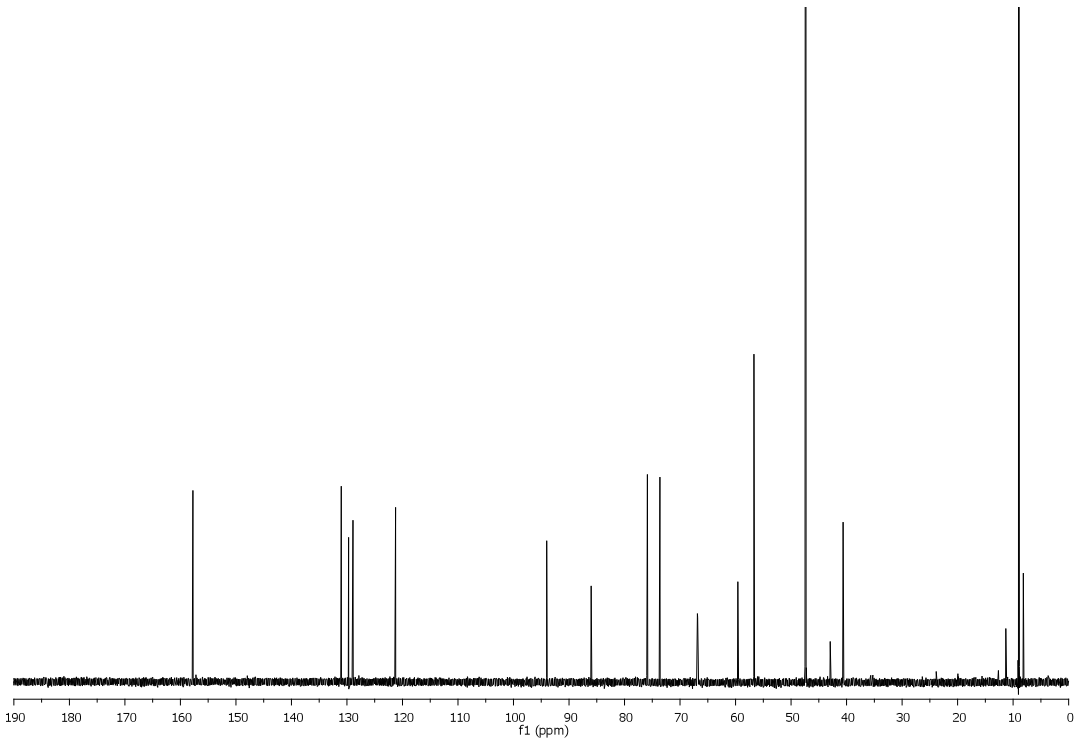


Figure S34: 151 MHz ^{13}C NMR spectra (D_2O) of **7**

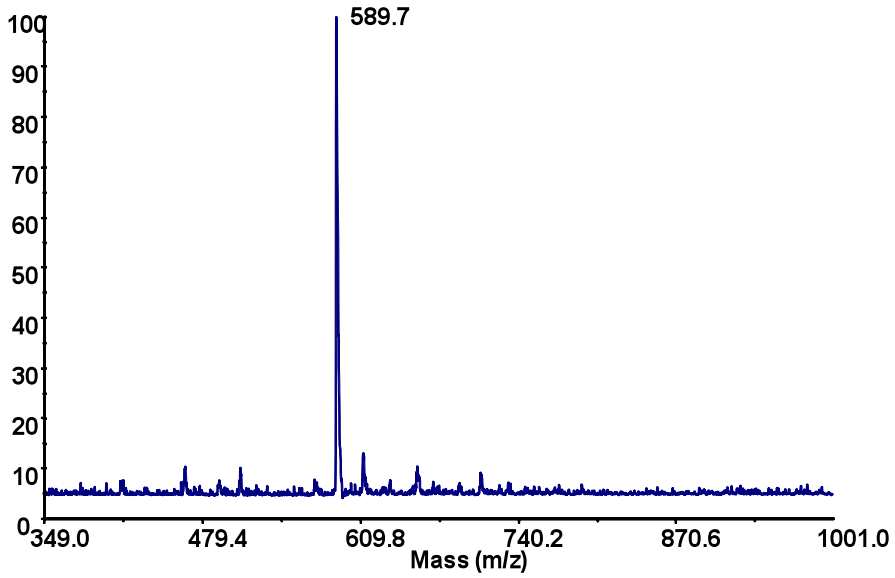


Figure S35: MALDI-TOF MS analysis of **7**

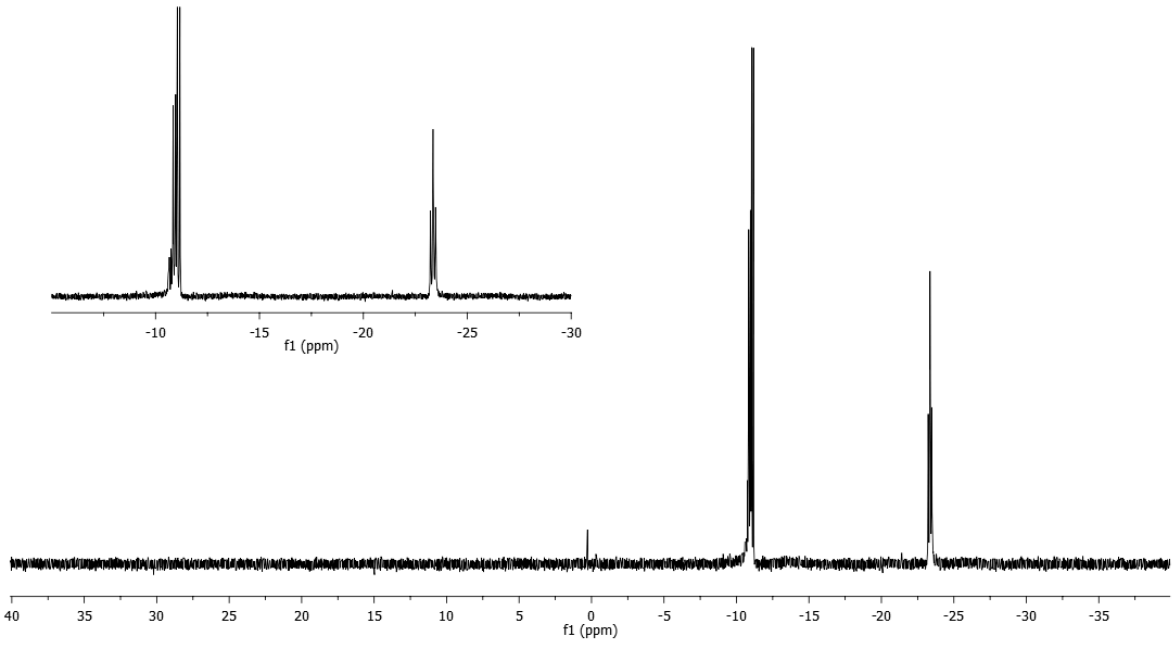


Figure S36: 162 MHz ^{31}P NMR spectra (D_2O) of **8**

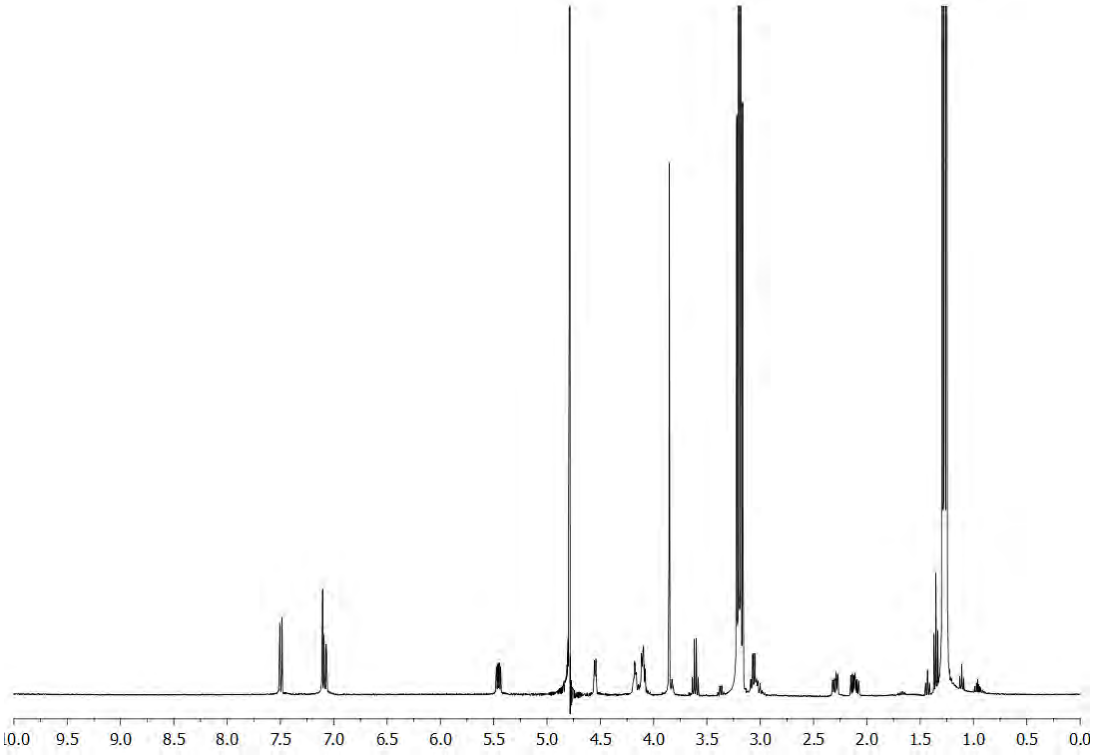


Figure S37: 400 MHz ^{1}H NMR spectra (D_2O) of **8**

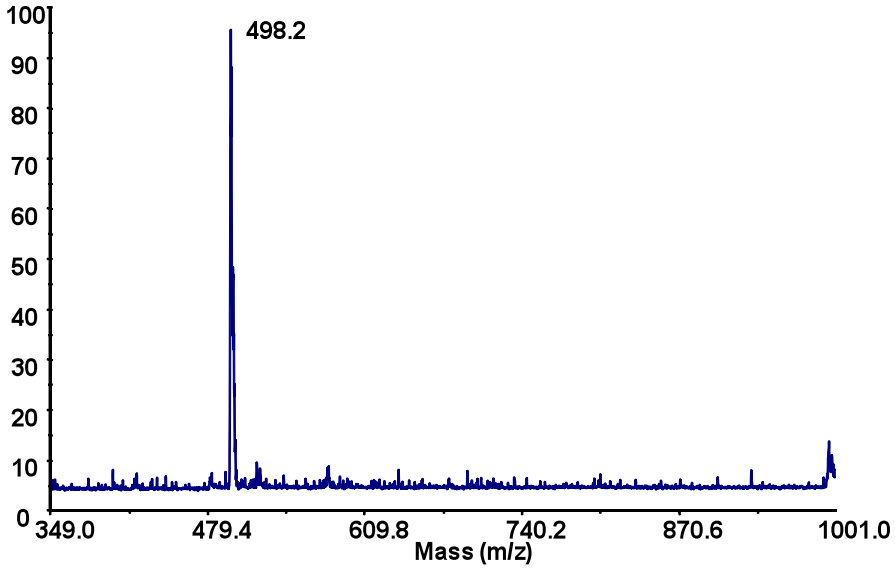


Figure S38: MALDI-TOF MS analysis of **8**

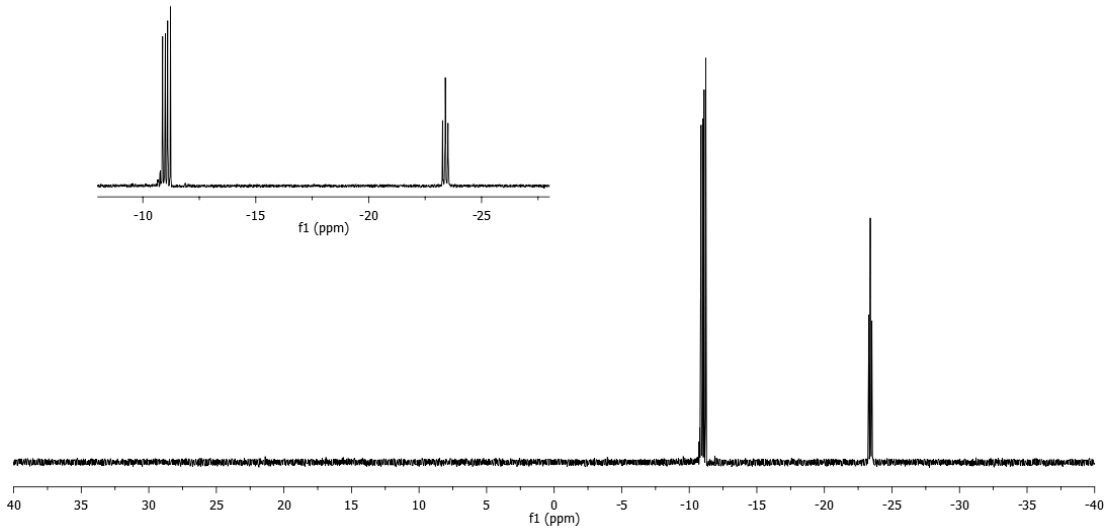


Figure S39: 162 MHz ^{31}P NMR spectra (D_2O) of **14**

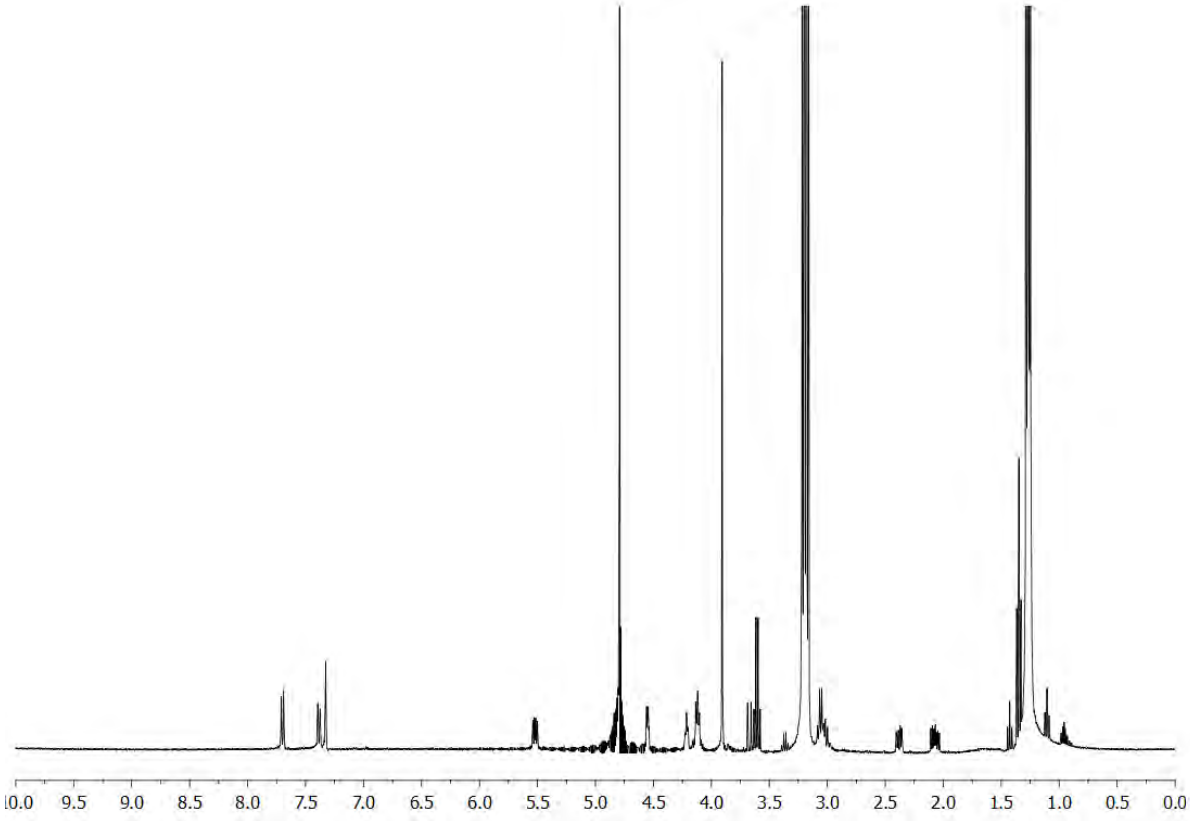


Figure S40: 400 MHz ^{1}H NMR spectra (D_2O) of **14**

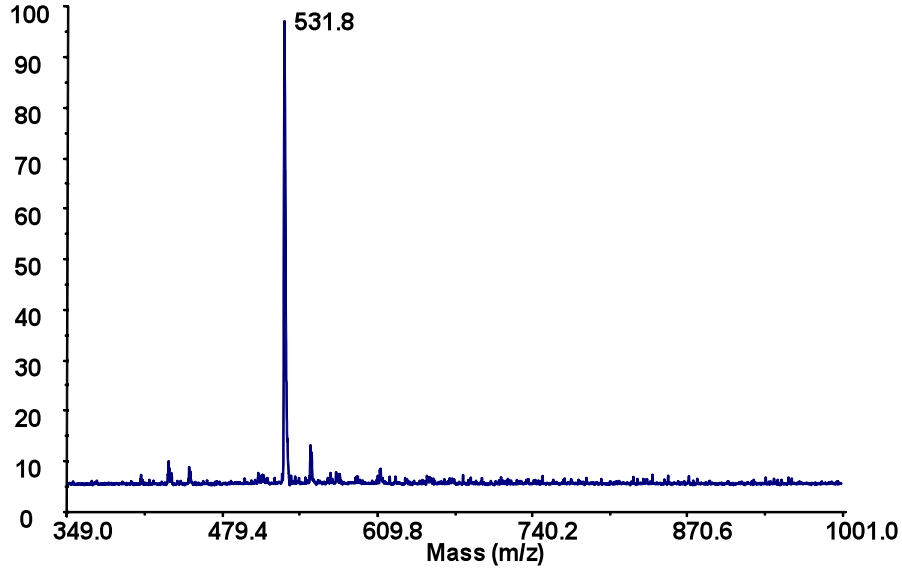


Figure S41: MALDI-TOF MS analysis of **14**

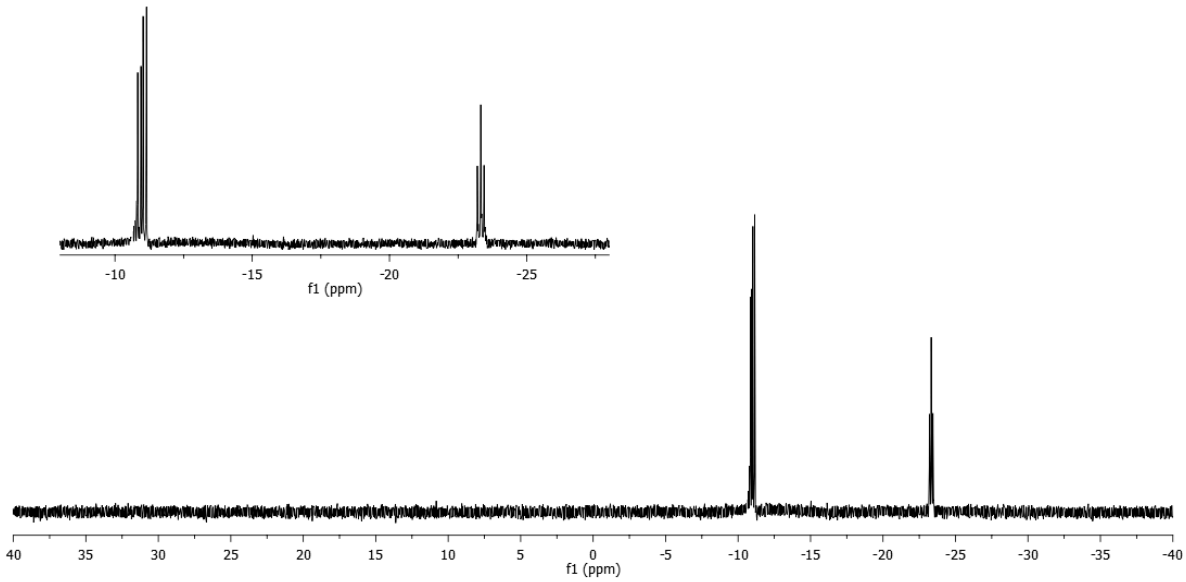


Figure S42: 162 MHz ^{31}P NMR spectra (D_2O) of **15**

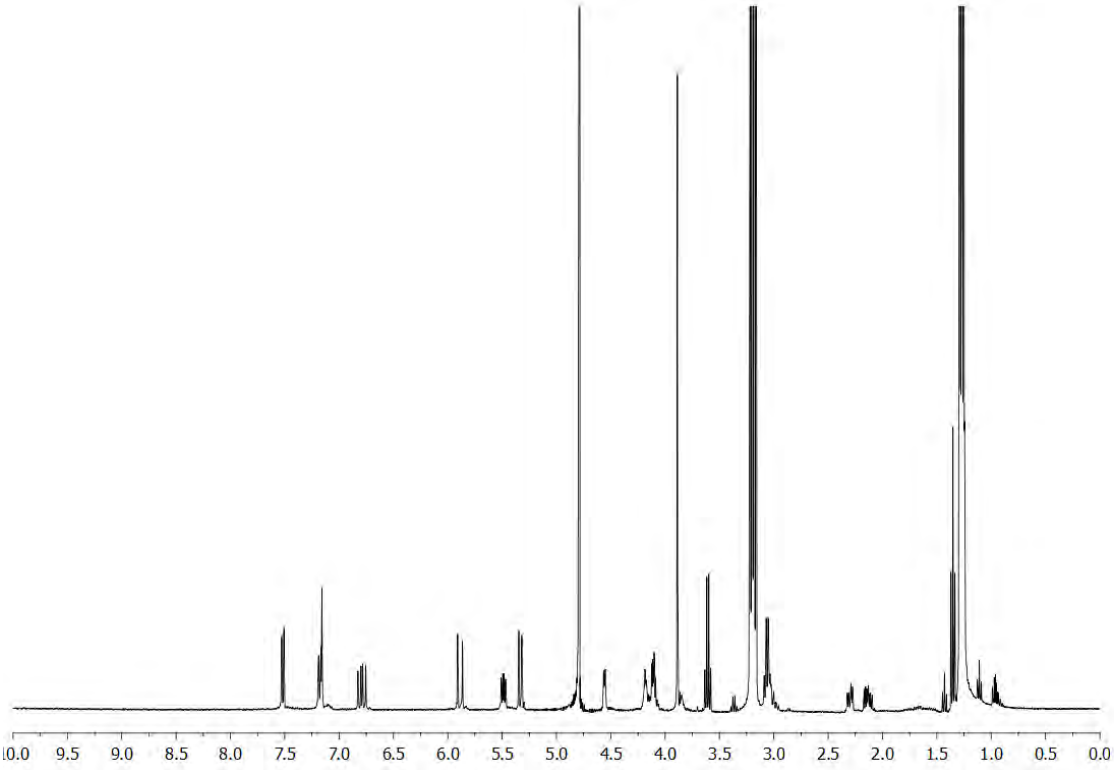


Figure S43: 400 MHz ^{1}H NMR spectra (D_2O) of **15**

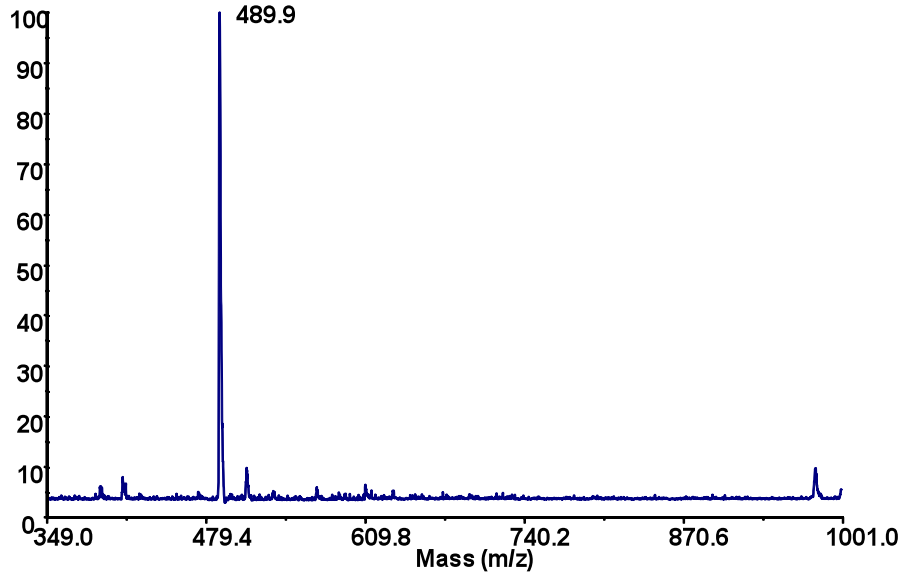


Figure S44: MALDI-TOF MS analysis of **15**

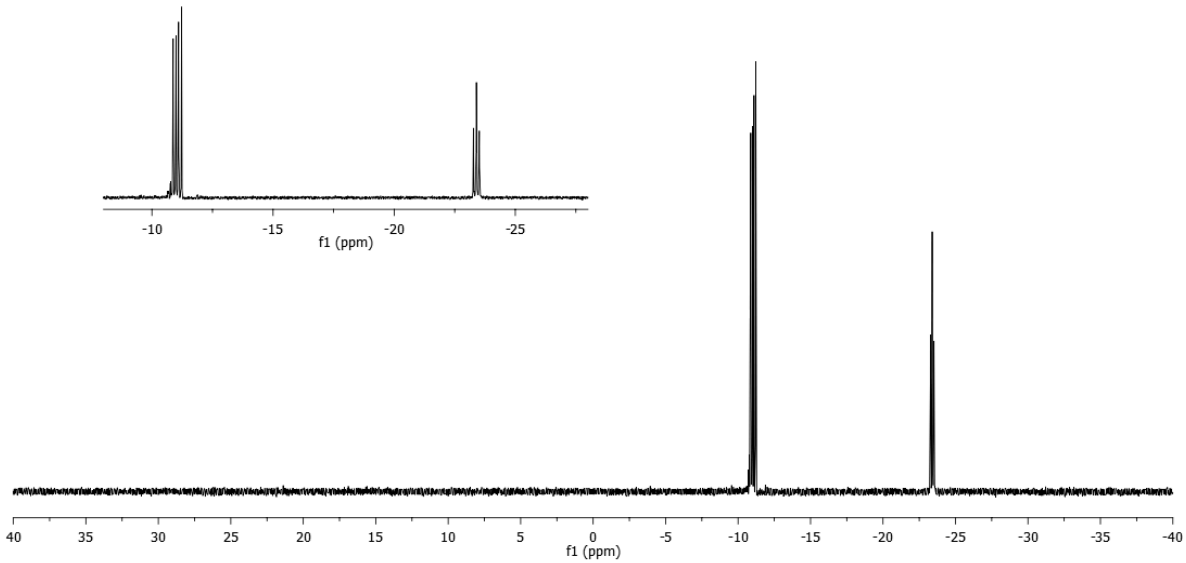


Figure S45: 162 MHz ^{31}P NMR spectra (D_2O) of **16**

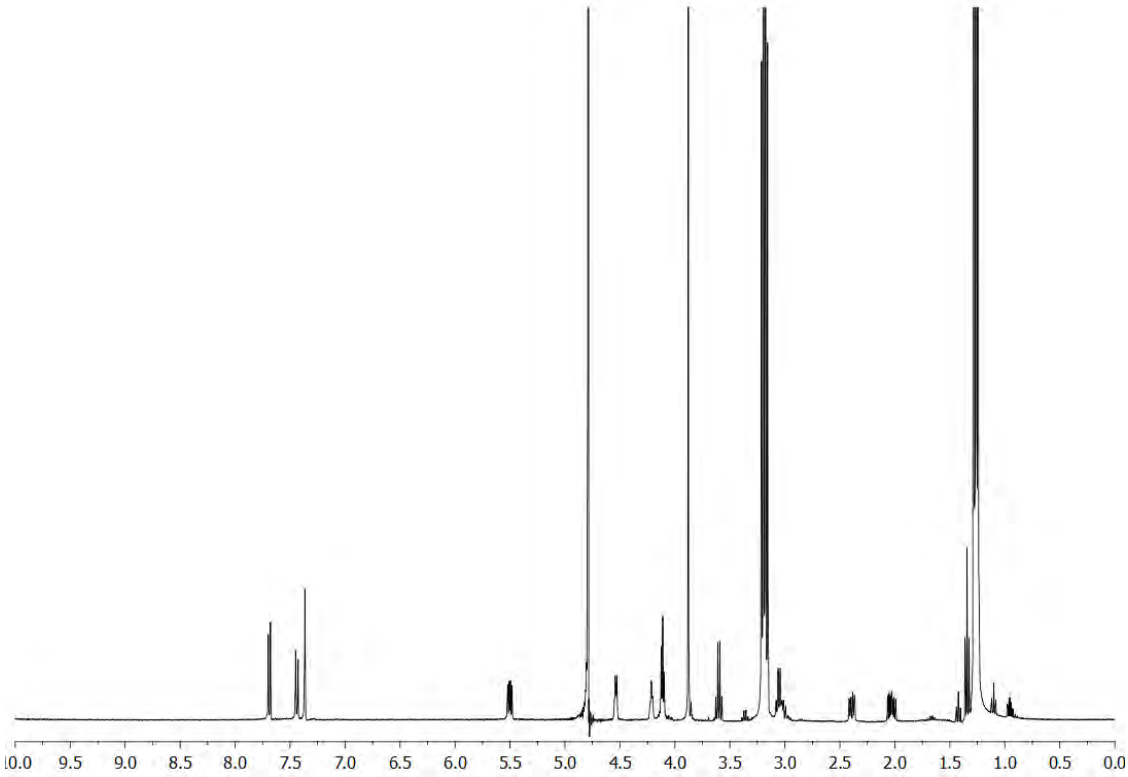


Figure S46: 400 MHz ^{1}H NMR spectra (D_2O) of **16**

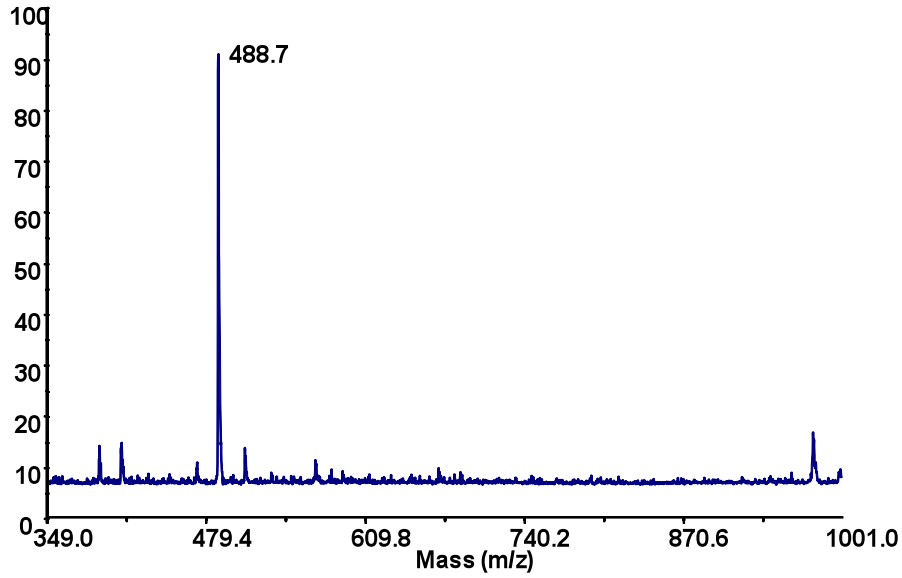


Figure S47: MALDI-TOF MS analysis of **16**

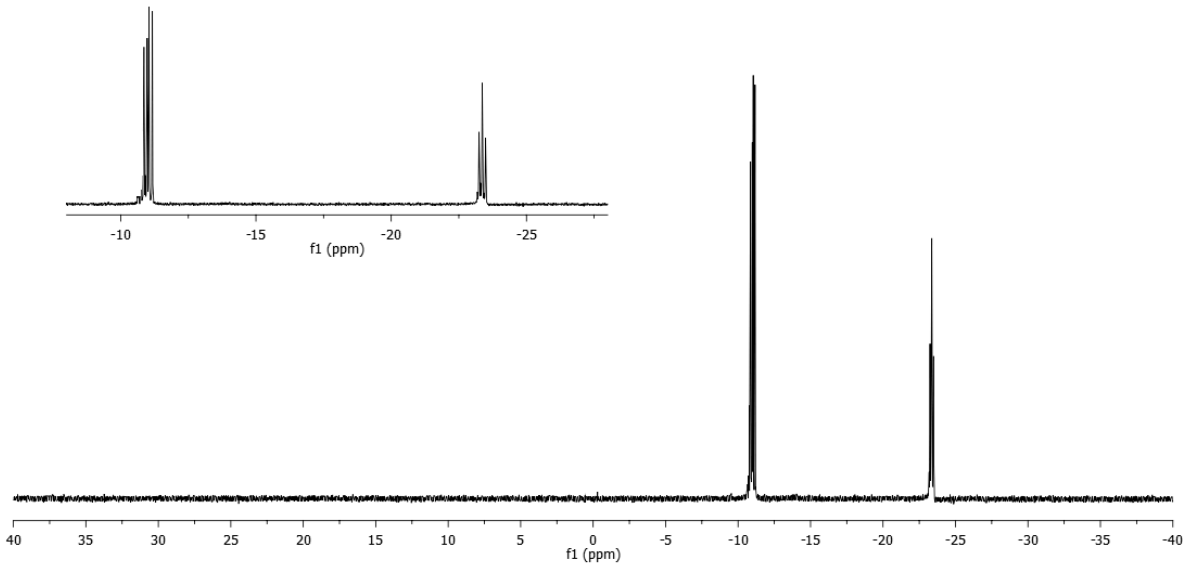


Figure S48: 162 MHz ^{31}P NMR spectra (D_2O) of **17**

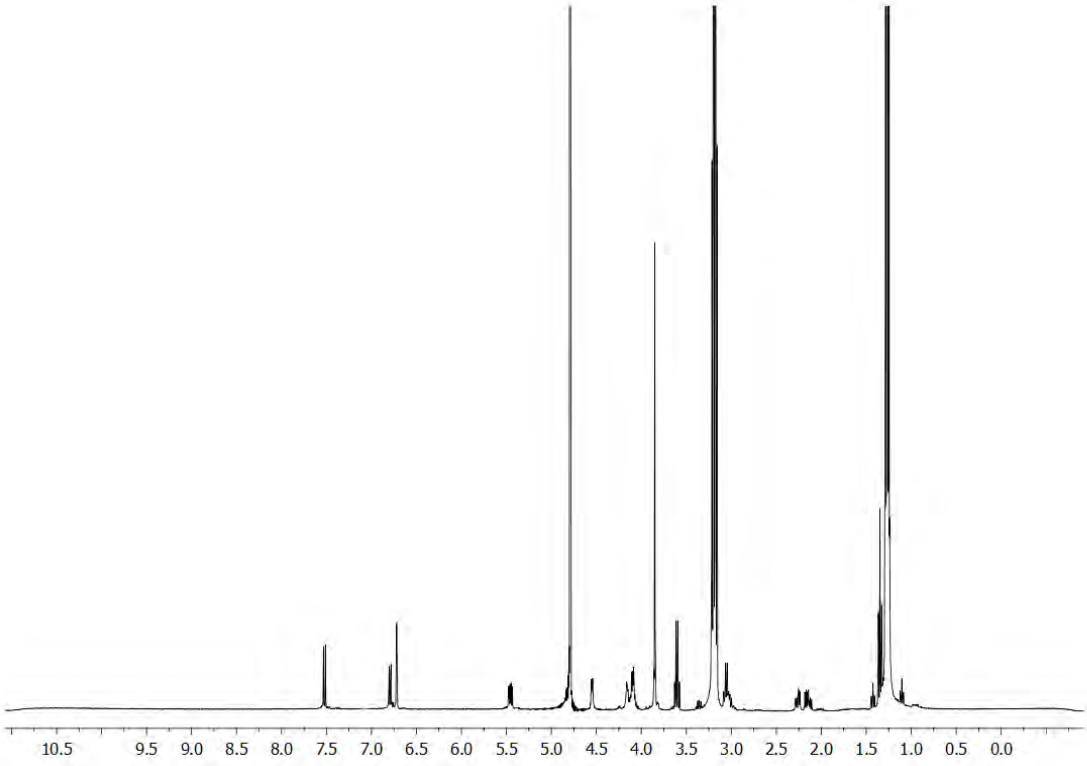


Figure S49: 400 MHz ^{1}H NMR spectra (D_2O) of **17**

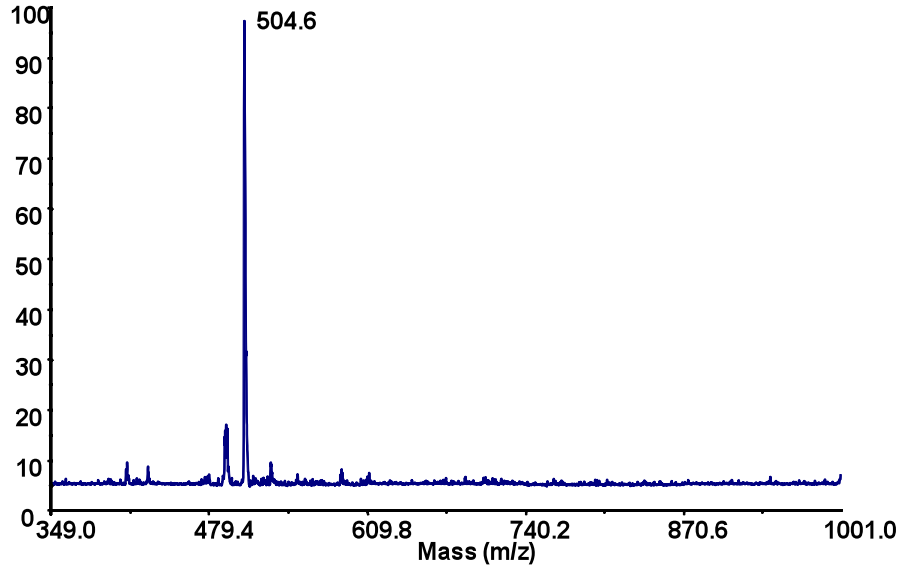


Figure S50: MALDI-TOF MS analysis of **17**

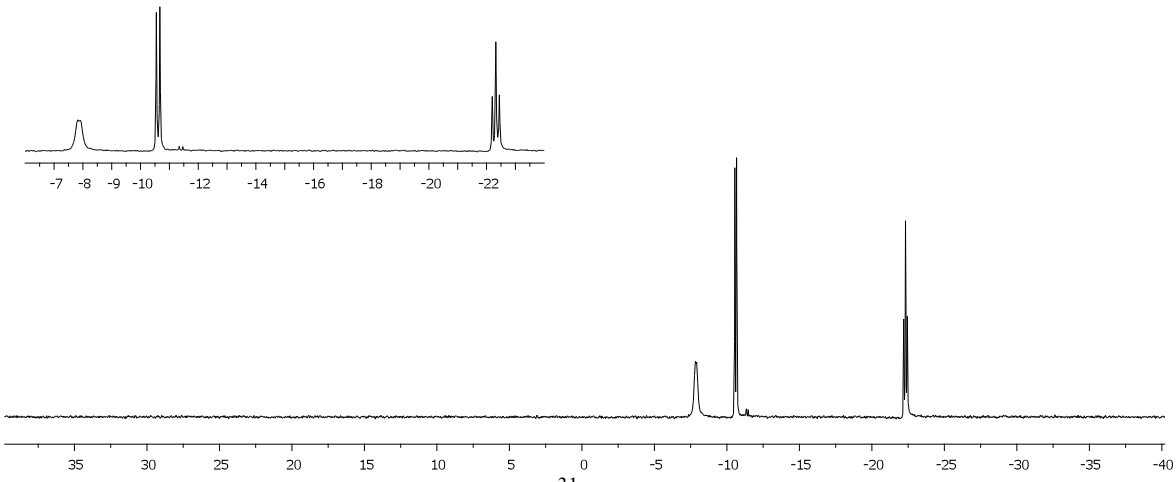


Figure S51: 162 MHz ^{31}P NMR spectra (D_2O) of **35**

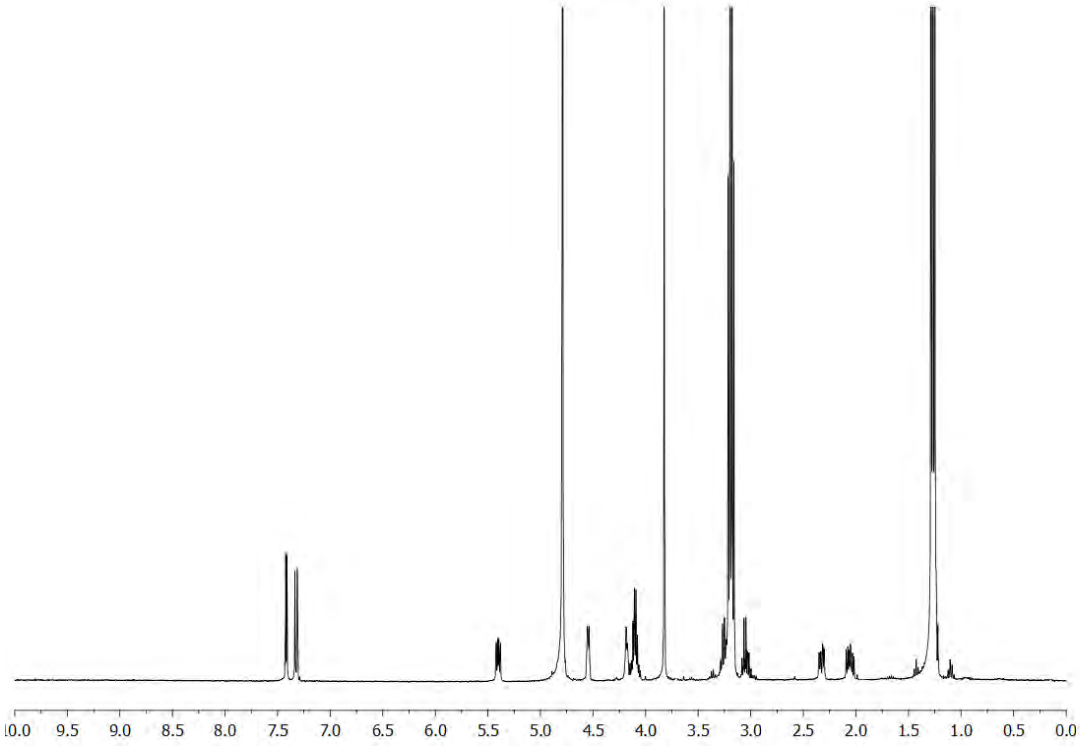


Figure S52: 400 MHz ^{1}H NMR spectra (D_2O) of **35**

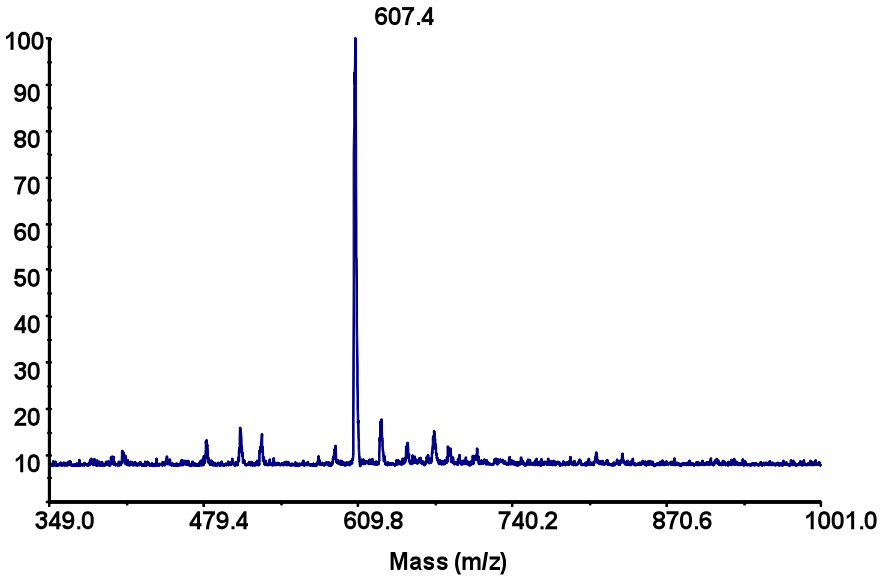


Figure S53: MALDI-TOF MS analysis of **35**

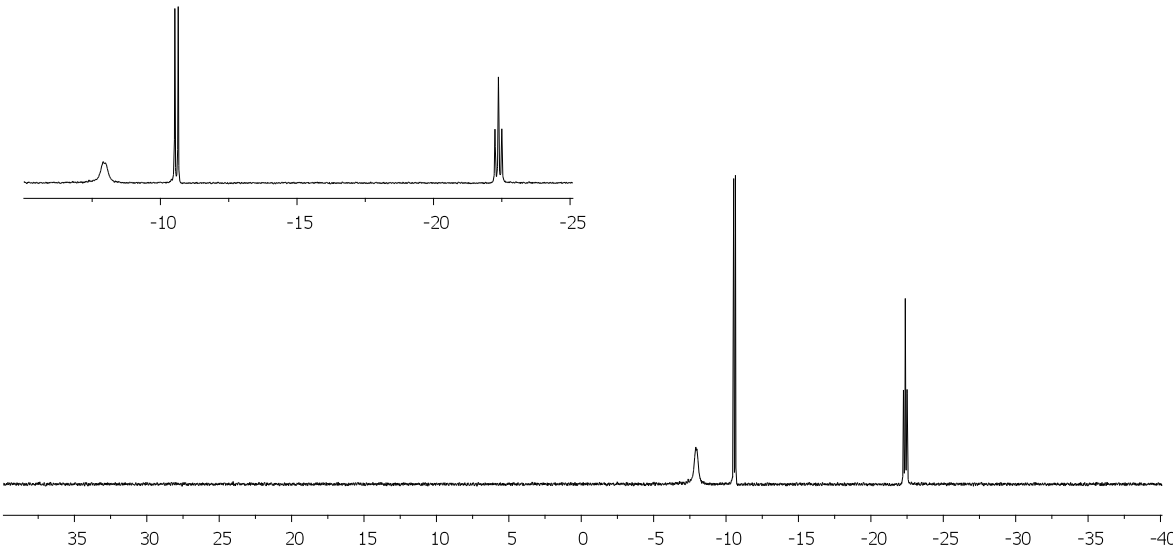


Figure S54: 162 MHz ^{31}P NMR spectra (D_2O) of **36**

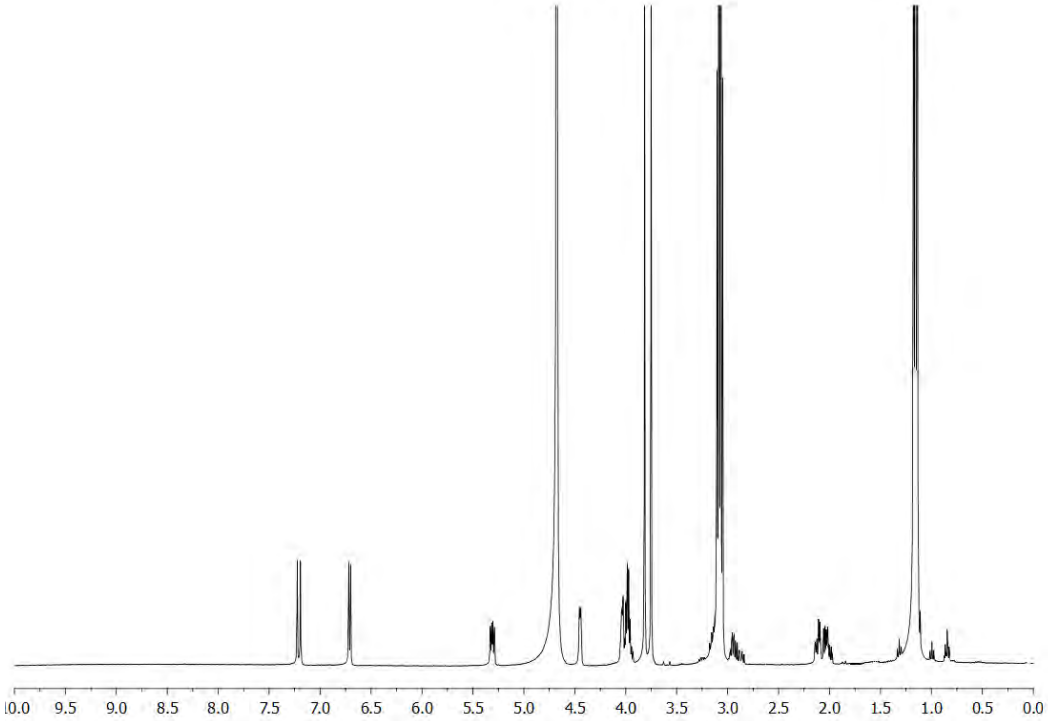


Figure S55: 400 MHz ^{1}H NMR spectra (D_2O) of **36**

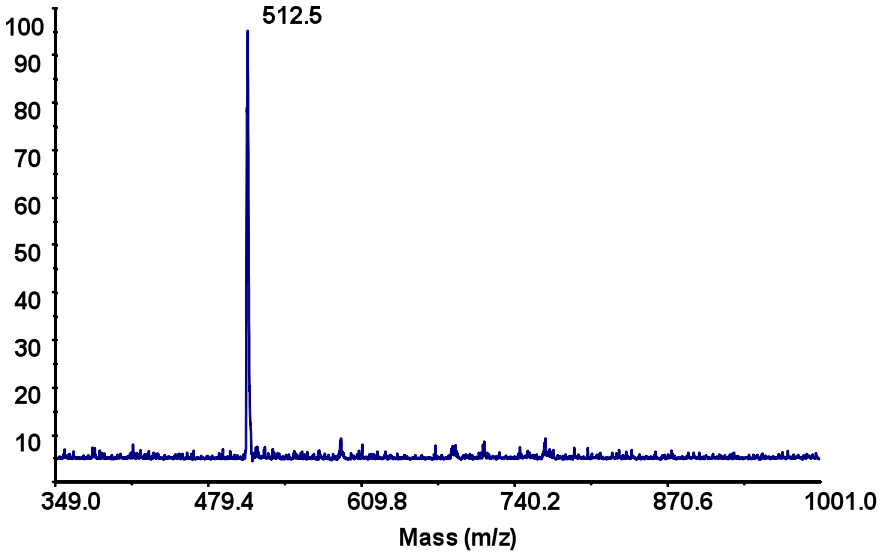


Figure S56: MALDI-TOF MS analysis of **36**

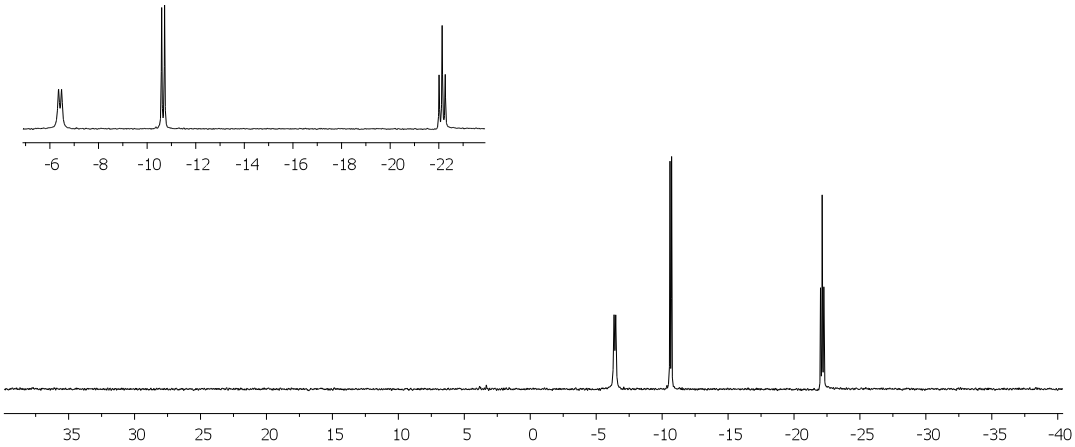


Figure S57: 162 MHz ^{31}P NMR spectra (D_2O) of **39**

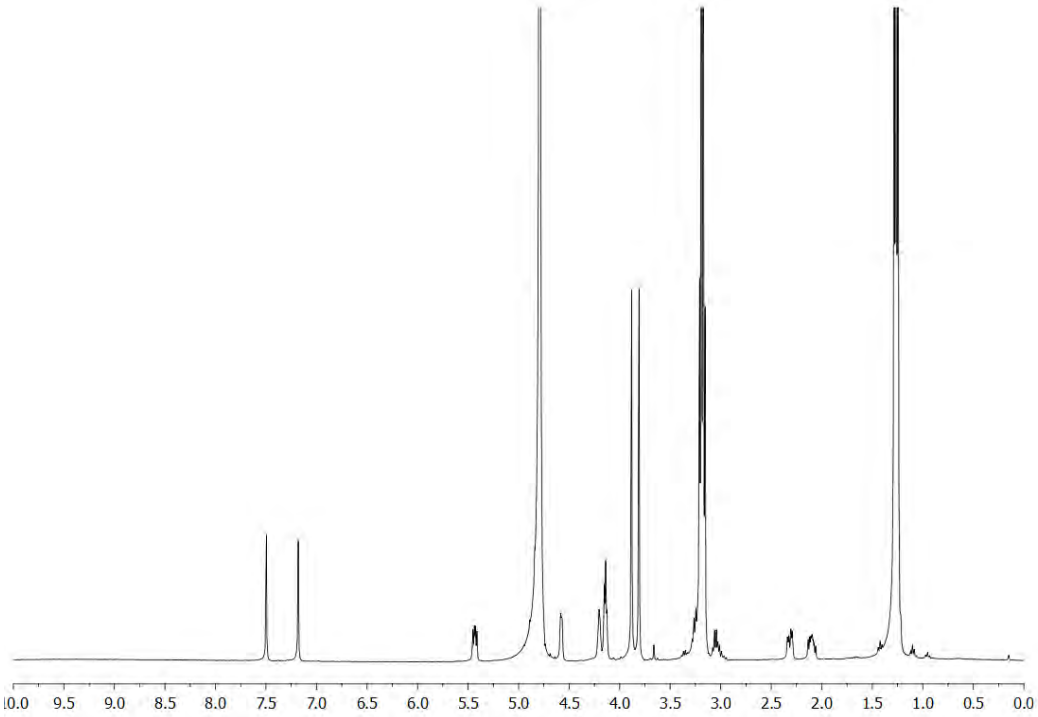


Figure S58: 400 MHz ^{1}H NMR spectra (D_2O) of **39**

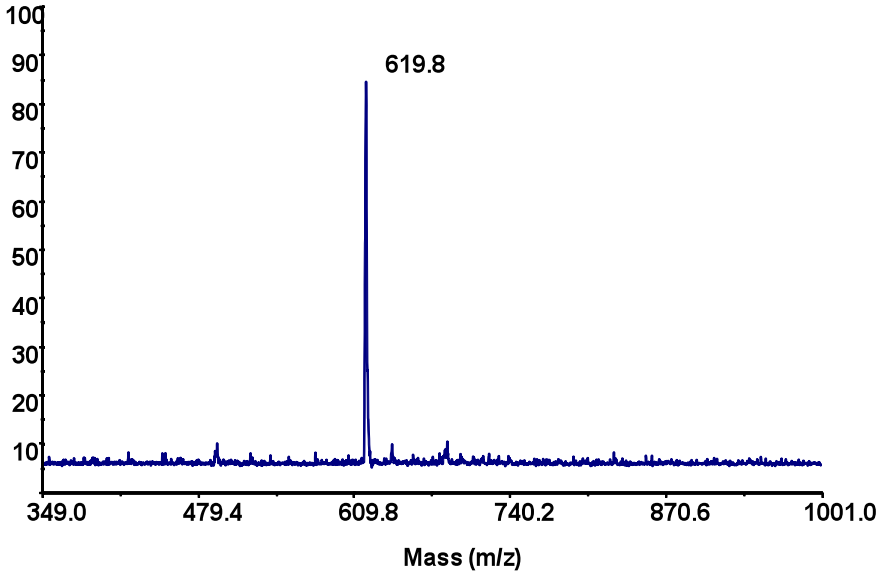


Figure S59: MALDI-TOF MS analysis of **39**

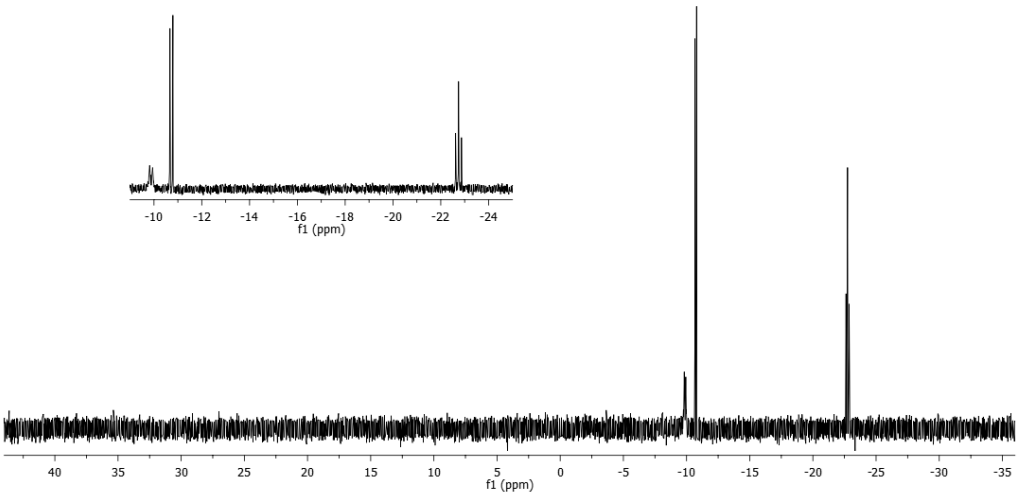


Figure S60: 162 MHz ^{31}P NMR spectra (D_2O) of **18**

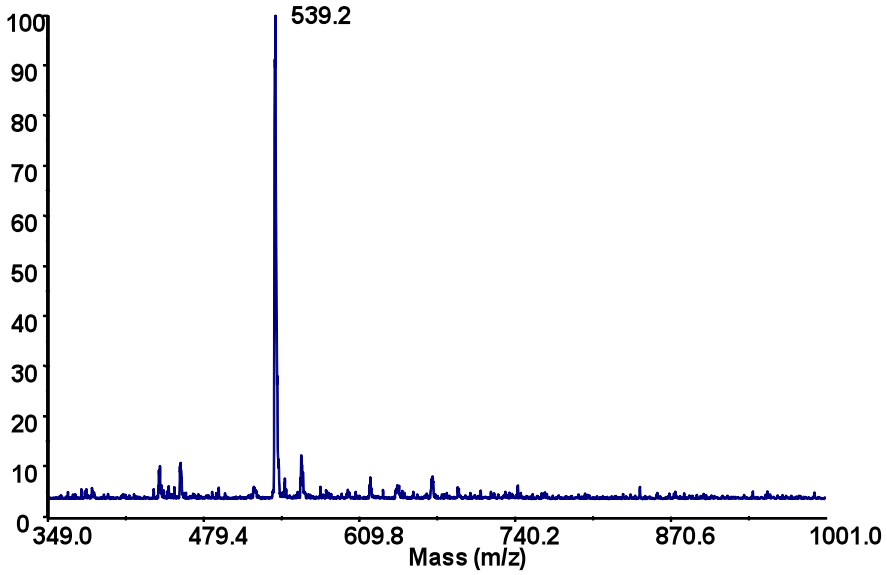


Figure S61: MALDI-TOF MS analysis of **18**

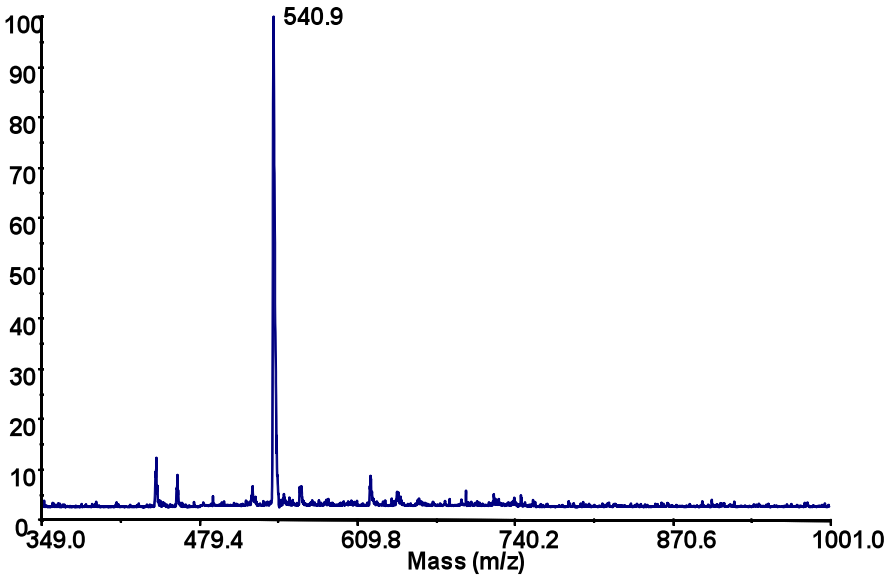


Figure S62: MALDI-TOF MS analysis of **19**

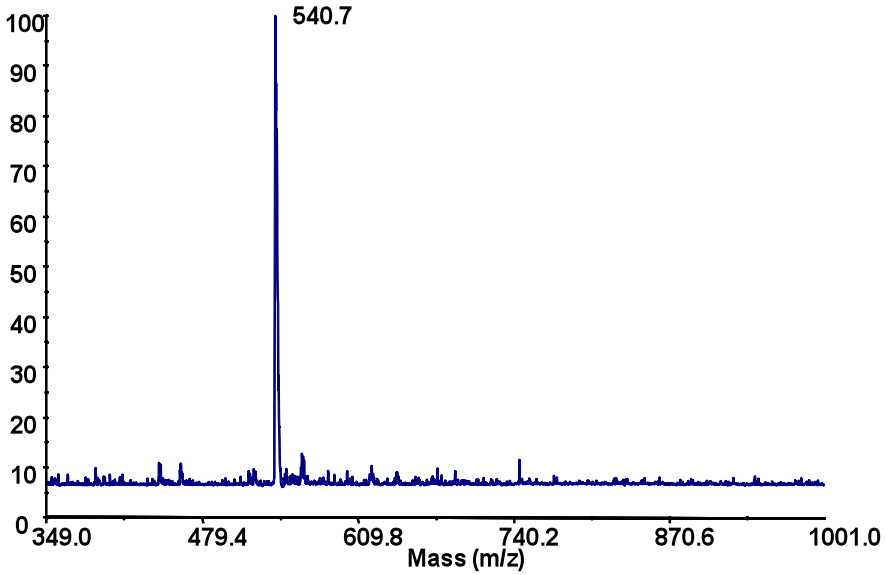


Figure S63: MALDI-TOF MS analysis of **20**

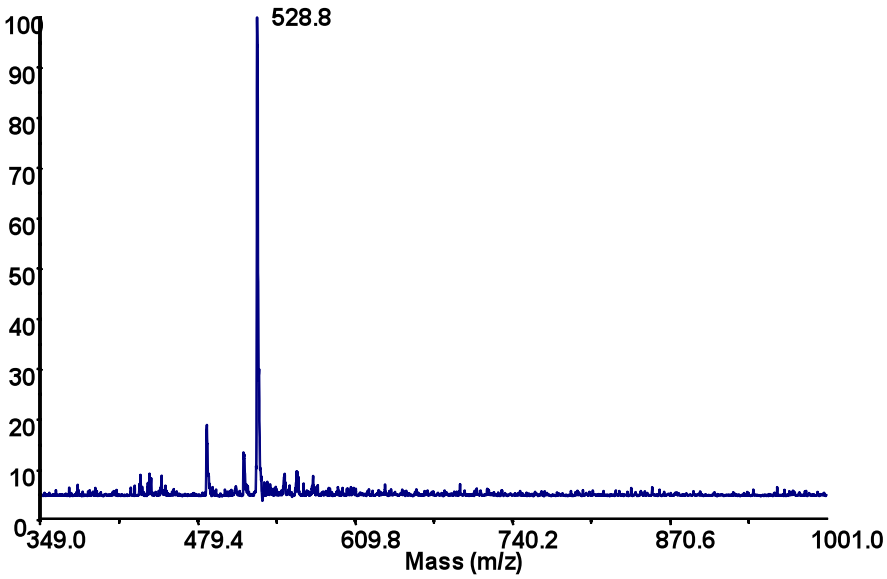


Figure S64: MALDI-TOF MS analysis of **21**

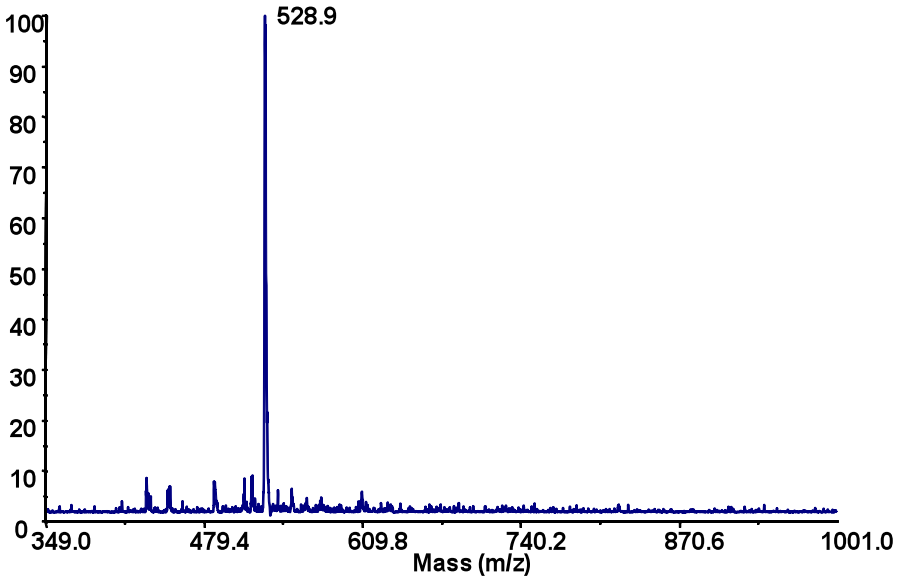


Figure S65: MALDI-TOF MS analysis of **22**

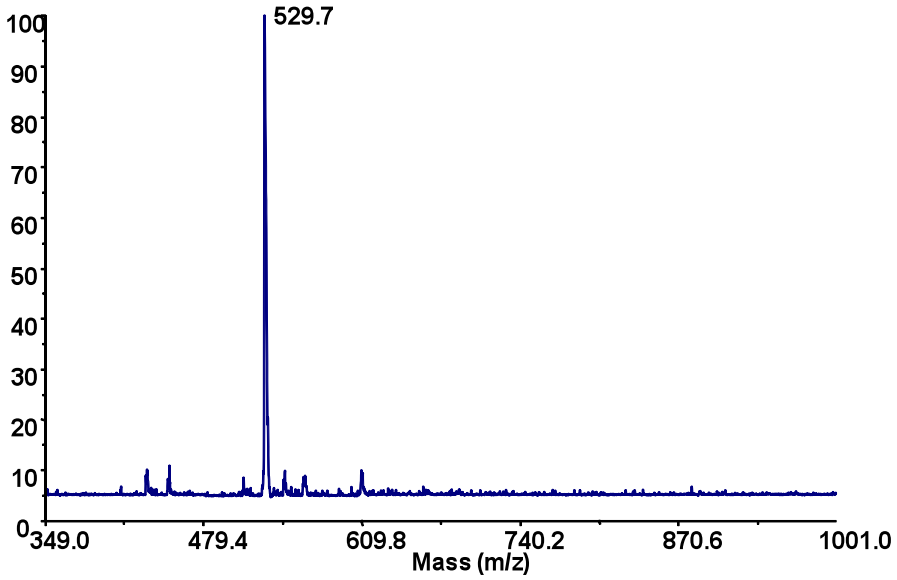


Figure S66: MALDI-TOF MS analysis of **23**

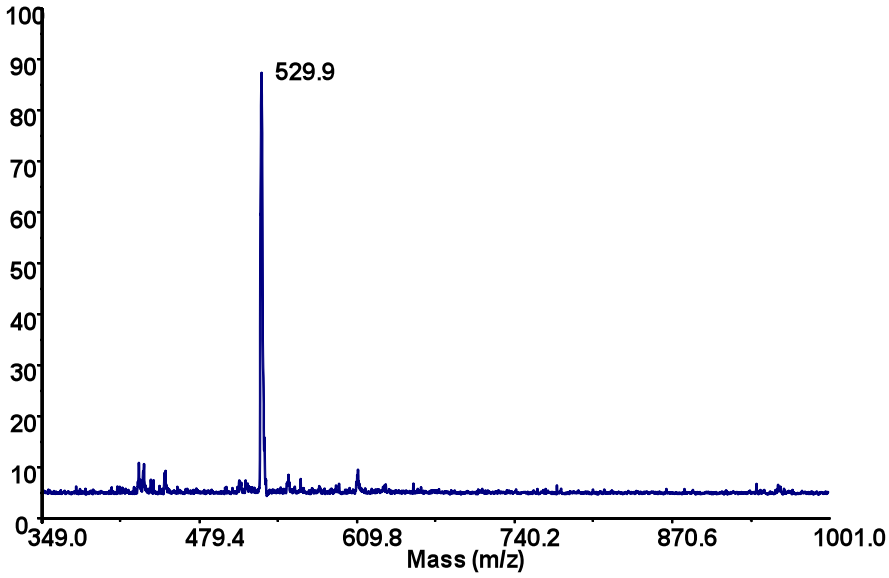


Figure S67: MALDI-TOF MS analysis of **24**

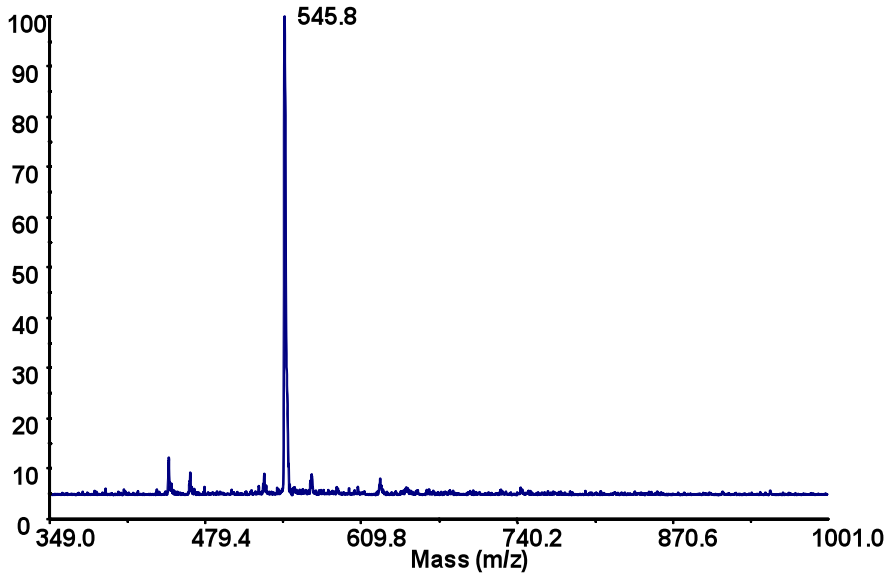


Figure S68: MALDI-TOF MS analysis of **25**

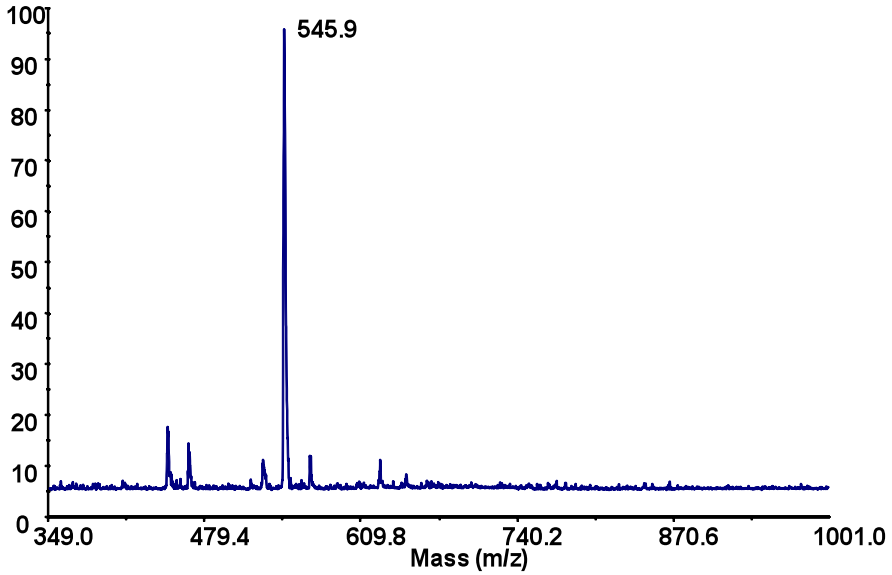


Figure S69: MALDI-TOF MS analysis of **26**

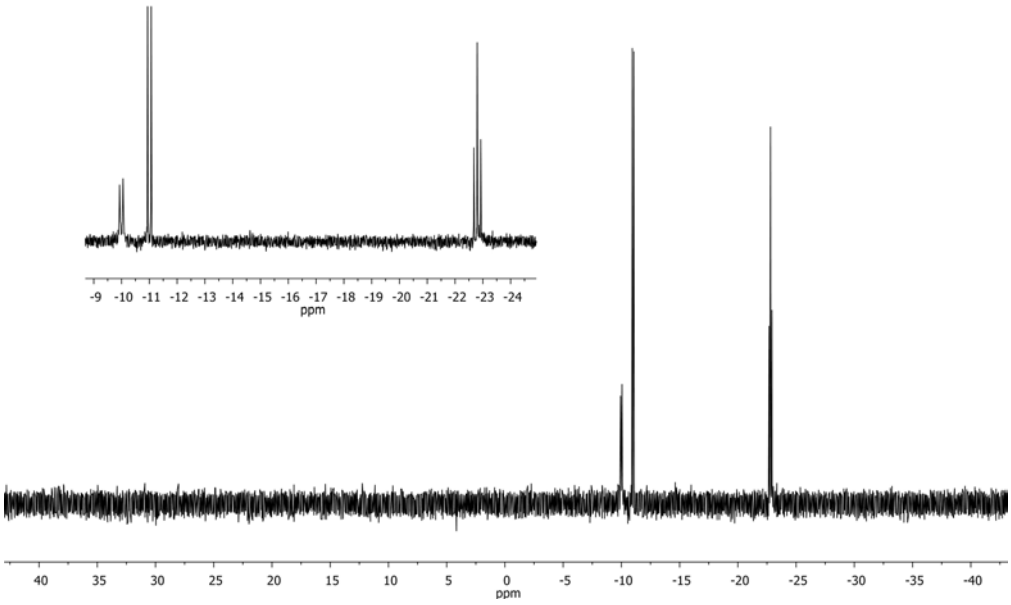


Figure S70: 162 MHz ^{31}P NMR spectra (D_2O) of **27**

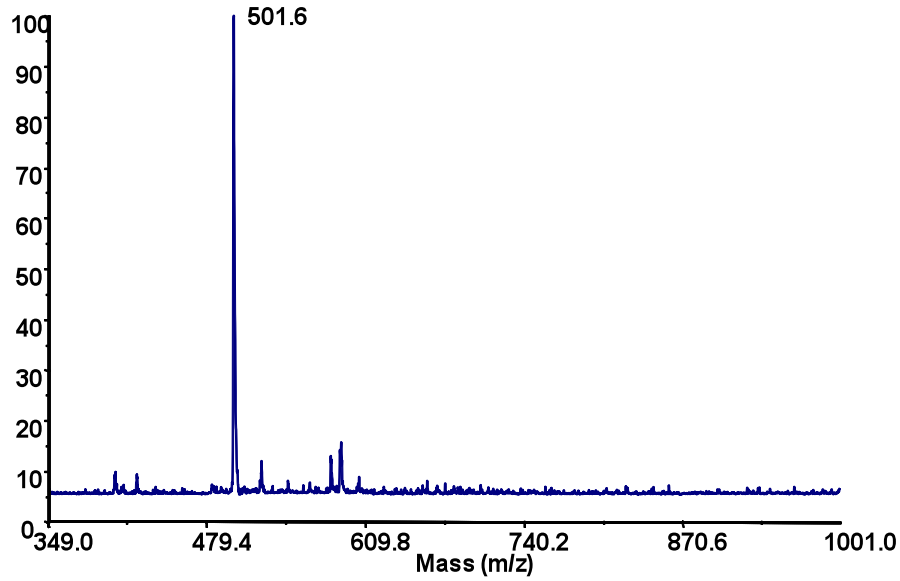


Figure S71: MALDI-TOF MS analysis of **27**

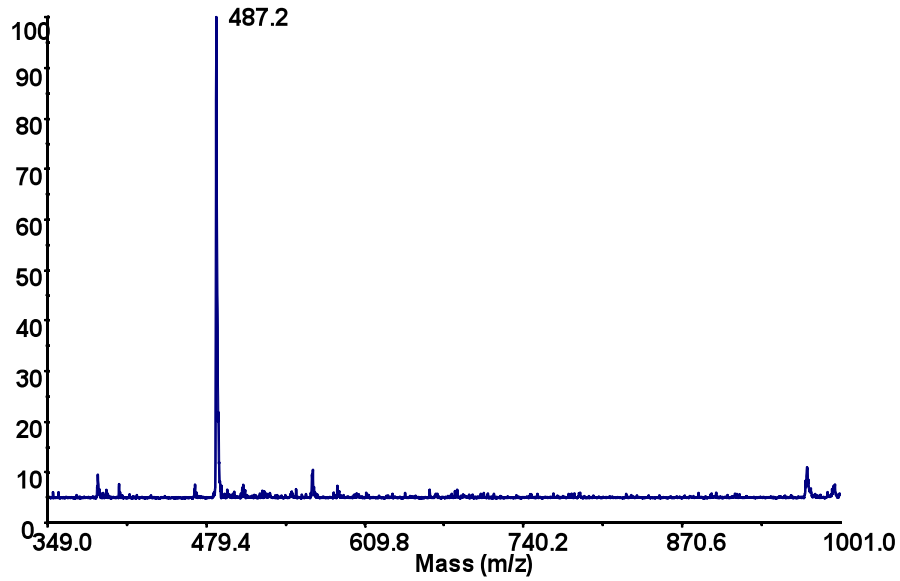


Figure S72: MALDI-TOF MS analysis of **28**

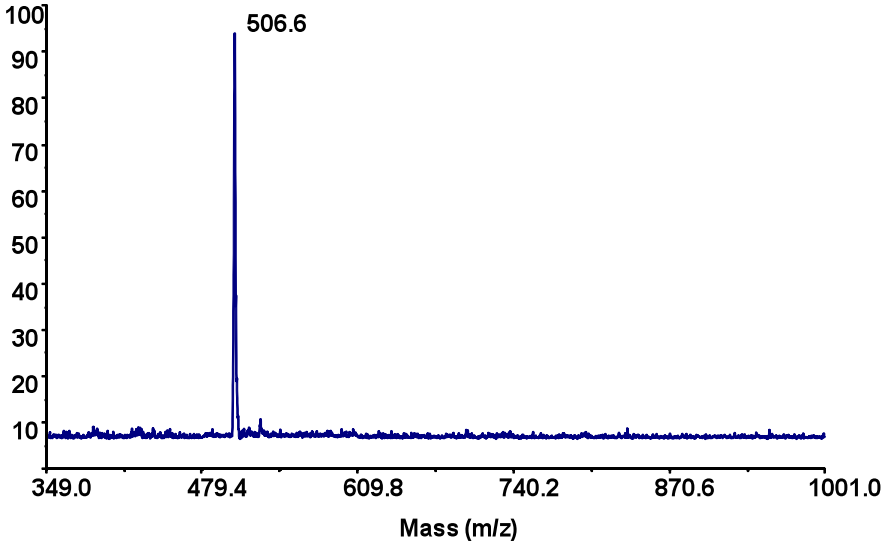


Figure S73: MALDI-TOF MS analysis of **37**

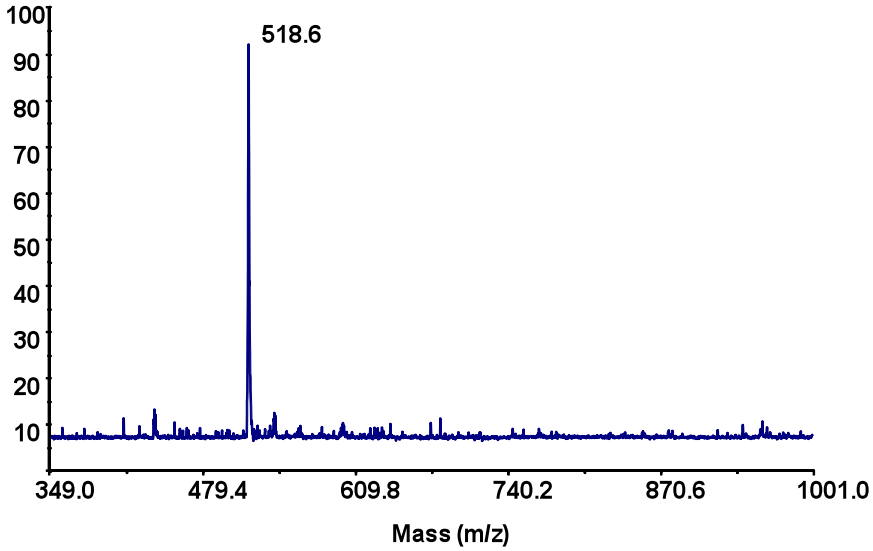


Figure S74: MALDI-TOF MS analysis of **40**

Table S1. %Incorporation-extension assays 20 μM dXTP, 20 μM dCTP, 10 second reaction times.[a]

X	%Incorporation	%Extension
MMO2	72.9	86.3
NaM	77.1	84.4
DMO	72.9	87.7
NMO1	72.1	41.7
PMO1	71.0	14.1
5FM	72.1	87.5
IMO	74.0	86.5
ClMO	74.0	87.8
QMO	72.9	47.9
CNMO	72.9	87.7
TfMO	72.9	69.9
VMO	70.0	87.1
ZMO	74.0	87.8
PrMO	72.9	89.0
EMO	72.9	89.0
PhMO	67.1	7.5
PyMO1	65.0	12.8
PyMO2	72.9	16.4
TpMO1	71.0	9.9
TpMO2	72.1	12.5
FuMO1	71.0	26.8
FuMO2	71.0	22.5
PMO2	70.0	17.1
PMO3	62.9	44.4
FlMO	74.0	86.5
FDMO	62.9	85.7
FEMO	74.0	87.5
MIMO	51.0	9.8
MEMO	52.9	37.7

[a]Incorporation and extension reactions were run with 20 μM d**MMO2** analog and 20 μM dCTP.

Table S2. %Incorporation-extension assays 1 μM dXTP, 1 μM dCTP, 10 second reaction times.[a]

X	%Incorporation	%Extension
MMO2	27.3 ± 1.7	49.8 ± 0.4
NaM	68.8 ± 1.7	33.2 ± 0.8
DMO	39.6 ± 2.5	68.4 ± 1.8
NMO1	30.6 ± 0.4	4.9 ± 0.7
PMO1	31.0 ± 3.5	3.8 ± 0.8
5FM	24.0 ± 1.7	71.4 ± 0.7
IMO	65.4 ± 1.3	19.8 ± 1.2
ClMO	55.4 ± 2.9	49.3 ± 1.2
QMO	57.1 ± 1.7	7.4 ± 0.9
CNMO	60.6 ± 1.7	33.9 ± 3.5
TfMO	43.8 ± 0.4	11.0 ± 0.7
VMO	23.3 ± 2.1	33.5 ± 0.6
ZMO	55.4 ± 3.8	53.0 ± 0.4
PrMO	47.9 ± 1.7	32.9 ± 2.4
EMO	55.0 ± 0.0	56.1 ± 1.0
PhMO	16.7 ± 1.3	2.5 ± 0.8
PyMO1	13.3 ± 1.7	2.1 ± 0.9
PyMO2	29.4 ± 1.3	4.4 ± 0.0
TpMO1	40.0 ± 2.5	2.5 ± 0.8
TpMO2	38.8 ± 1.7	2.9 ± 0.0
FuMO1	30.4 ± 1.7	4.4 ± 0.0
FuMO2	38.8 ± 2.9	3.9 ± 0.8
PMO2	17.9 ± 0.8	4.4 ± 0.0
PMO3	11.3 ± 1.3	6.6 ± 0.1
FIMO	68.1 ± 1.5	25.2 ± 0.2
FDMO	15.6 ± 1.3	29.1 ± 0.9
FEMO	67.7 ± 0.4	70.3 ± 2.5
MIMO	11.0 ± 0.2	6.7 ± 1.2
MEMO	10.4 ± 0.4	8.6 ± 0.1

[a]Incorporation reactions were run with 1 μM d**MMO2** analog and 1 μM dCTP. Extension reactions were run with 20 μM d**MMO2** analog and 1 μM dCTP.

Table S3. %Incorporation-extension assays 1 μM dXTP, 1 μM dCTP, 5 second reaction times.[a]

X	%Incorporation	%Extension
MMO2	16.7 ± 0.5	30.2 ± 1.9
NaM	64.0 ± 0.8	22.3 ± 1.2
DMO	21.7 ± 3.6	51.9 ± 1.1
5FM	15.0 ± 0.0	51.8 ± 1.2
IMO	51.7 ± 1.3	13.8 ± 1.9
ClMO	39.0 ± 0.0	33.3 ± 0.5
CNMO	41.4 ± 1.7	21.8 ± 1.8
ZMO	39.7 ± 0.5	35.7 ± 0.5
PrMO	34.7 ± 1.3	20.1 ± 2.0
EMO	37.3 ± 1.3	39.1 ± 0.3
FIMO	61.0 ± 2.5	17.2 ± 1.1
FEMO	56.7 ± 0.9	51.9 ± 2.5

[a]Incorporation reactions were run with 1 μM d**MMO2** analog and 1 μM dCTP. Extension reactions were run with 20 μM d**MMO2** analog and 1 μM dCTP.

Table S4. %Incorporation-extension assays 0.2 μM d**X**TP, 0.5 μM dCTP, 10 second reaction times[a]

X	%Incorporation	%Extension
MMO2	10.4 ± 0.4	34.7 ± 2.2
NaM	46.3 ± 0.4	21.5 ± 1.3
DMO	13.3 ± 0.4	52.4 ± 2.3
5FM	8.3 ± 1.0	53.5 ± 0.5
IMO	22.7 ± 1.3	12.5 ± 2.2
ClMO	17.7 ± 0.4	34.4 ± 1.9
CNMO	17.9 ± 0.8	24.5 ± 2.4
ZMO	12.3 ± 0.4	36.2 ± 2.5
PrMO	14.0 ± 0.0	20.7 ± 1.5
EMO	17.1 ± 0.8	38.7 ± 0.9
FIMO	34.4 ± 0.4	16.7 ± 0.9
FEMO	31.3 ± 0.4	51.0 ± 0.8

[a]Incorporation reactions were run with 0.2 μM d**MMO2** analog and 0.5 μM dCTP. Extension reactions were run with 20 μM d**MMO2** analog and 0.5 μM dCTP.

PCR amplification: determination of fidelity

The retention of an unnatural base pair (*f*, percent retention of an unnatural base pair) was measured using raw sequencing data and normalized to fidelities per doubling, as described.[6,7] Briefly, the presence of an unnatural nucleotide leads to a sharp termination of the sequencing profile, while mutation to a natural nucleotide results in "read-through" (compare sequencing traces of d**NaM**/d**5SICS** and d**MEMO**/d**5SICS** in **Fig. S62**). The extent of the "read-through" is thus inversely correlated with the retention of the unnatural base pair. To use the sequencing data as a quantitative measurement of PCR fidelity, we performed calibration experiments in the range of 50-100% retention of the unnatural base pair (see the Supporting Information and Malyshev, 2009[6] for a complete description). Therefore, low retention (<50%) and high "read-through" make the quantification inaccurate and thus f the threshold for fidelity quantification.

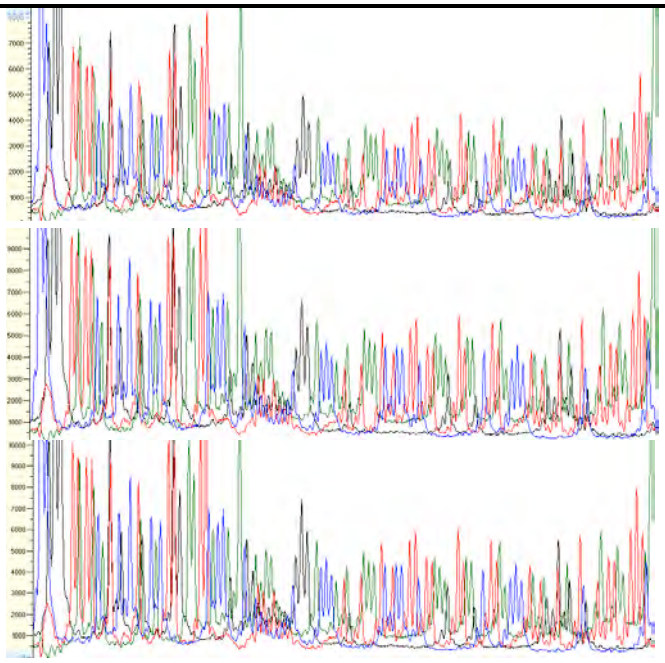
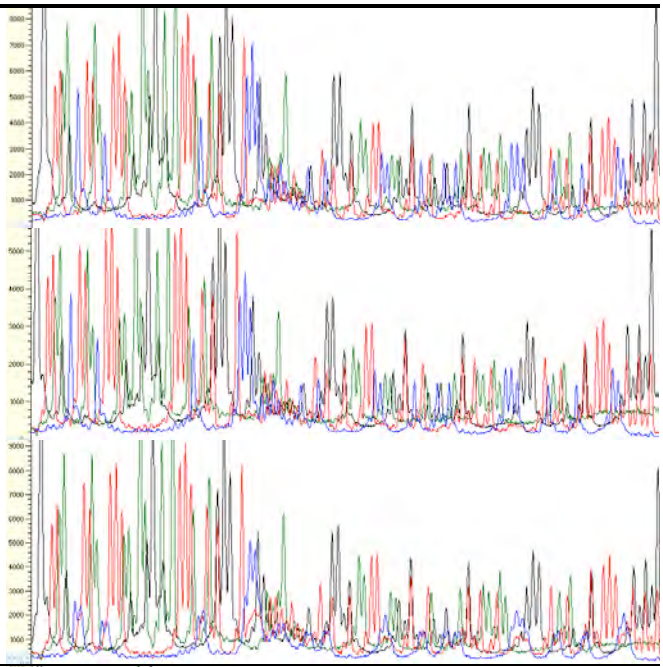
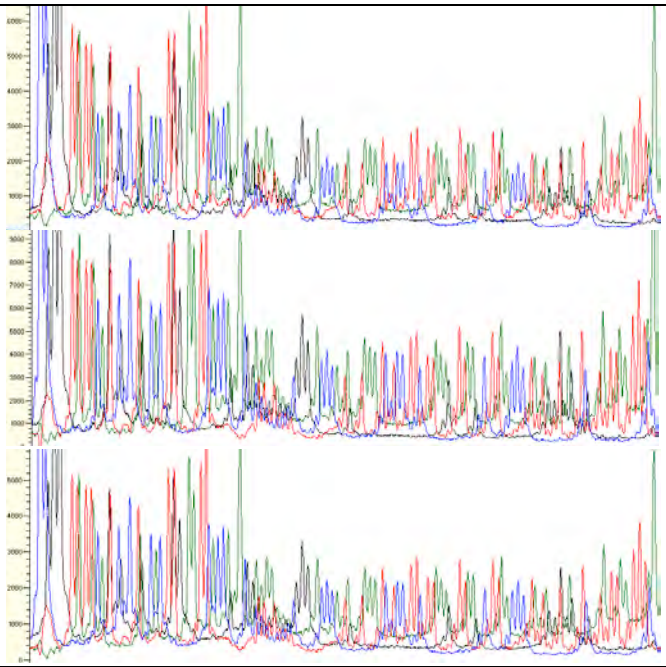
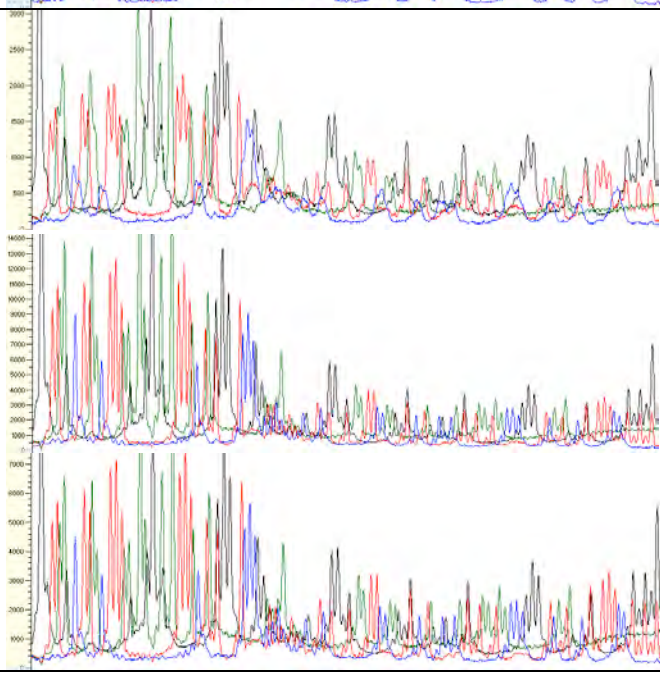
Quantification of the high retention (>50%) was performed by adjusting the start and stop points for the Sequencing Analysis software (Applied Biosystems) and then determining the average signal intensity individually for each channel (A, C, G and T) for peaks within those defined points (35-45 nucleotides in length) before (section L) and after (section R) the unnatural nucleotide. The R/L ratio was normalized using sequencing calibration plots to account for both noise in the sequencing chromatograms and the read-through in the control samples. (see Malyshev, 2009[6] for details). The R/L ratio of after normalization (R/L_{norm}) corresponds to the percentage of the natural sequences in the pool. Finally, the retention of the unnatural base pair (F) was calculated as $1 - (R/L)_{norm}$ and the retention of the unnatural base pair per doubling (fidelity, f) was calculated as $F^{\frac{1}{\log_2 A}}$, where A is an amplification and $\log_2 A$ is the number of doublings. Each sample before and PCR amplification was sequenced in triplicate in each direction to minimize sequencing error (Fig. S62-65).

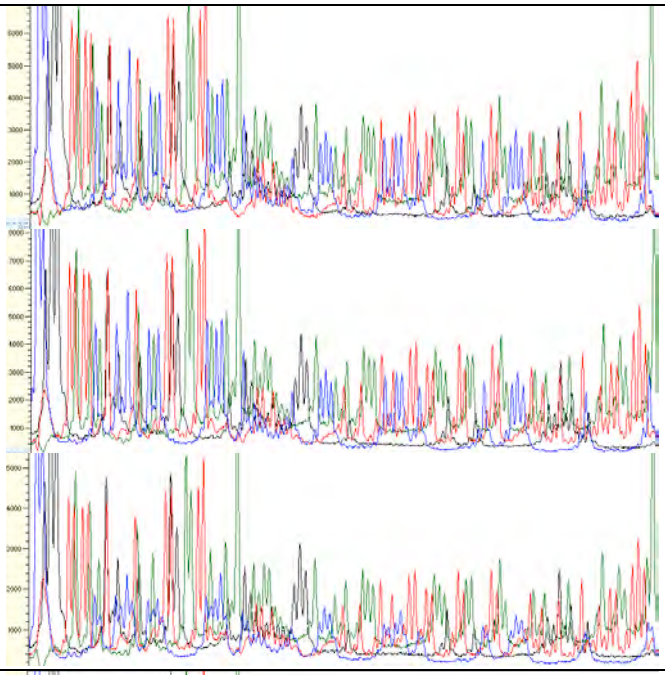
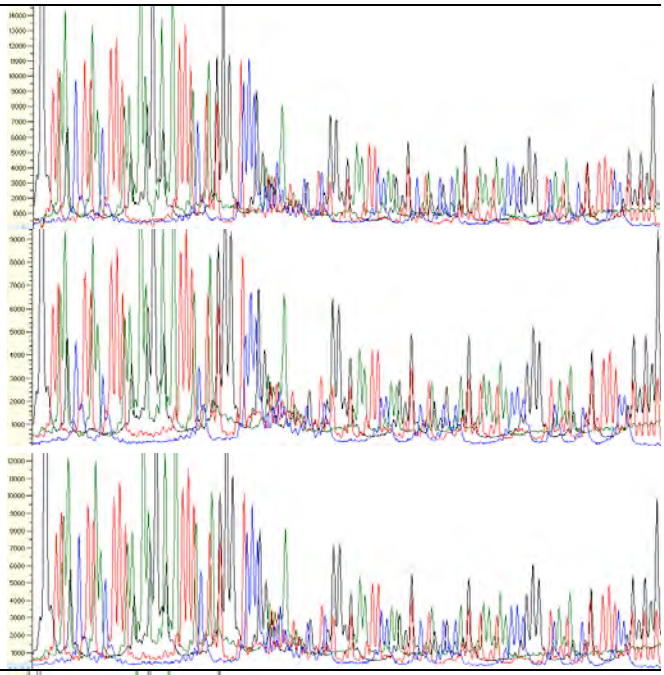
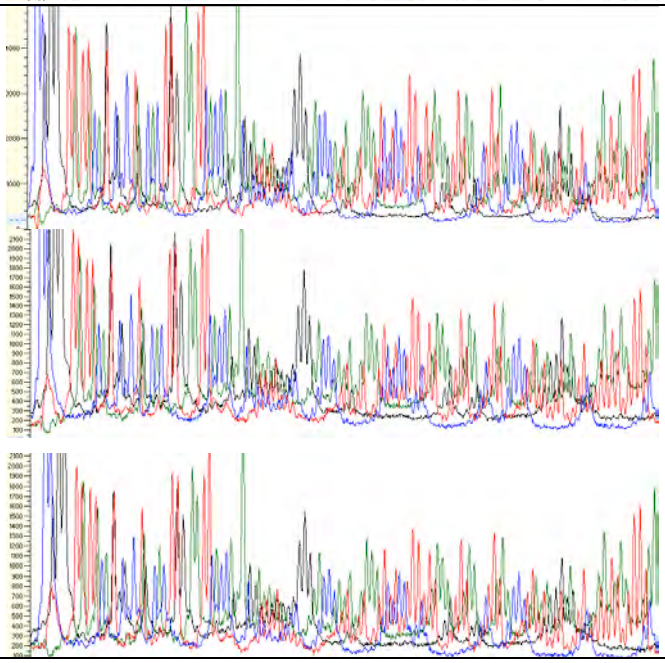
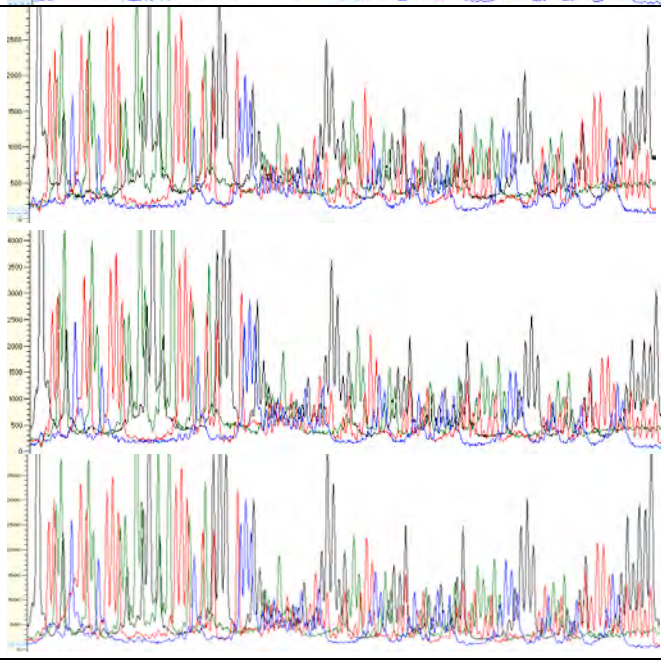
Table S5. PCR conditions

	OneTaq	*Taq*	OneTaq 10^{13} fold	KOD
Buffer	1×OneTaq buffer	1×Taq buffer	1×OneTaq buffer	1× KOD Hot Start DNAP buffer
Enzyme concentration, U/μL	0.02	0.02	0.02	0.02
Template	D6 (0.1 ng)	D6 (0.01 ng)	D6 (0.01 ng)[1]	D6 (0.1 ng)
dNTPs, μM	200	200	200	700
d**5SICS**TP, μM	100	100	100	100
d**X**TP, μM	100	100	100	100
Mg^{2+}, mM	3	3	3	6
Primers, μM	1	1	1	1
Thermal conditions				
Initial denaturing	-	-	-	98°C 30 s
Denaturing	96°C 10 s	96°C 10 s	96°C 10 s	96°C 10 s
Annealing	60°C 15 s	60°C 15 s	60°C 15 s	60°C 15 s
Extension	68°C 60 s	68°C 15 s	68°C 60 s	68°C 60 s
# of cycles	14	17	16+16+16[1]	16

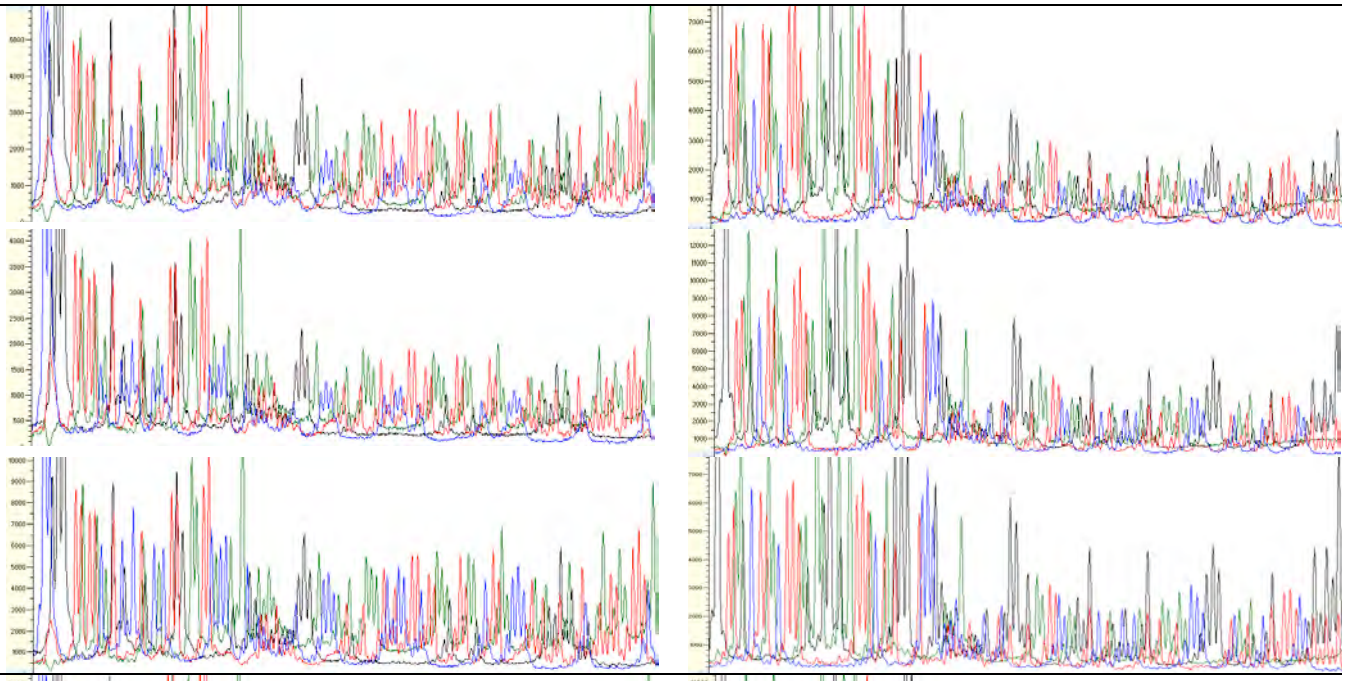
[1] Initial amount of template was 0.01 ng. PCR mixture was amplified over 16 cycles, diluted 40 000 fold and amplified over another 16 cycles. Such dilution/amplification step was repeated resulting in 48 total cycles of amplification.

Figure S75. Raw sequencing data after PCR amplification by OneTaq polymerase in the presence of d**5SICS**TP and d**X**TP. Triplicate data shown.

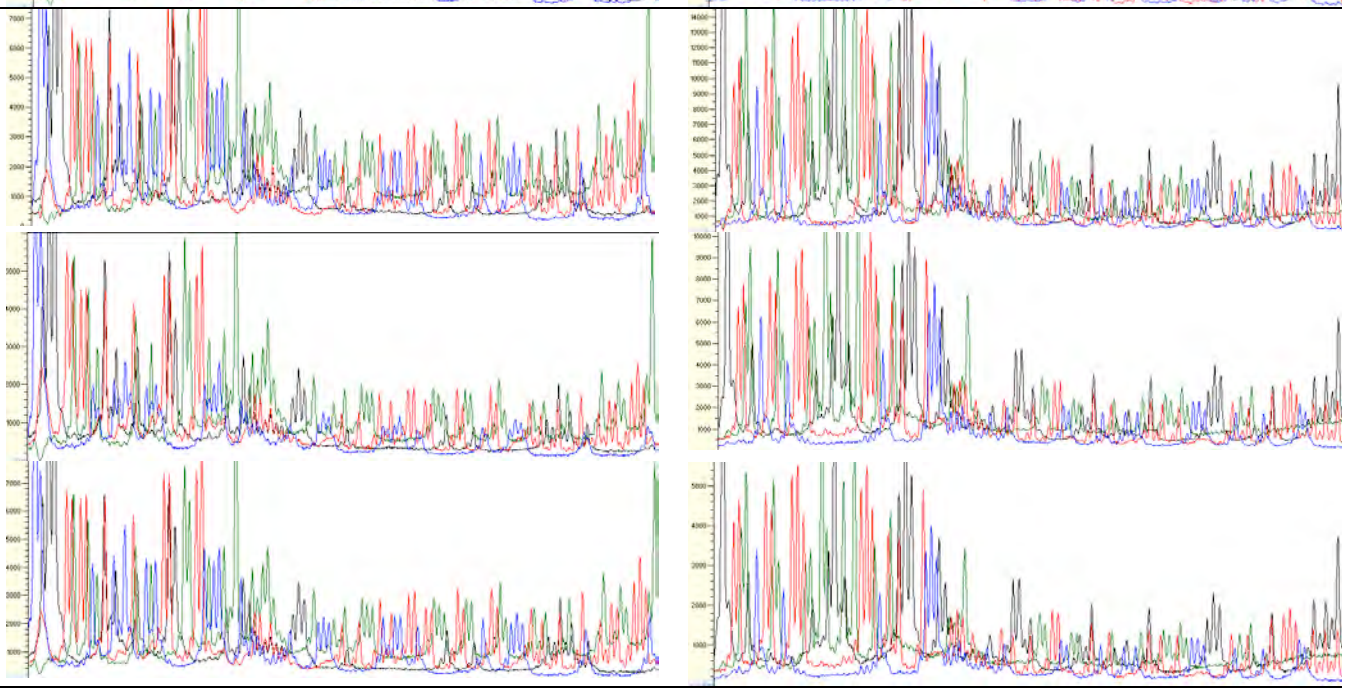
X	Primer 1	Primer 2
PhMO		
PyMO1		

PyMO2		
TpMO1		

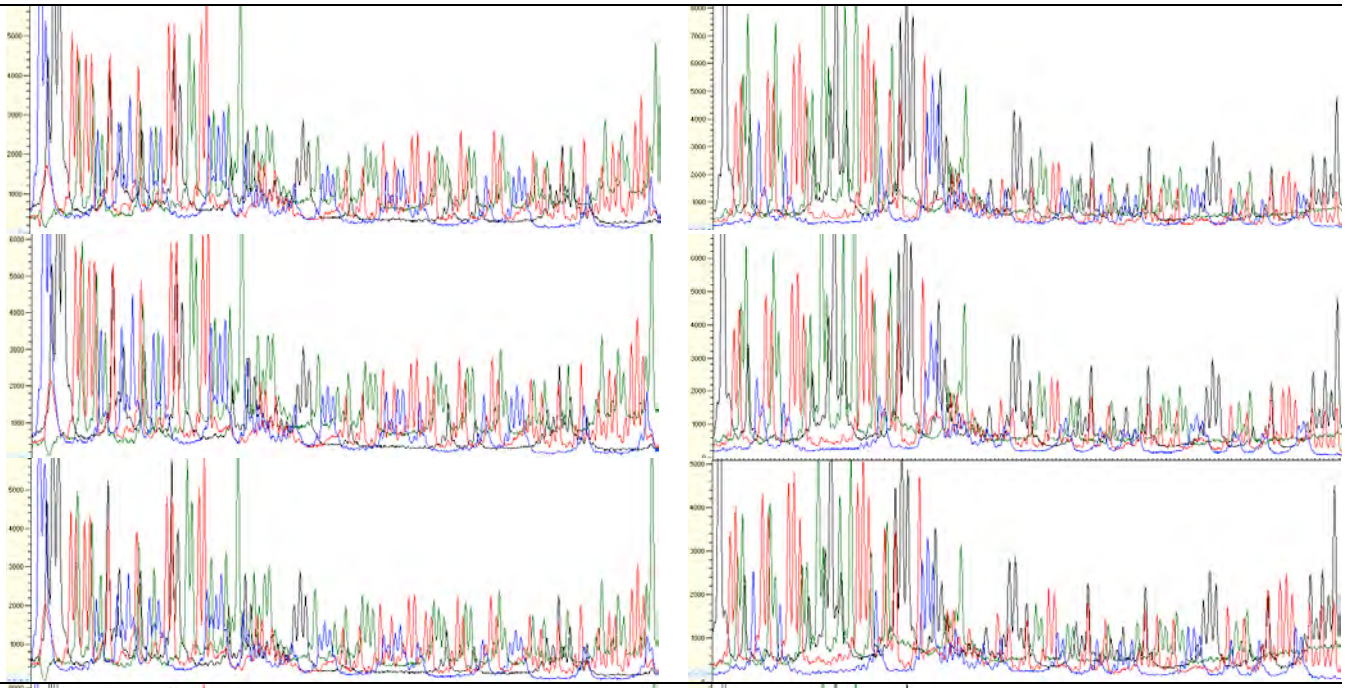
TpMO2



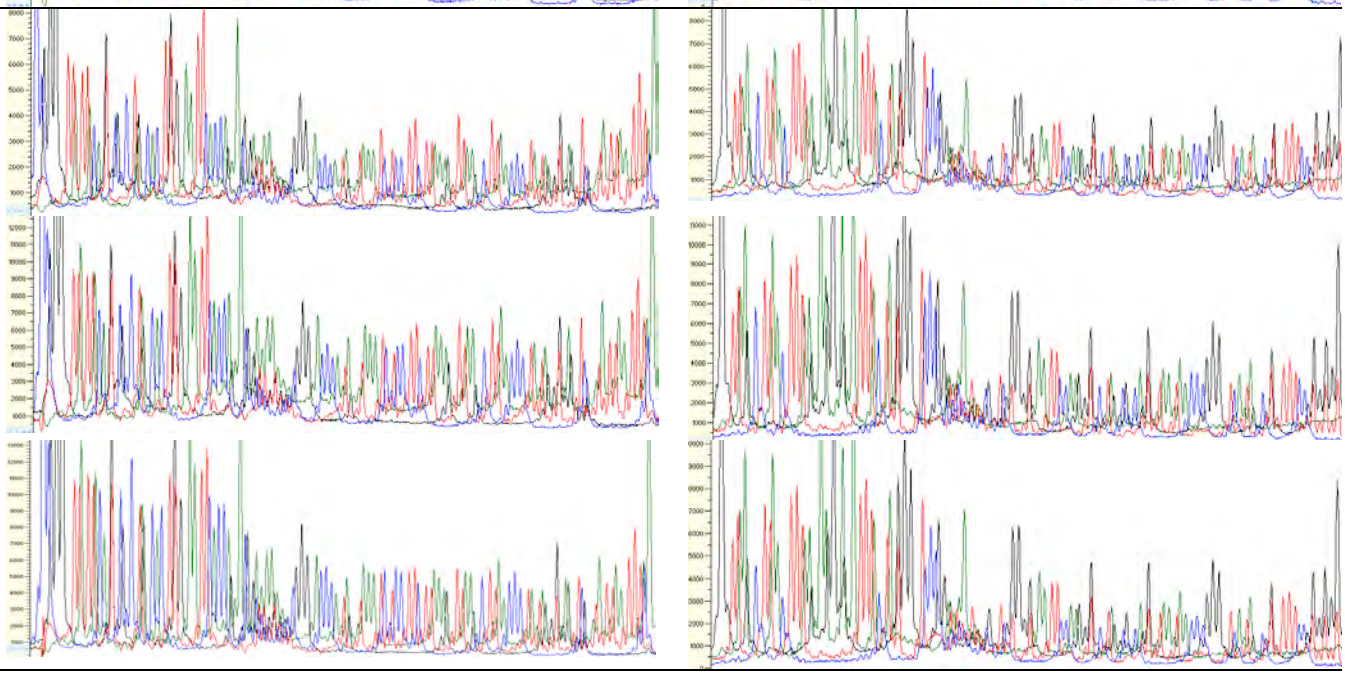
FuMO1

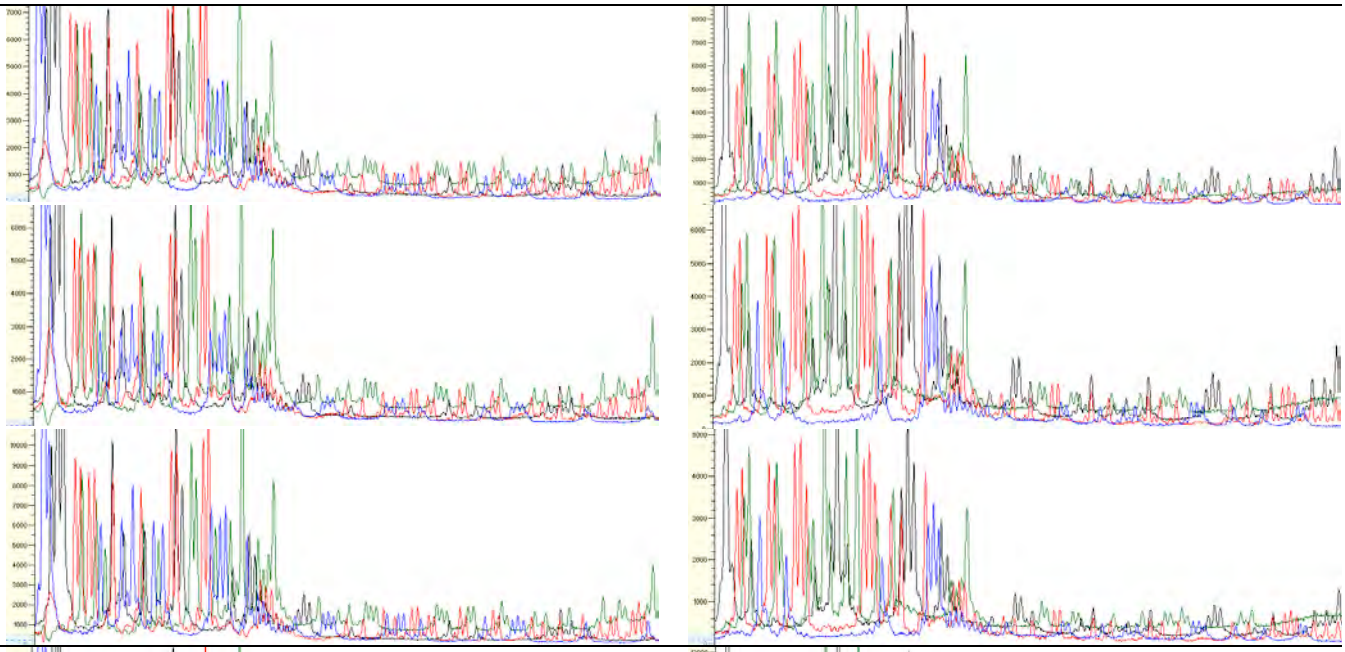
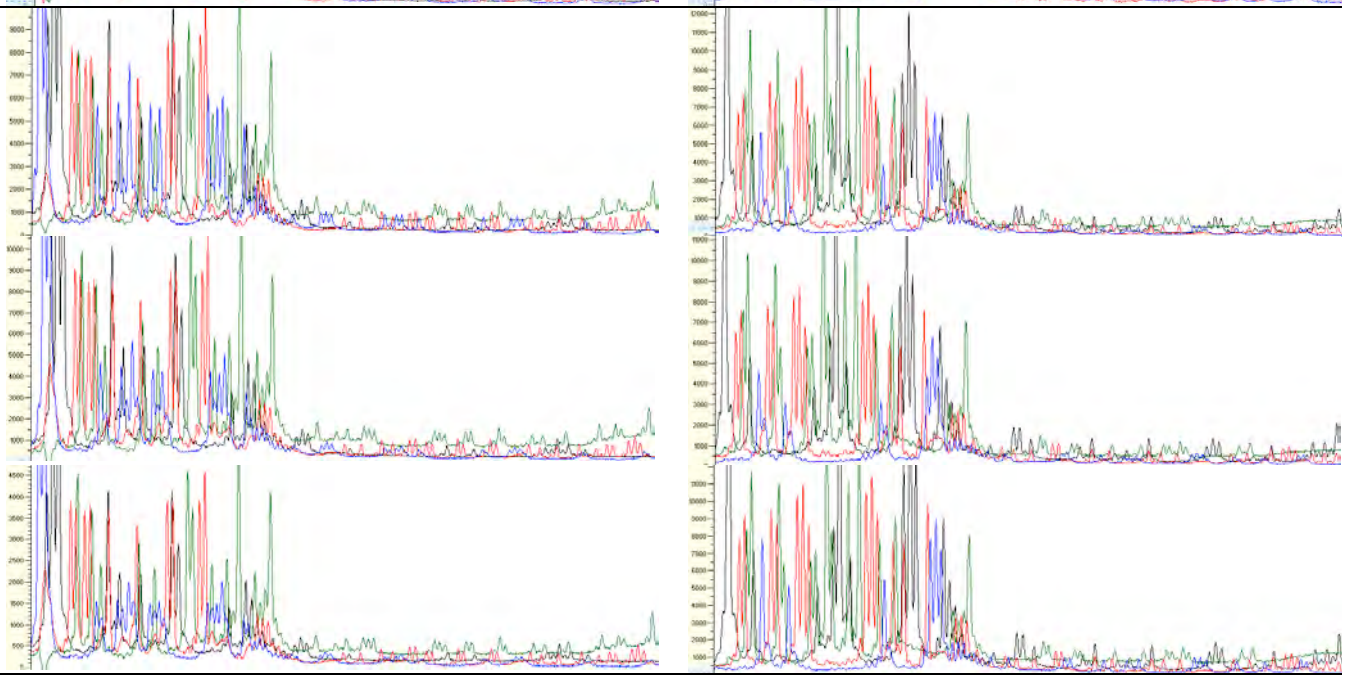


FuMO2

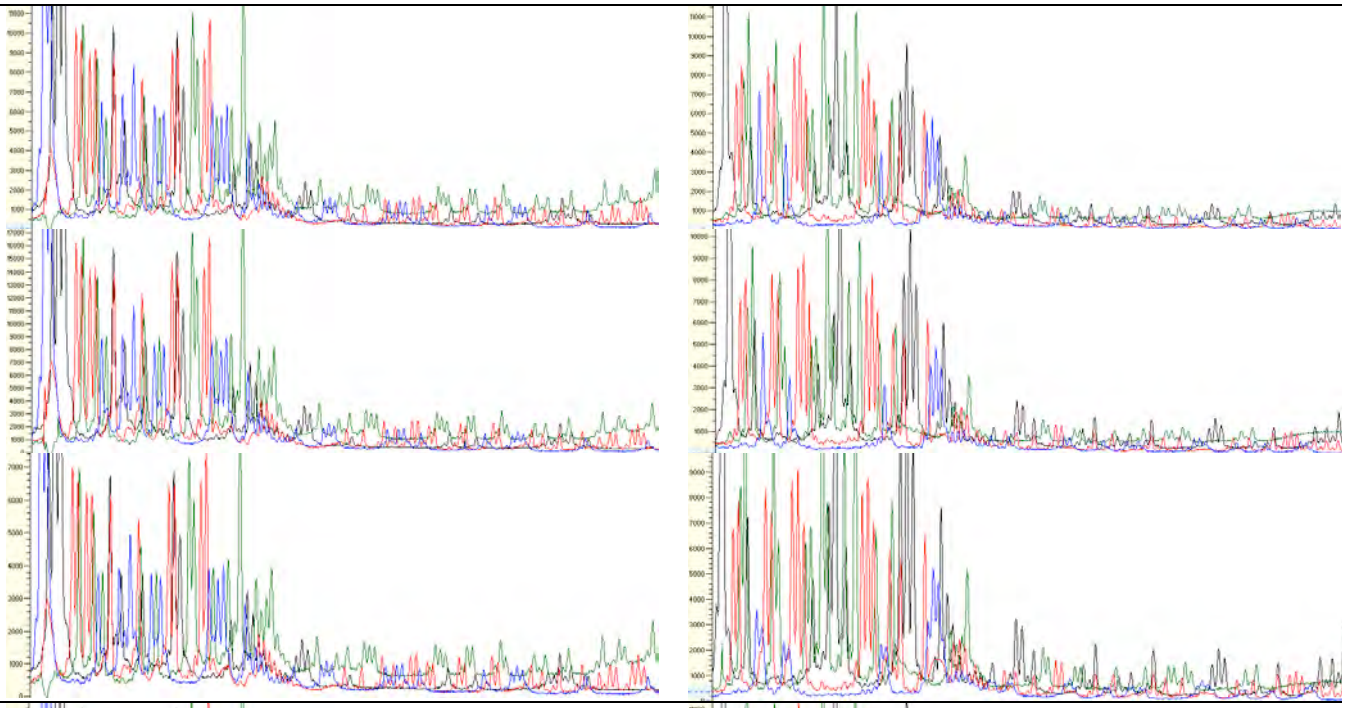


PMO2

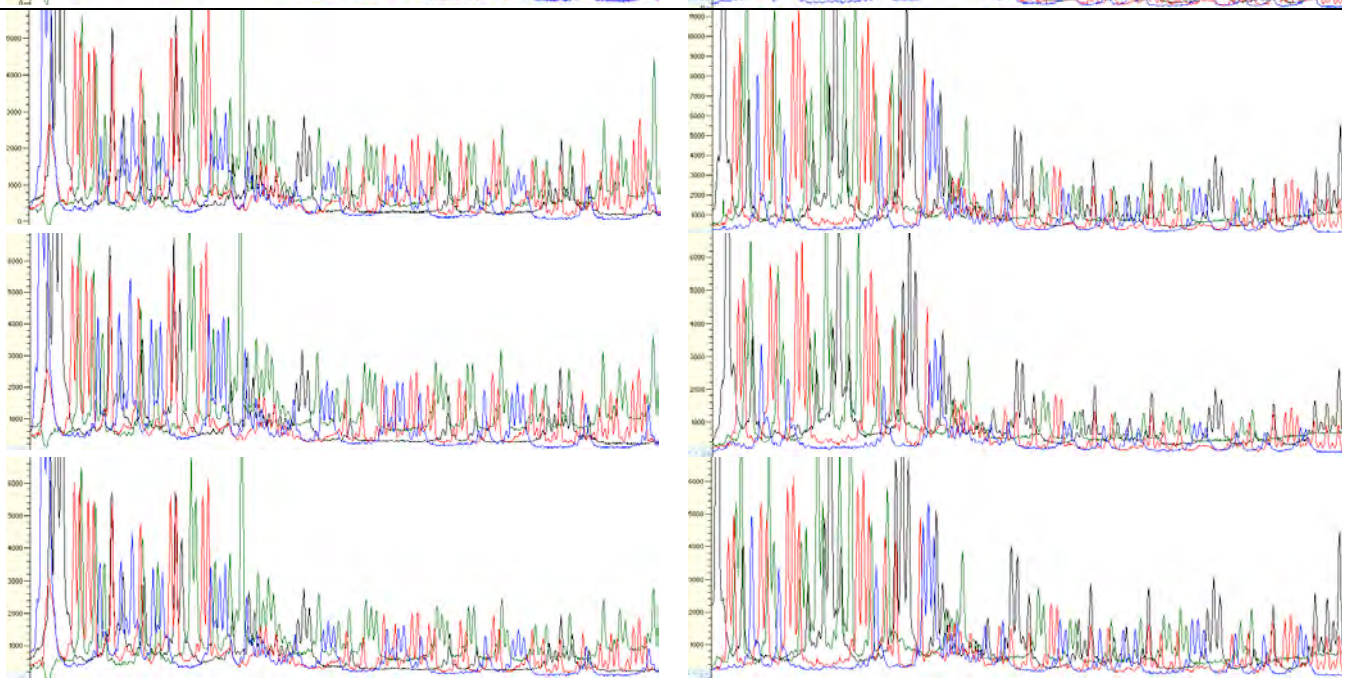


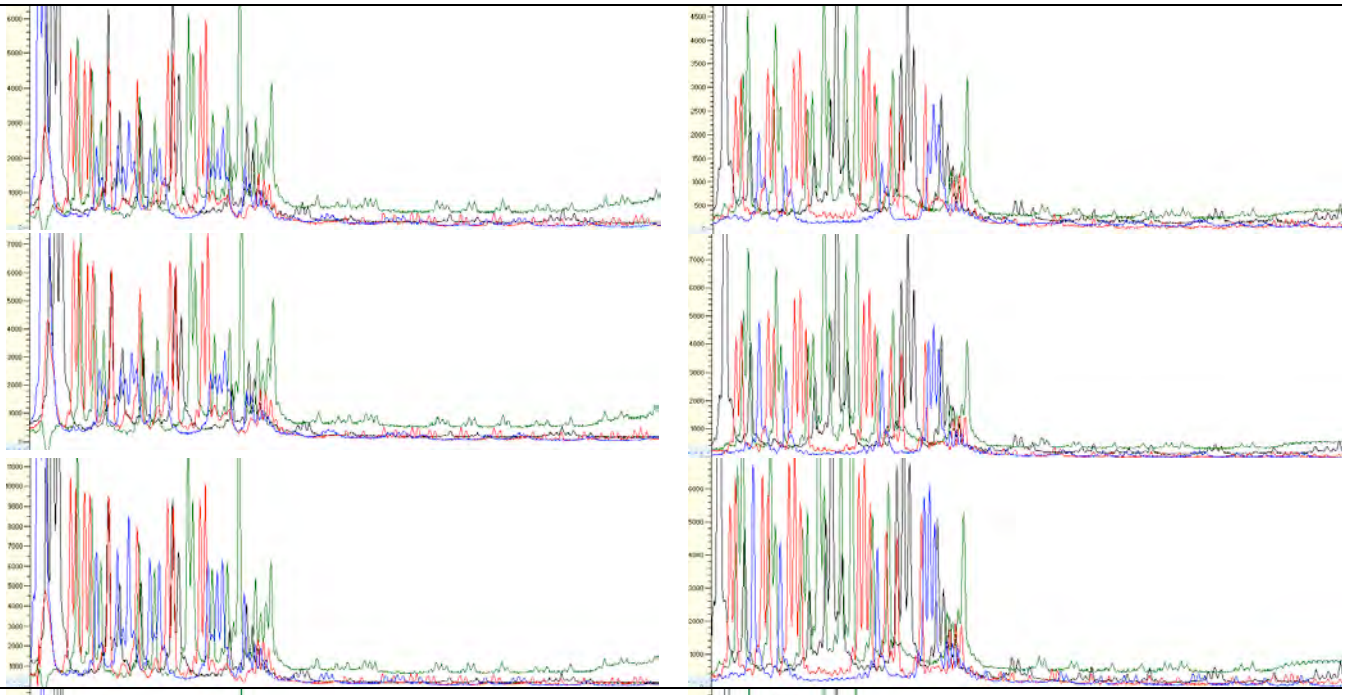
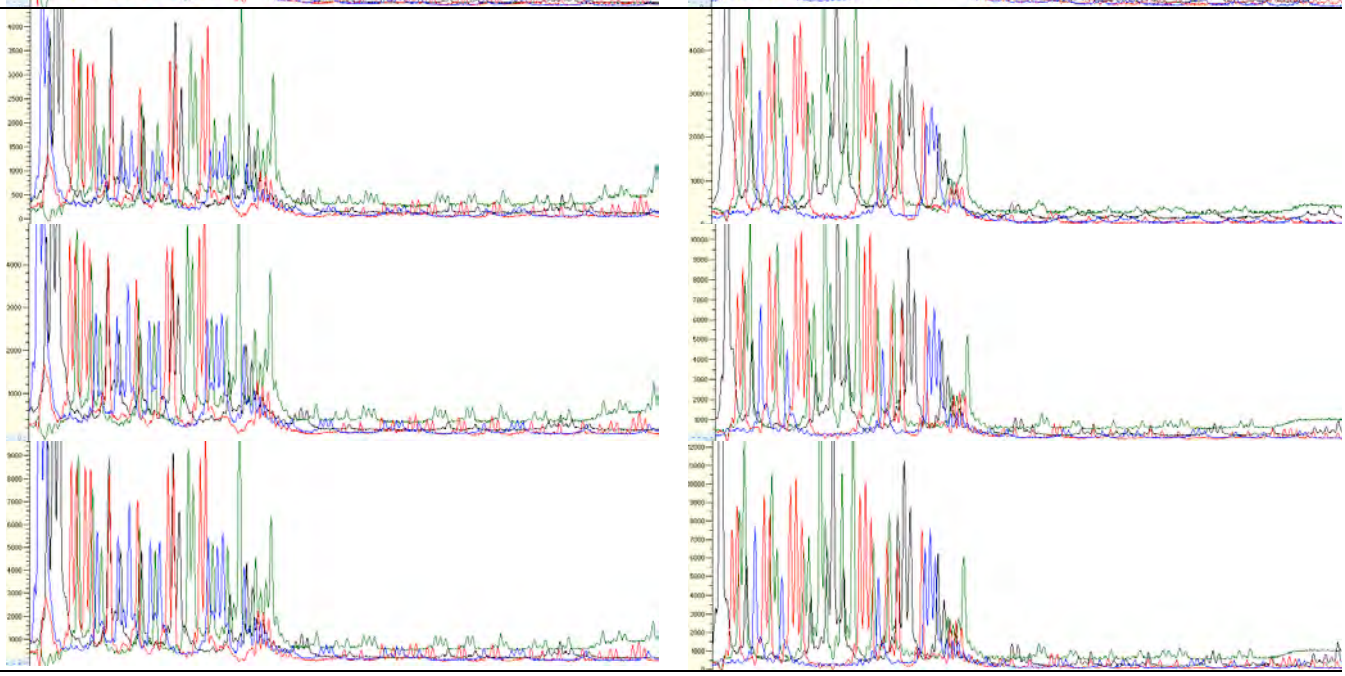
PrMO	
EMO	

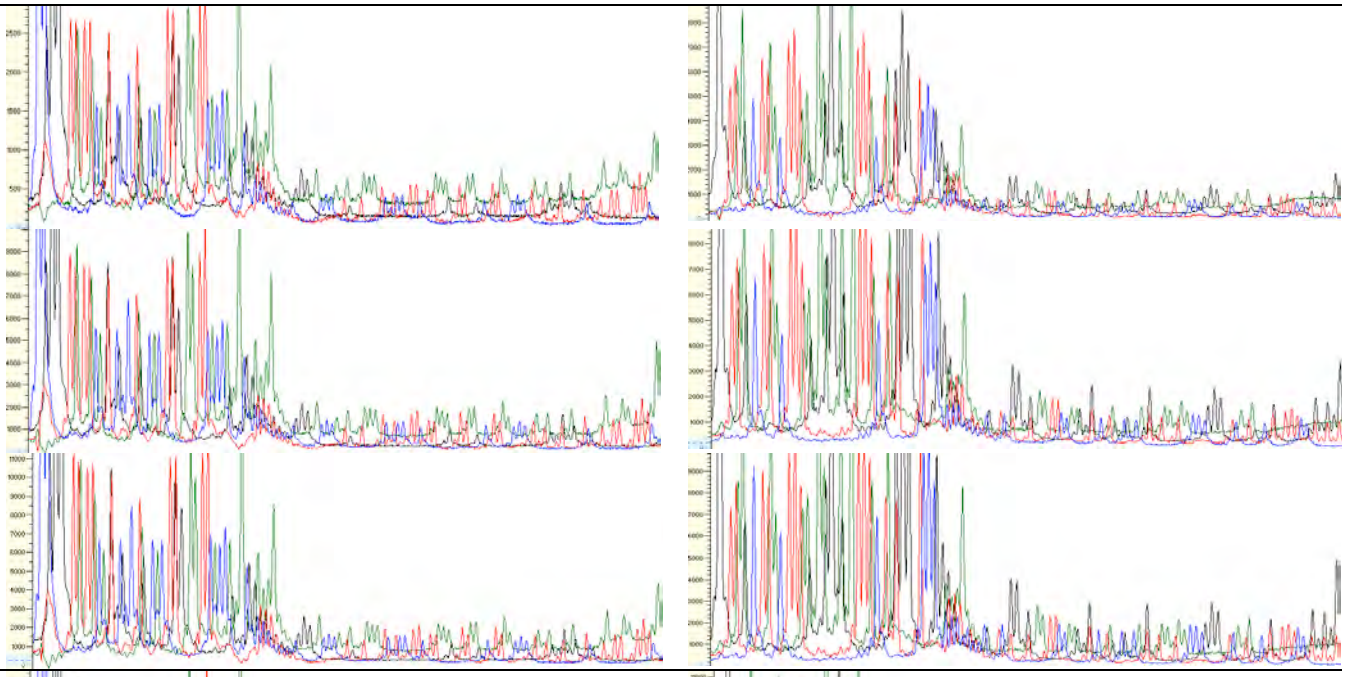
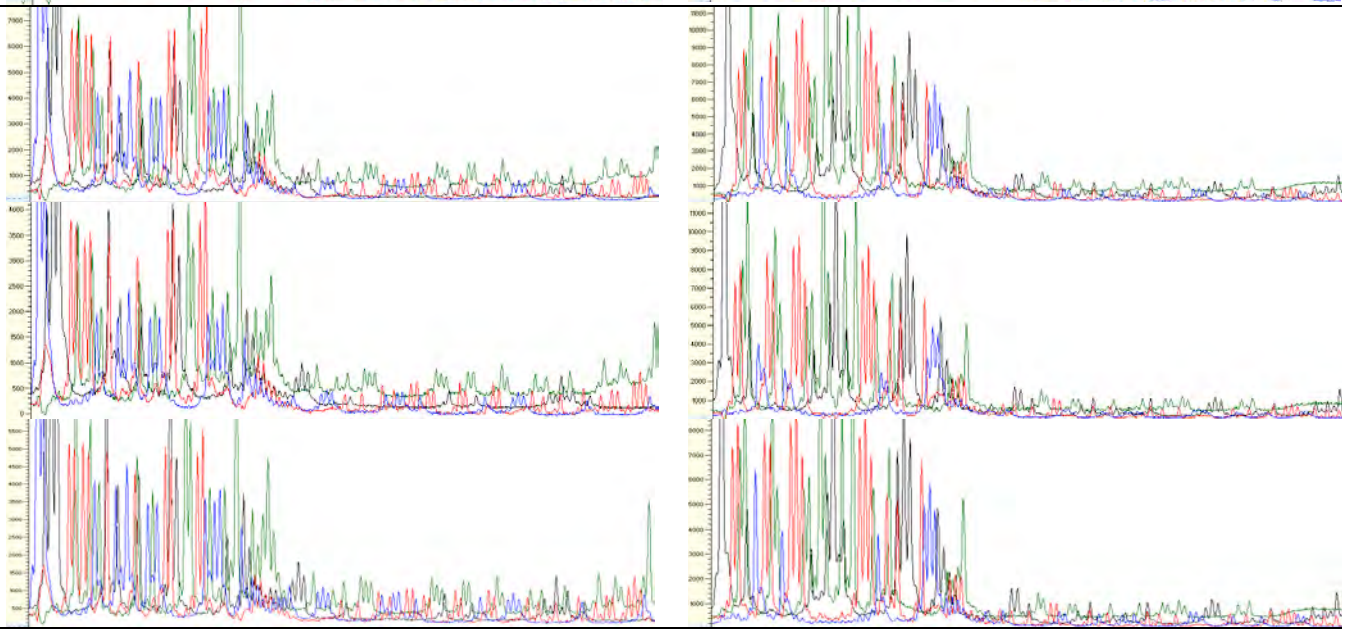
NMO1



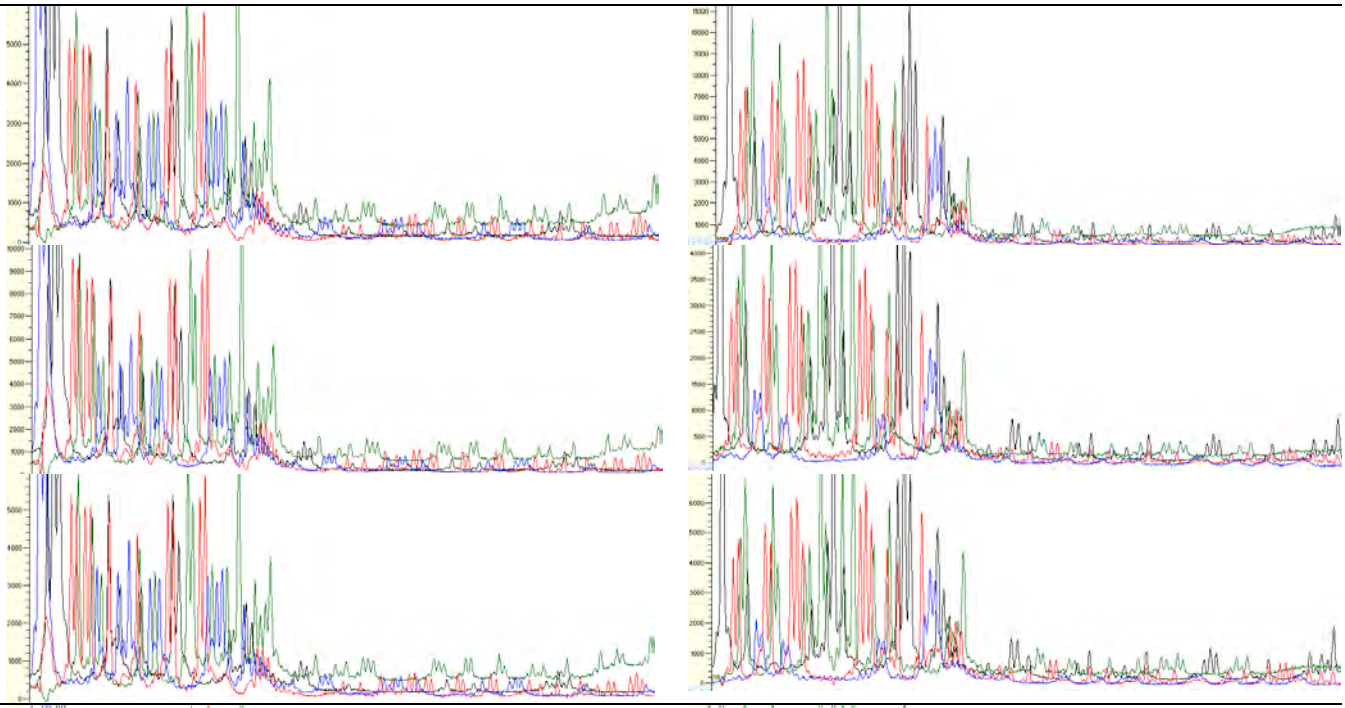
PMO1



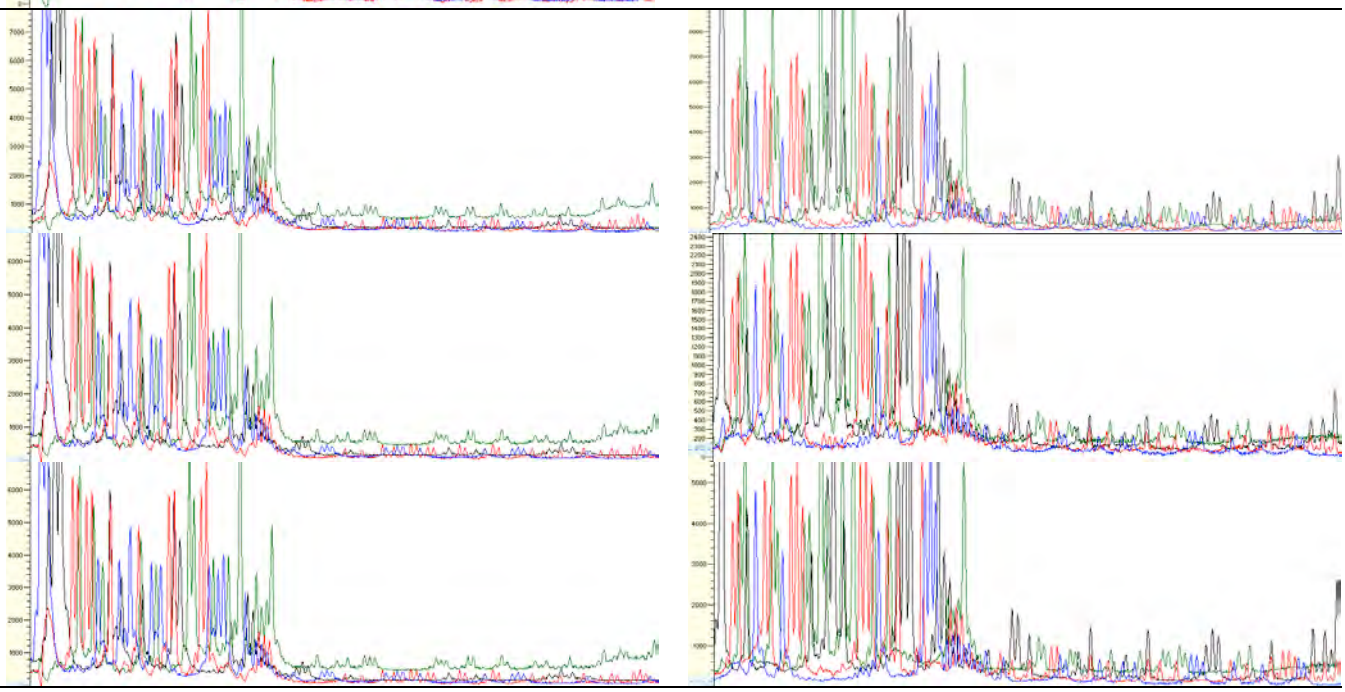
IMO	
ClMO	

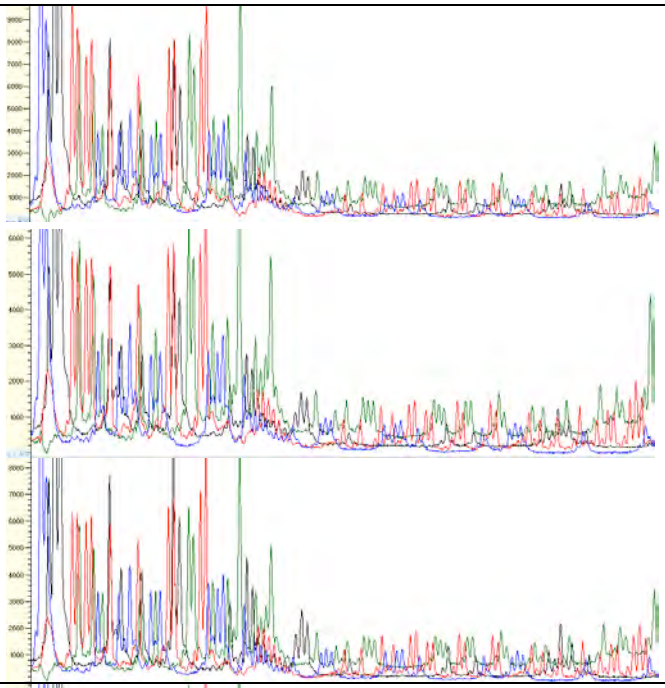
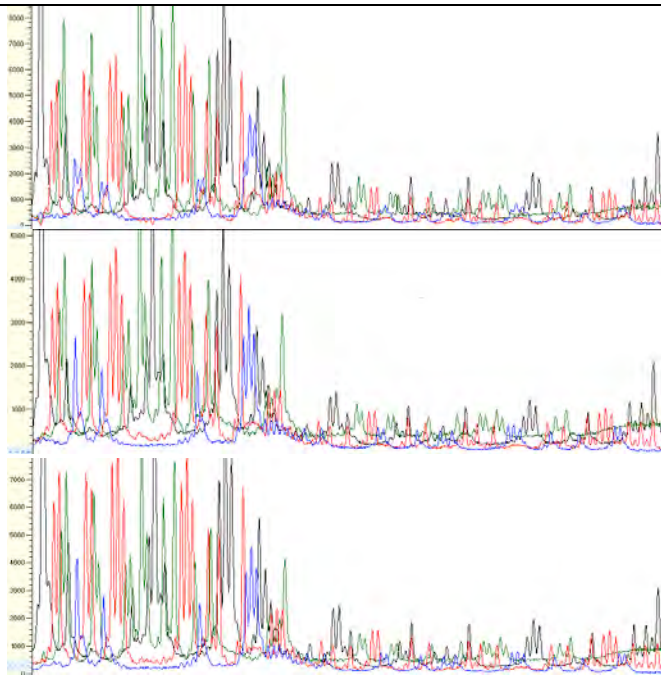
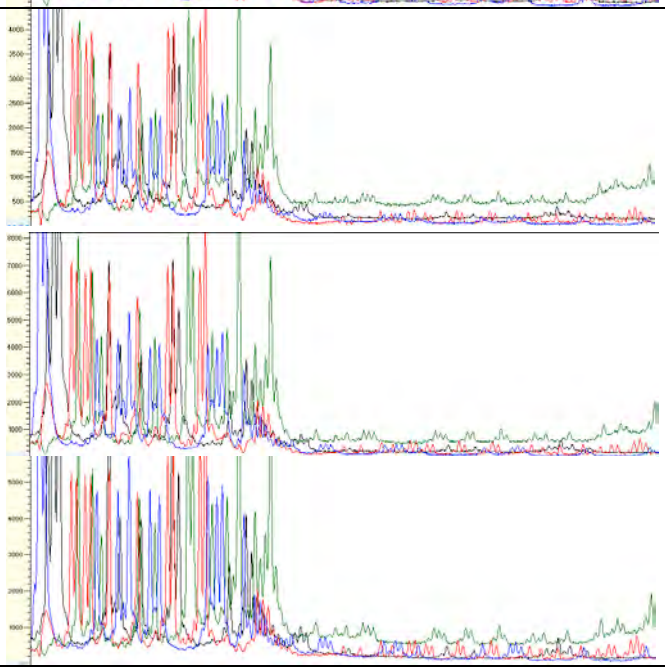
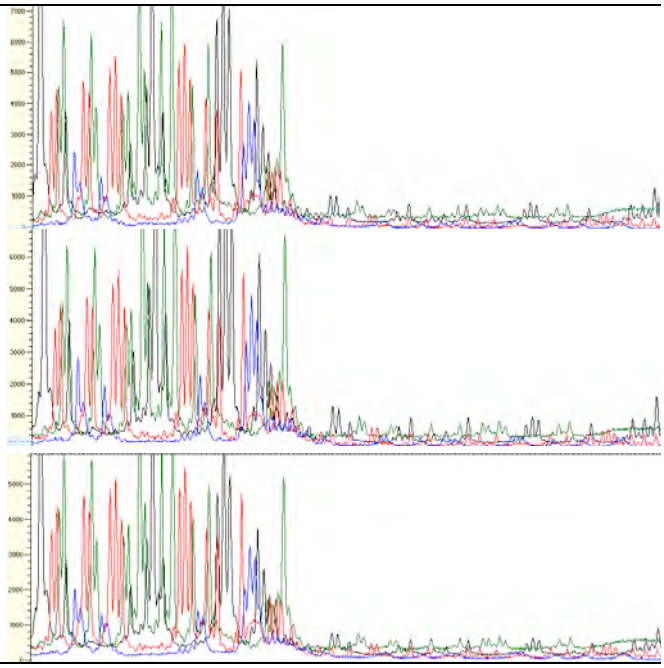
CNMO	
TfMO	

VMO

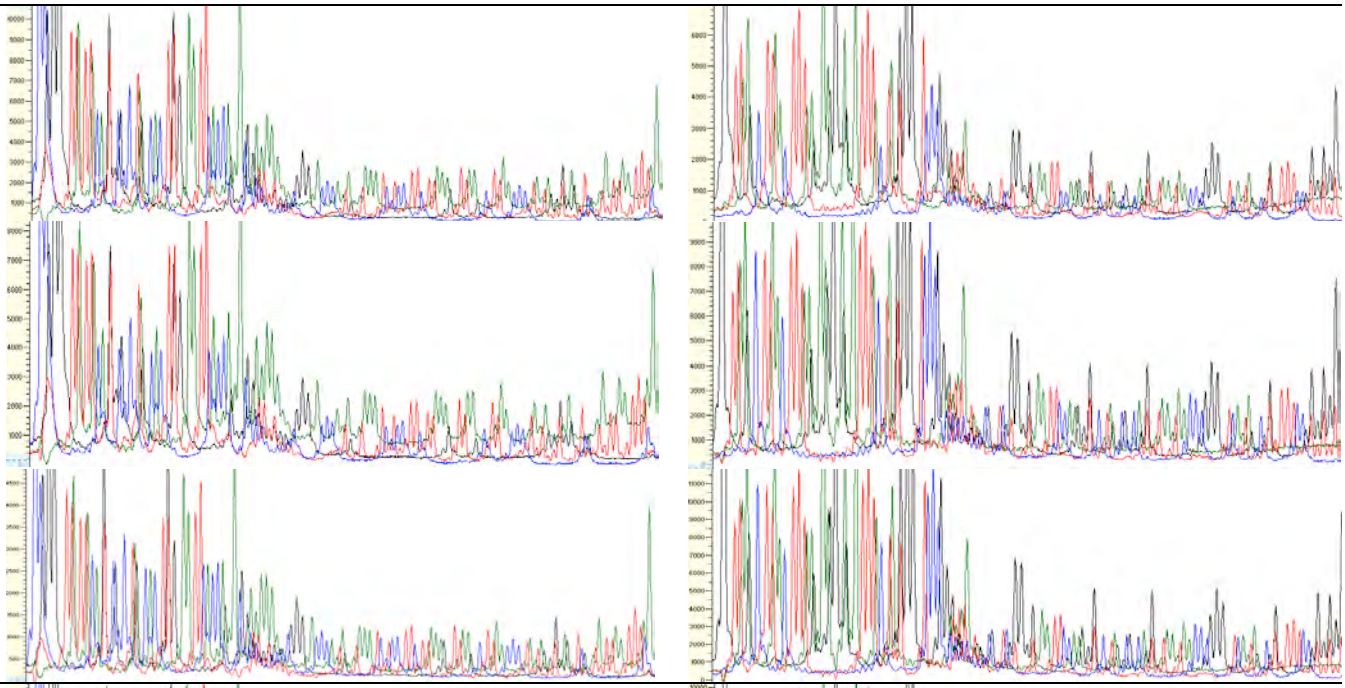


ZMO

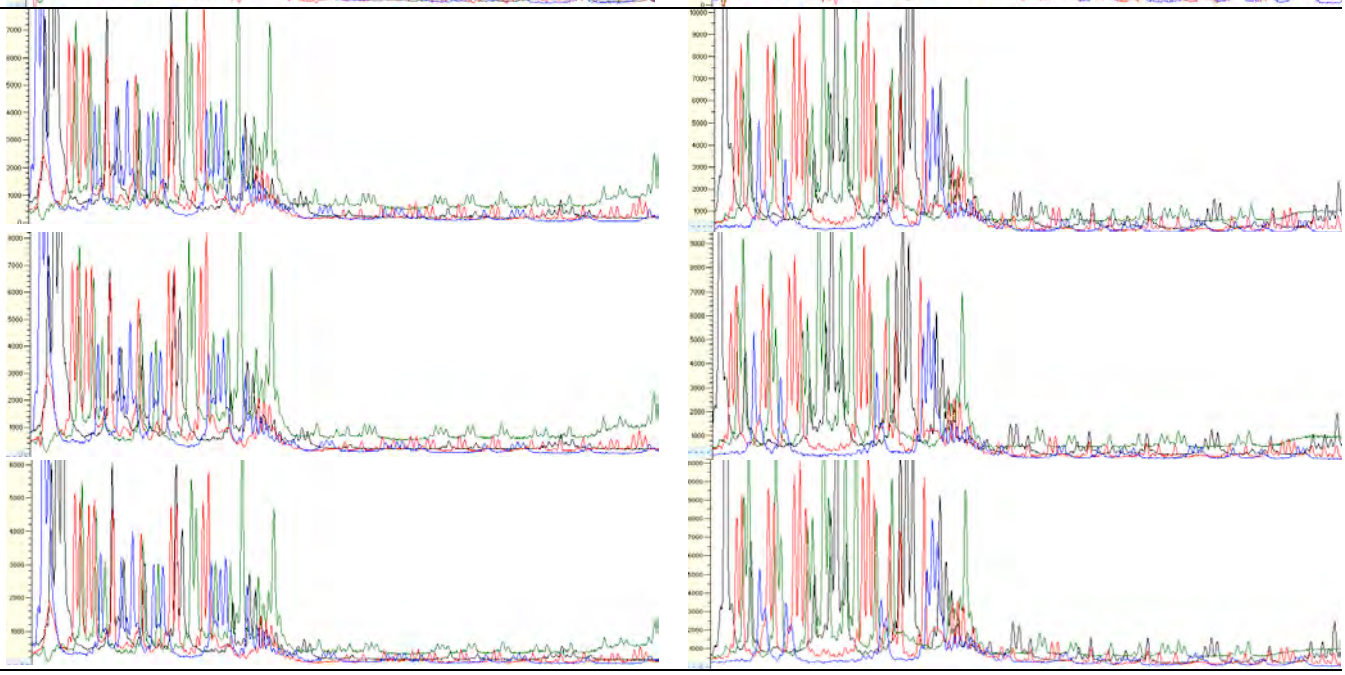


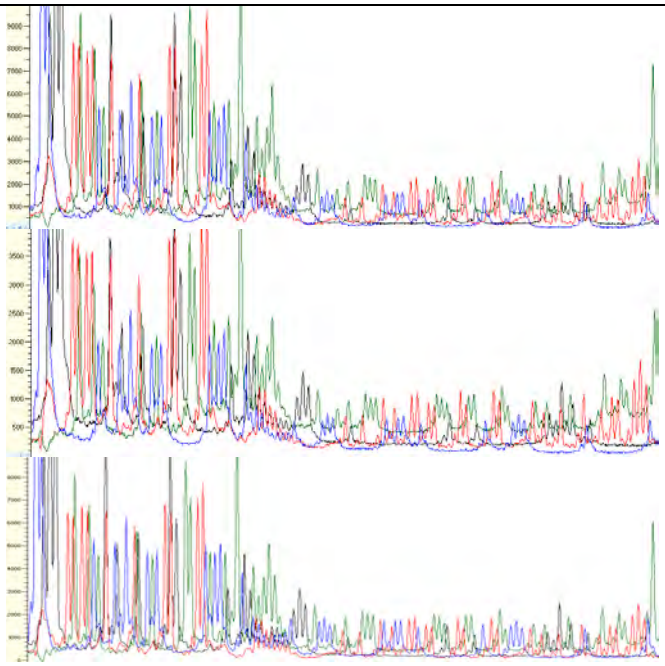
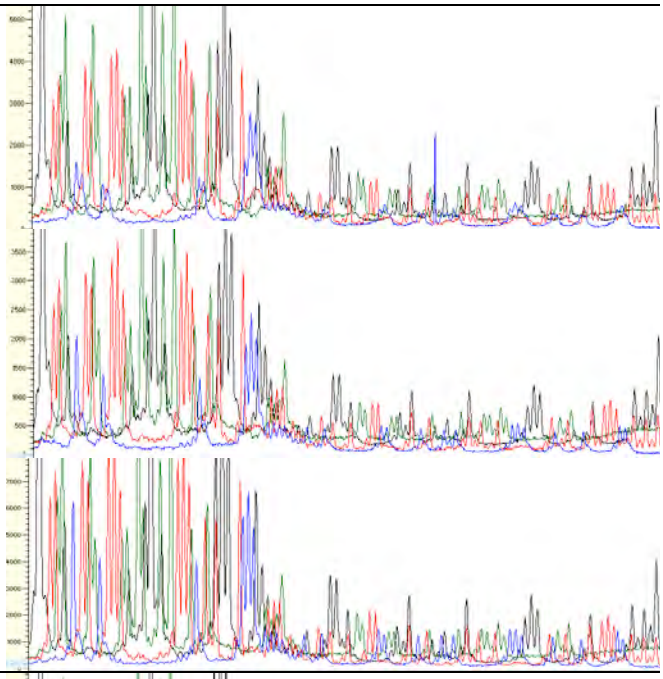
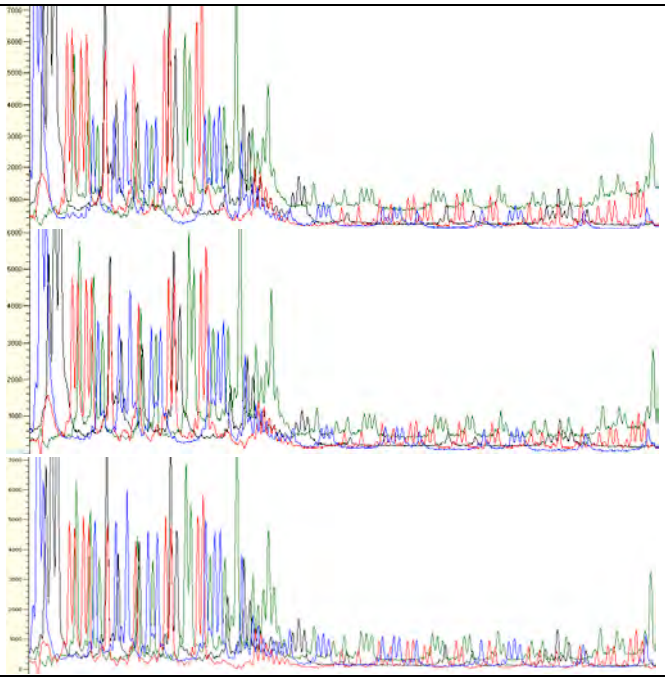
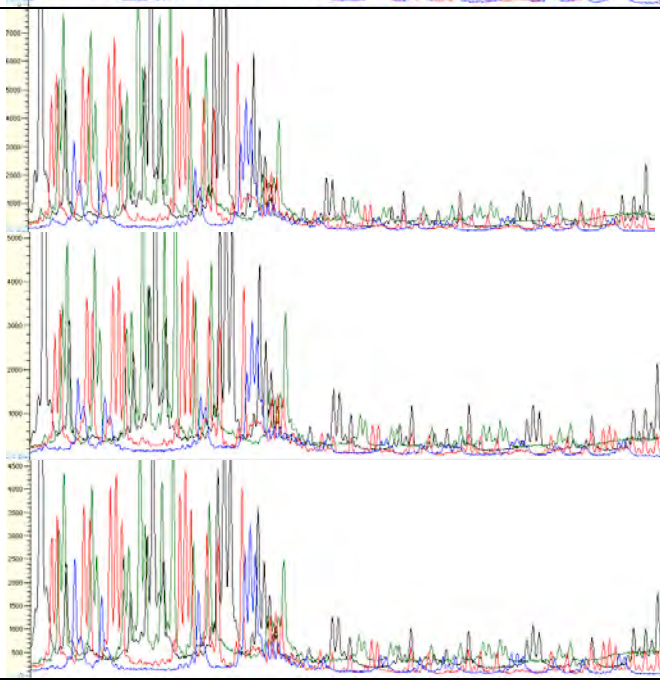
QMO		
FIMO		

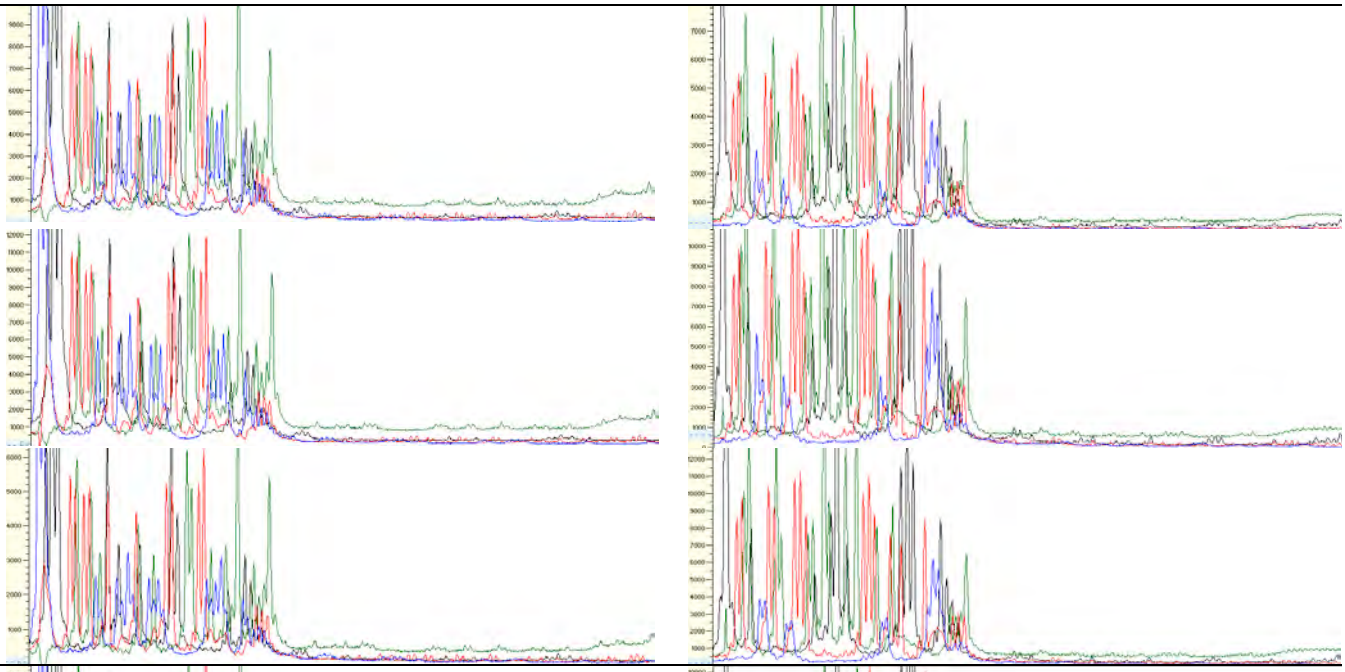
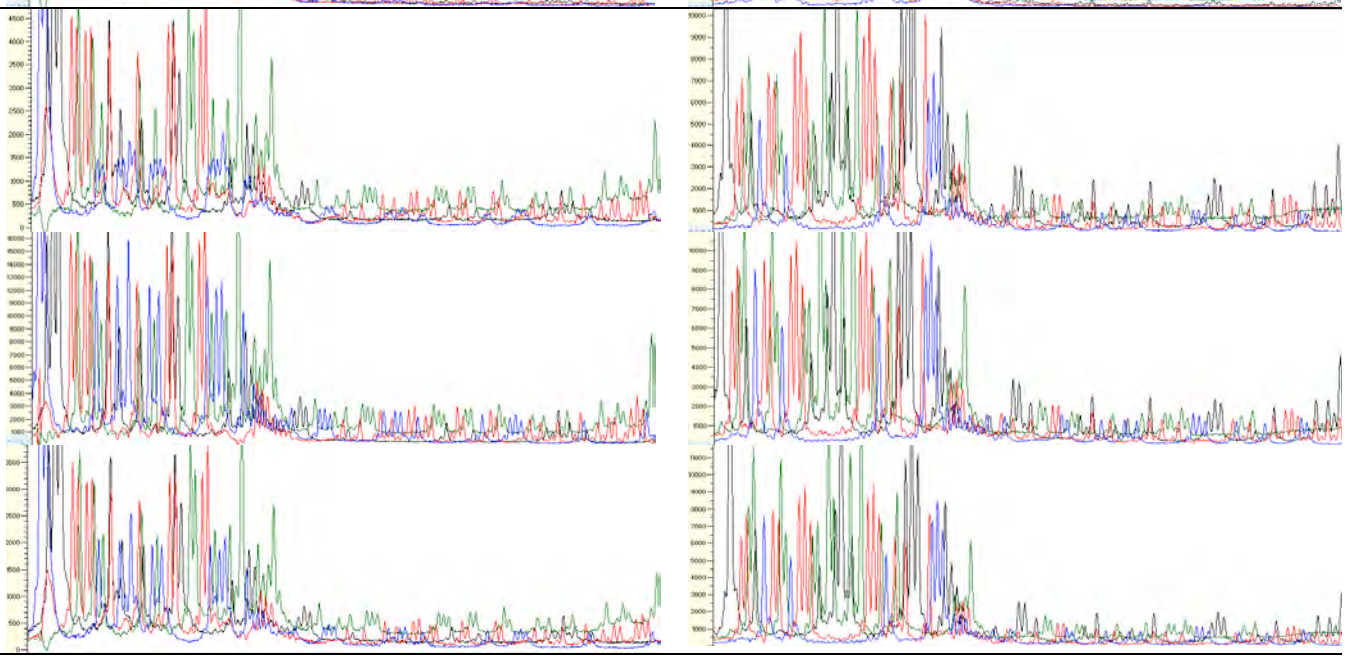
MIMO

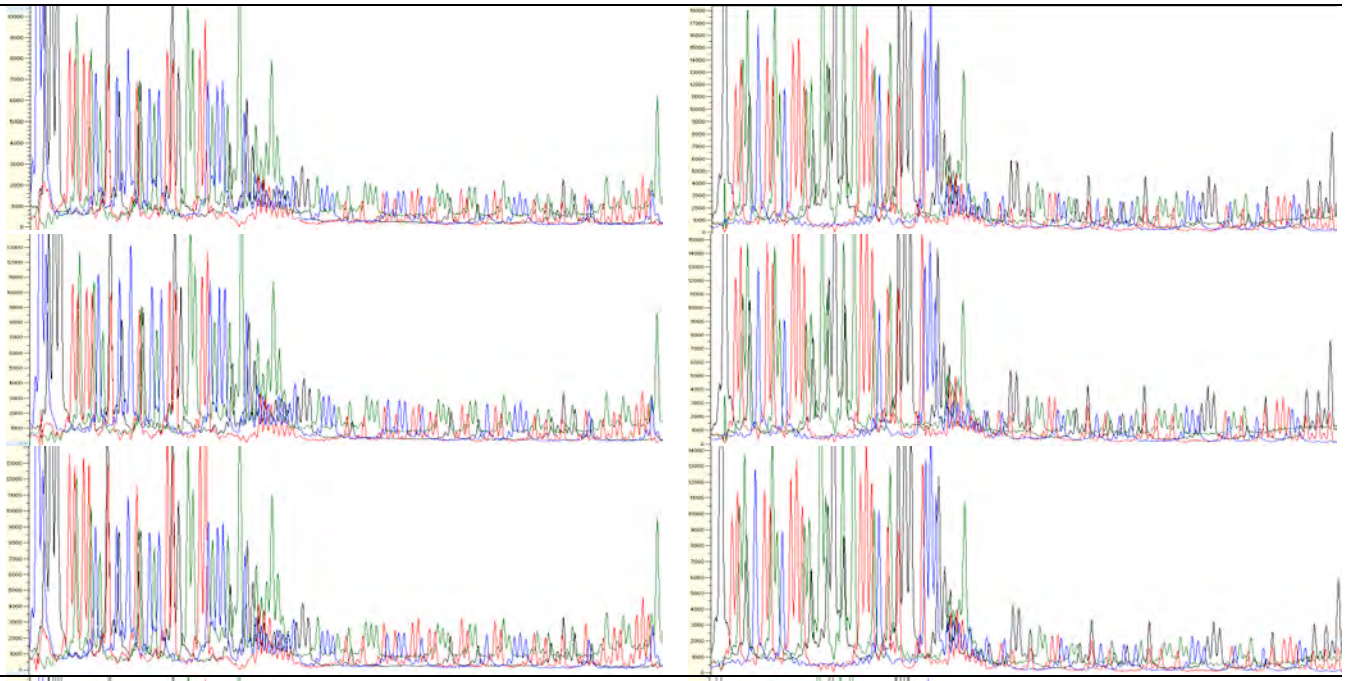
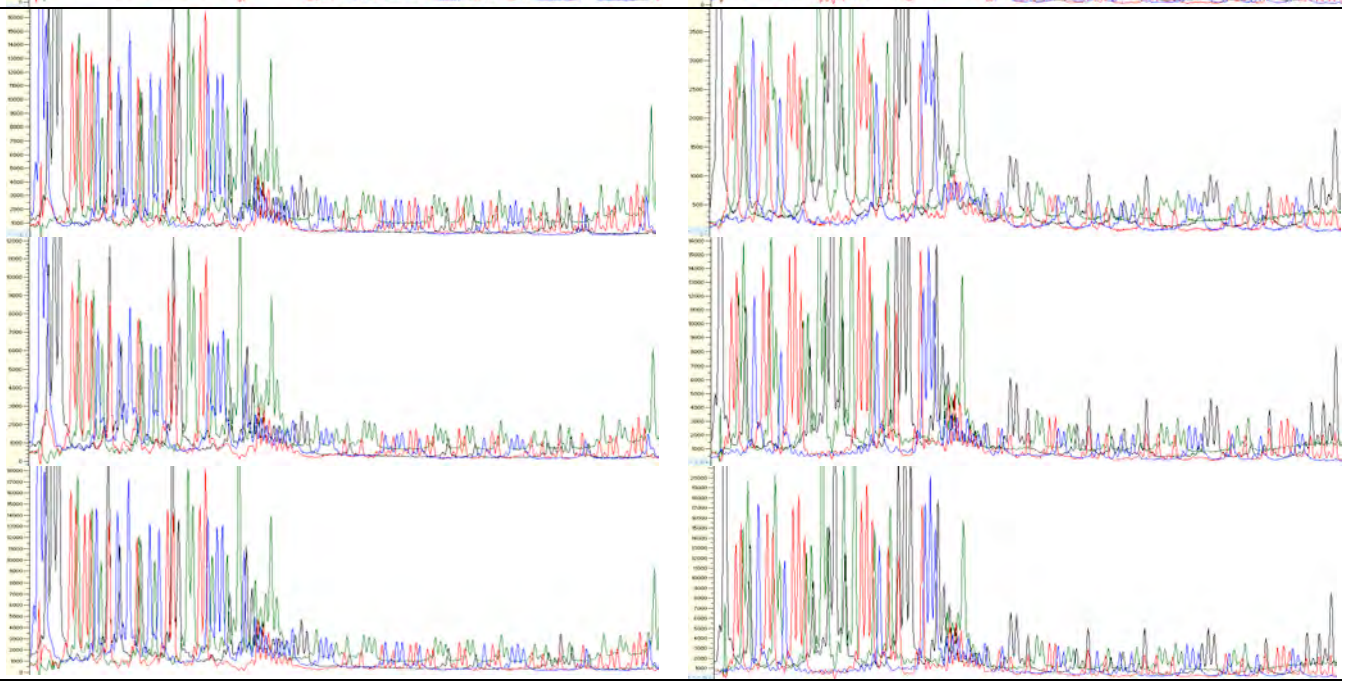


FEMO



MEMO		
FDMO		

NaM	
MMO2	

DMO	
5FM	

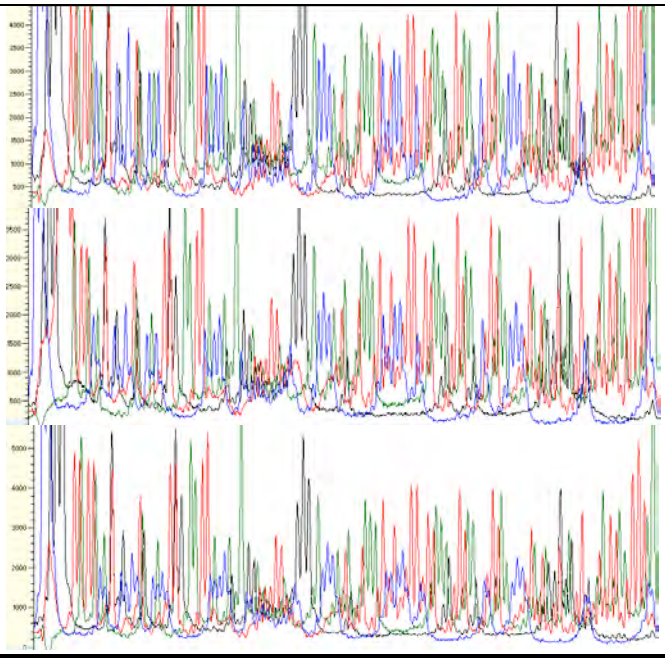
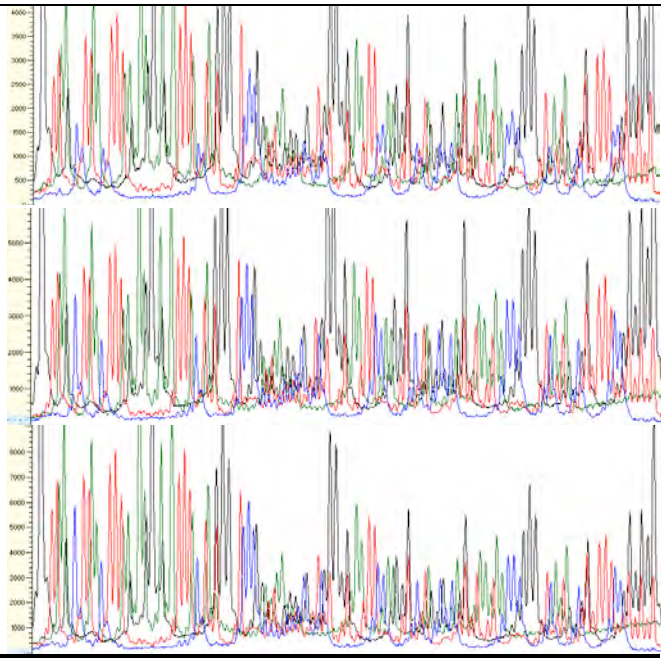
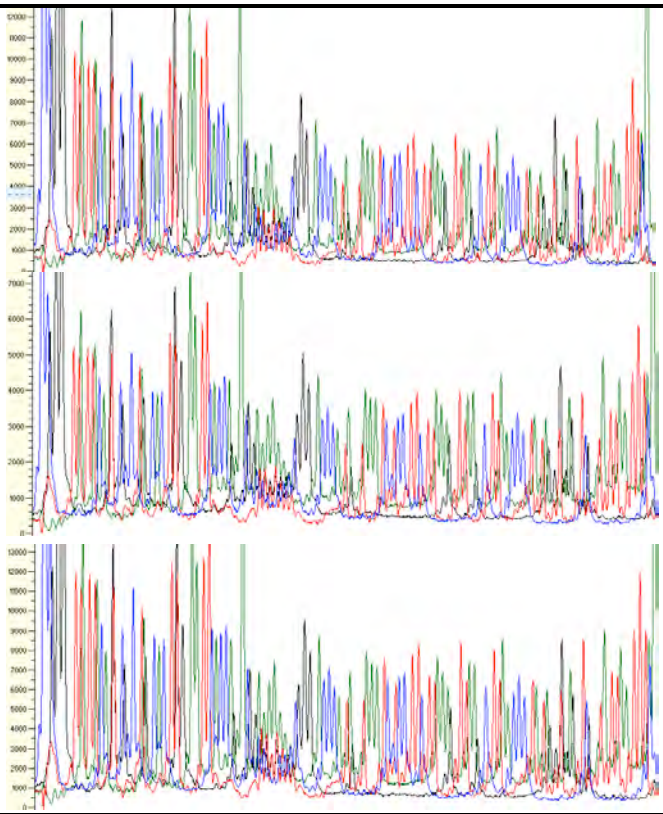
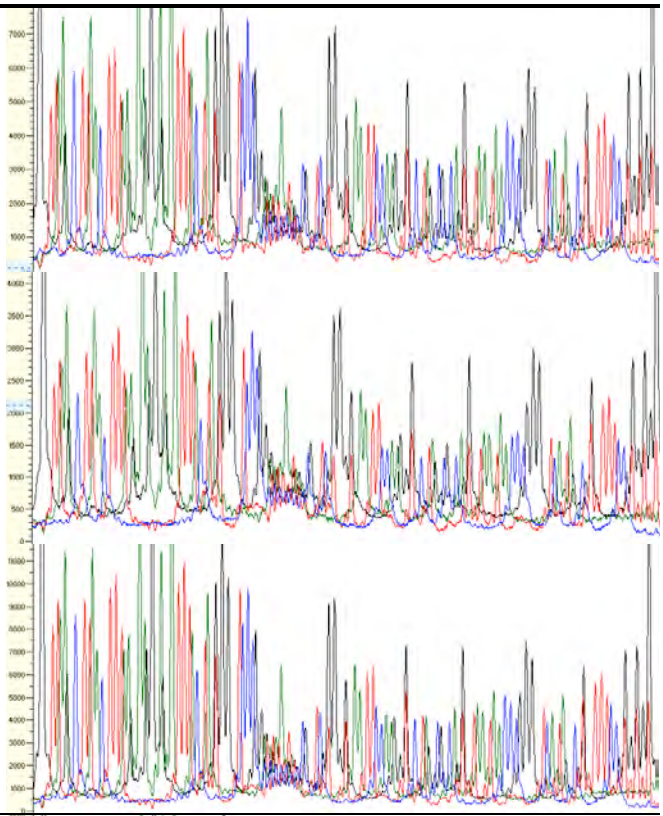
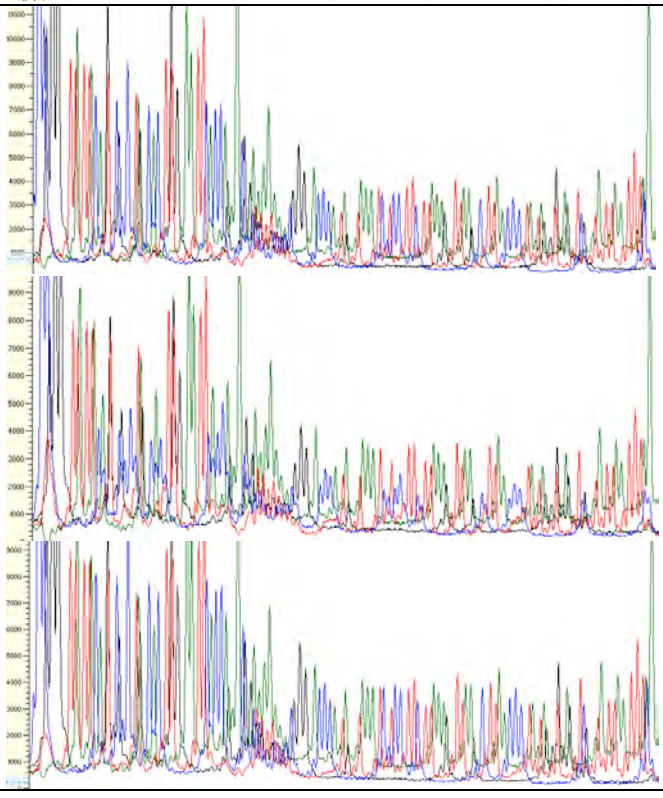
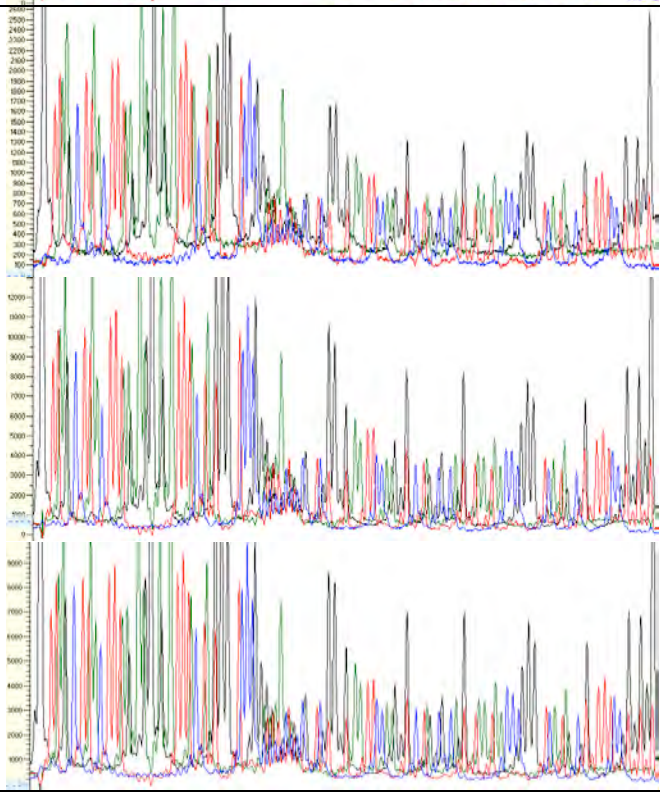
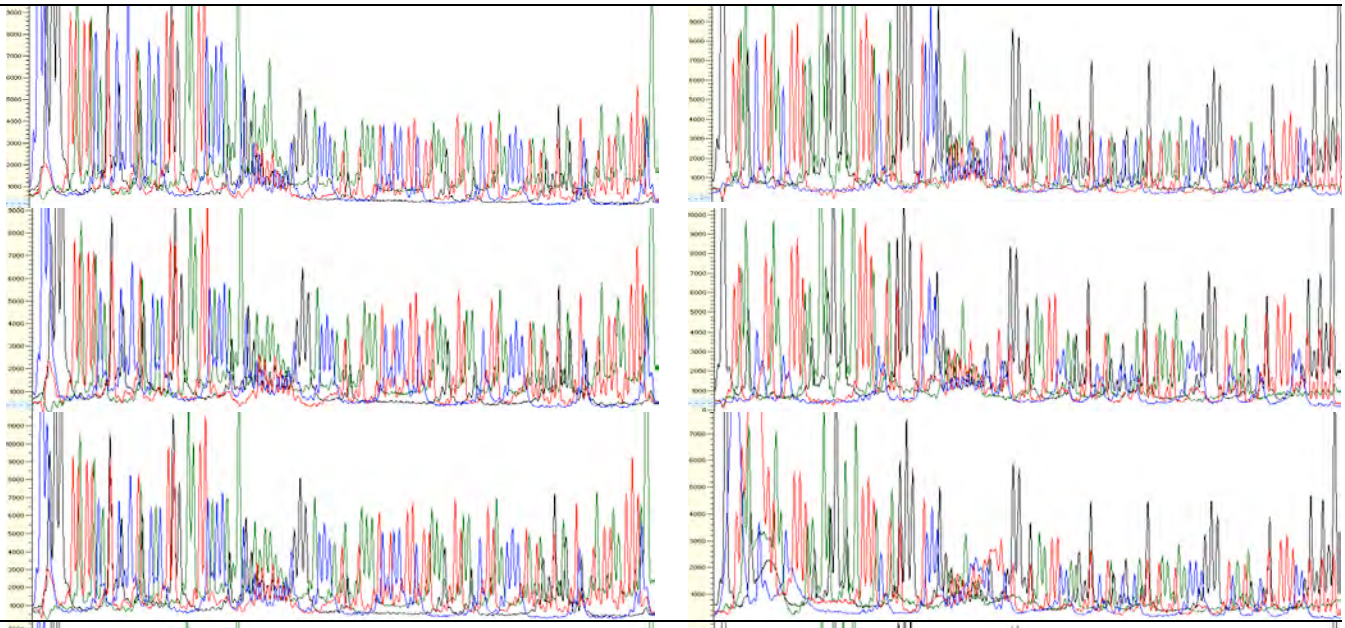
Only natural dNTPs		

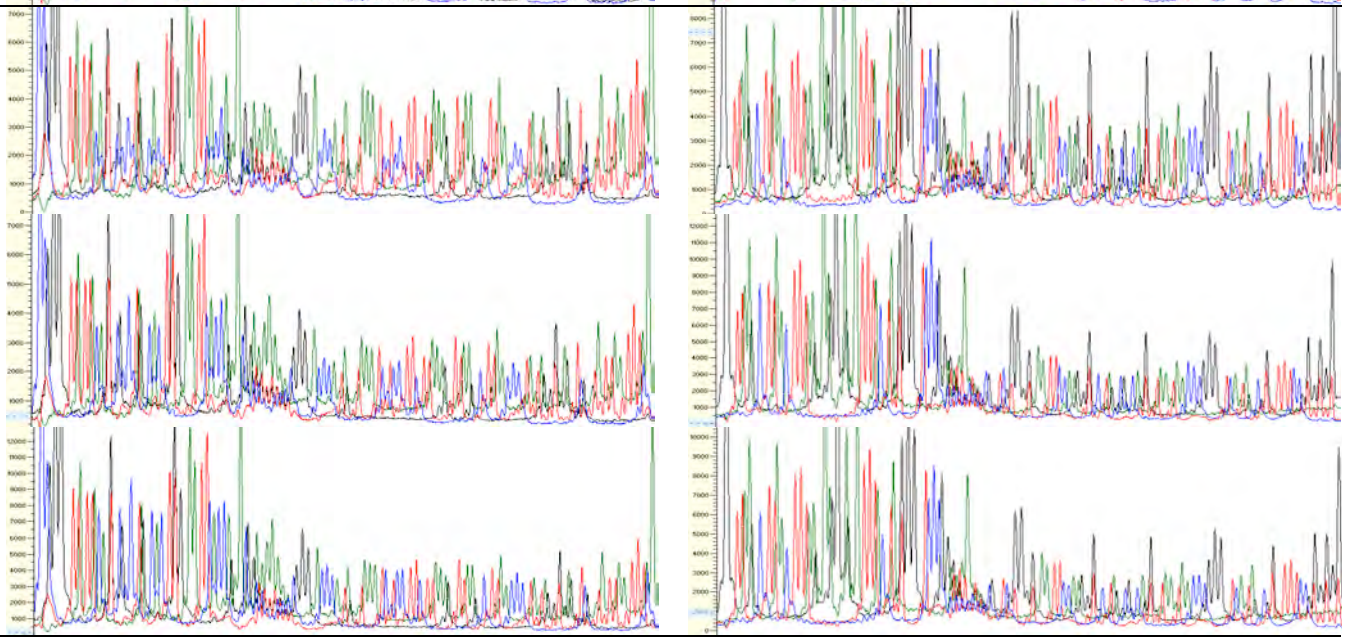
Figure S76. Raw sequencing data after PCR amplification by *Taq* polymerase in the presence of d**5SICS**TP and d**X**TP. Triplicate data shown.

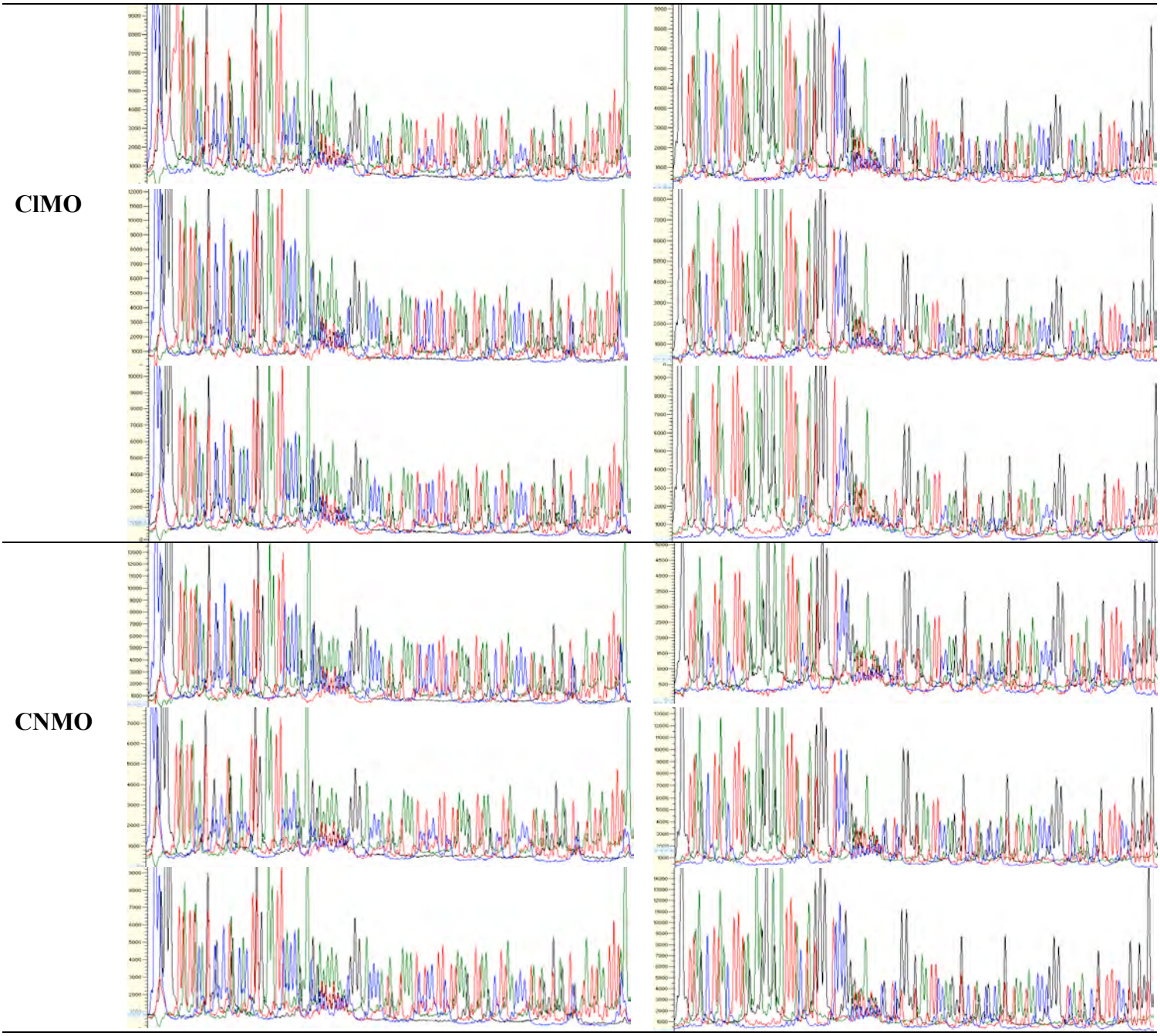
X	Primer 1	Primer 2
PrMO		
EMO		

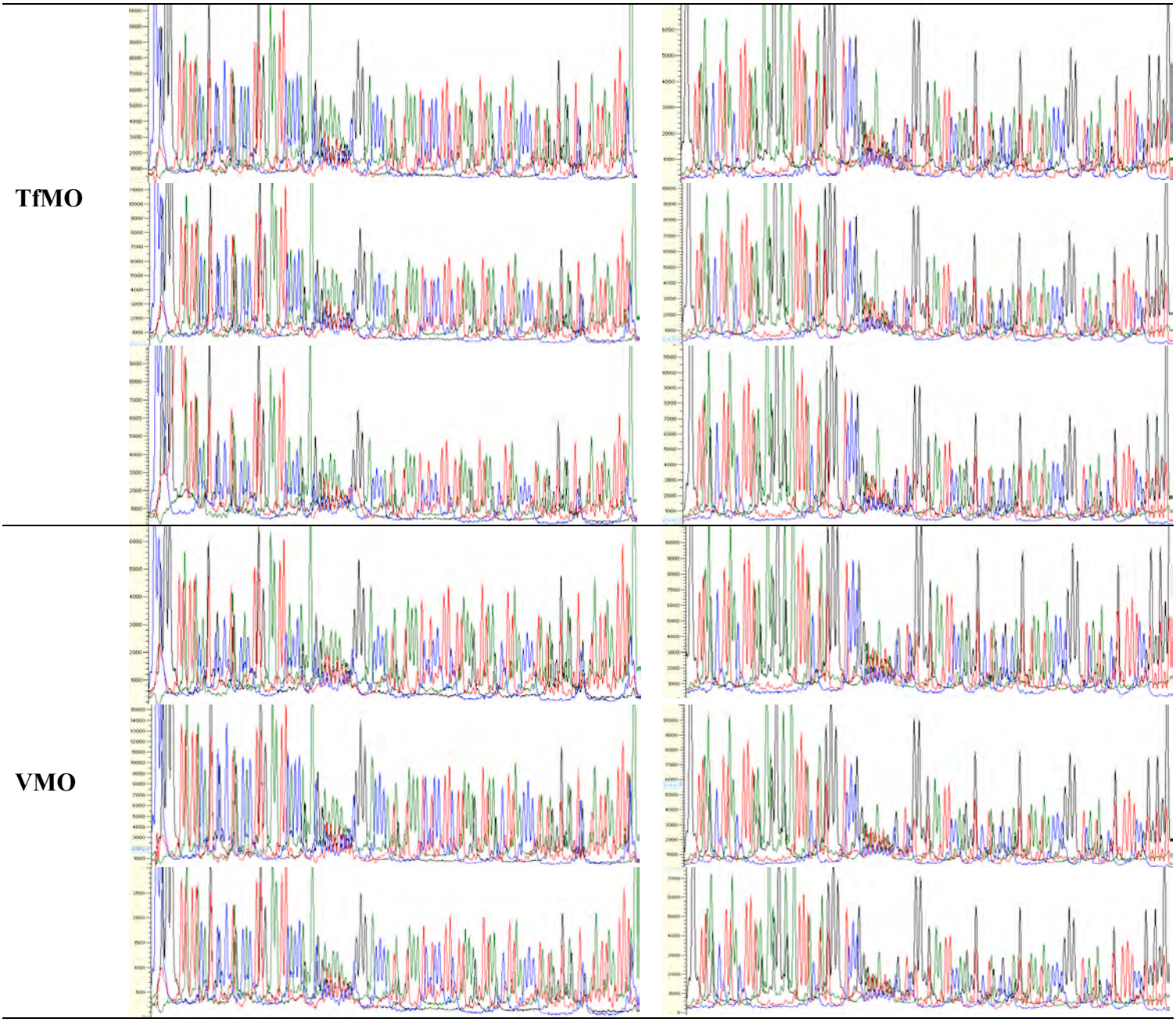
NMO1

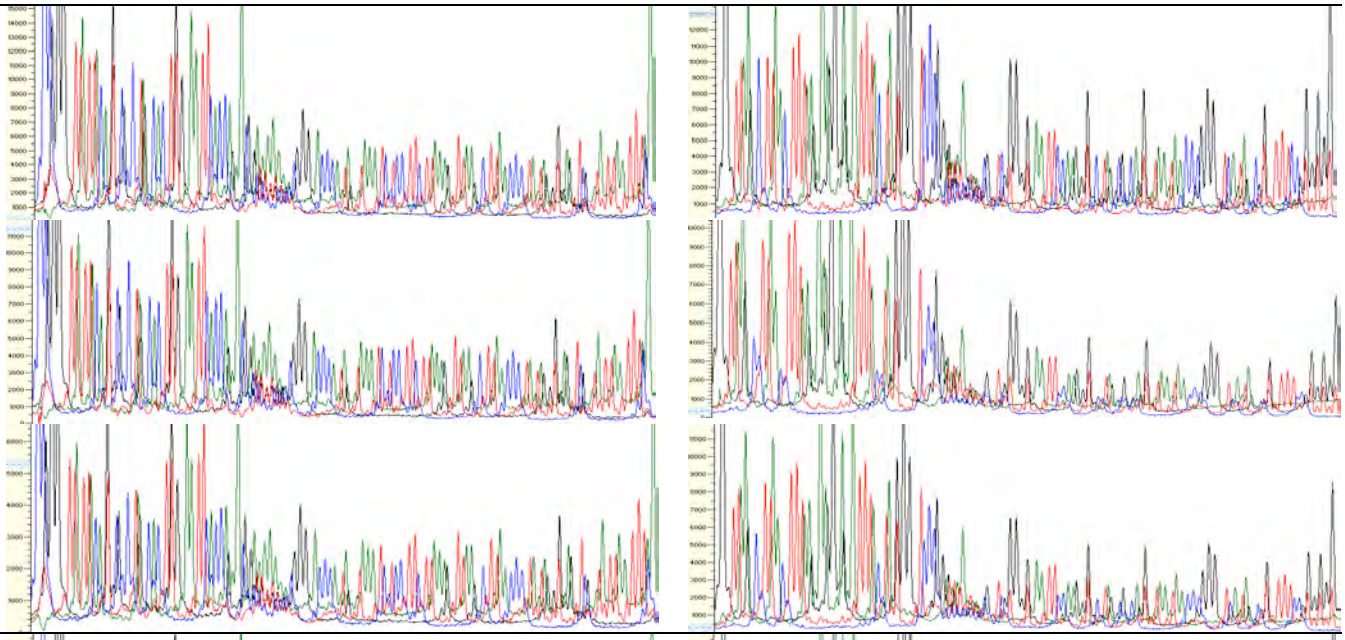
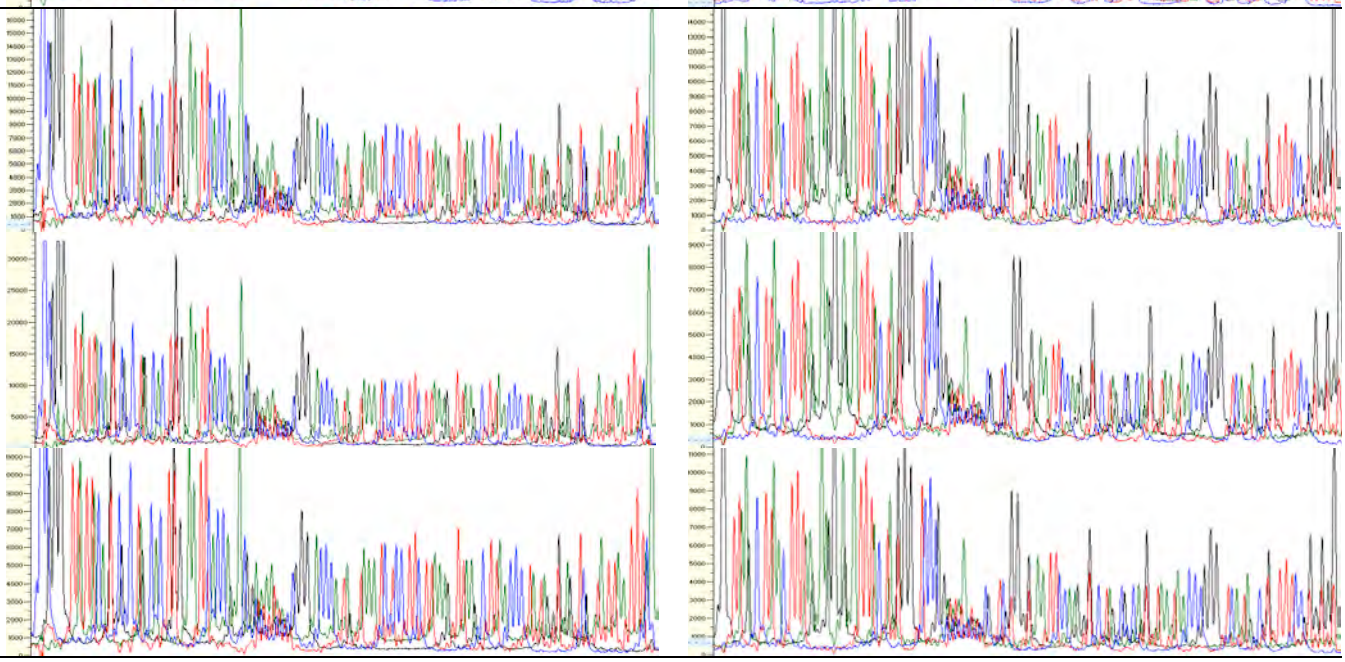


IMO

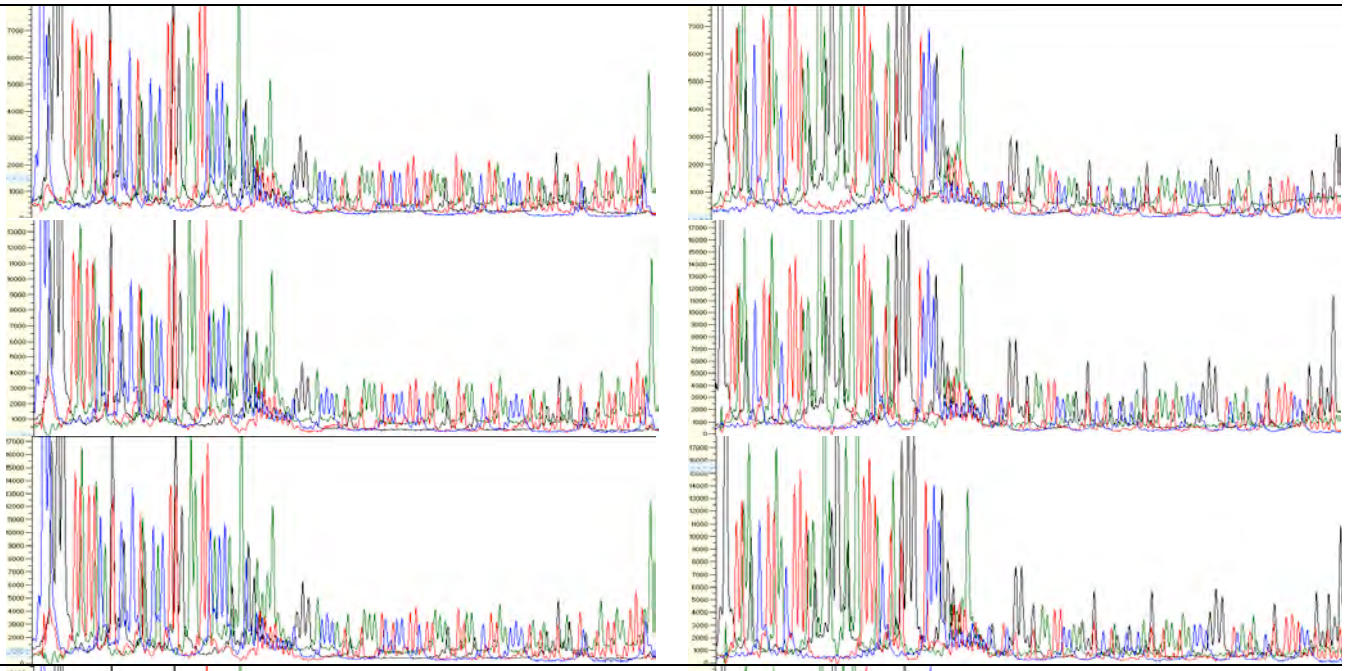



ClMO
CNMO

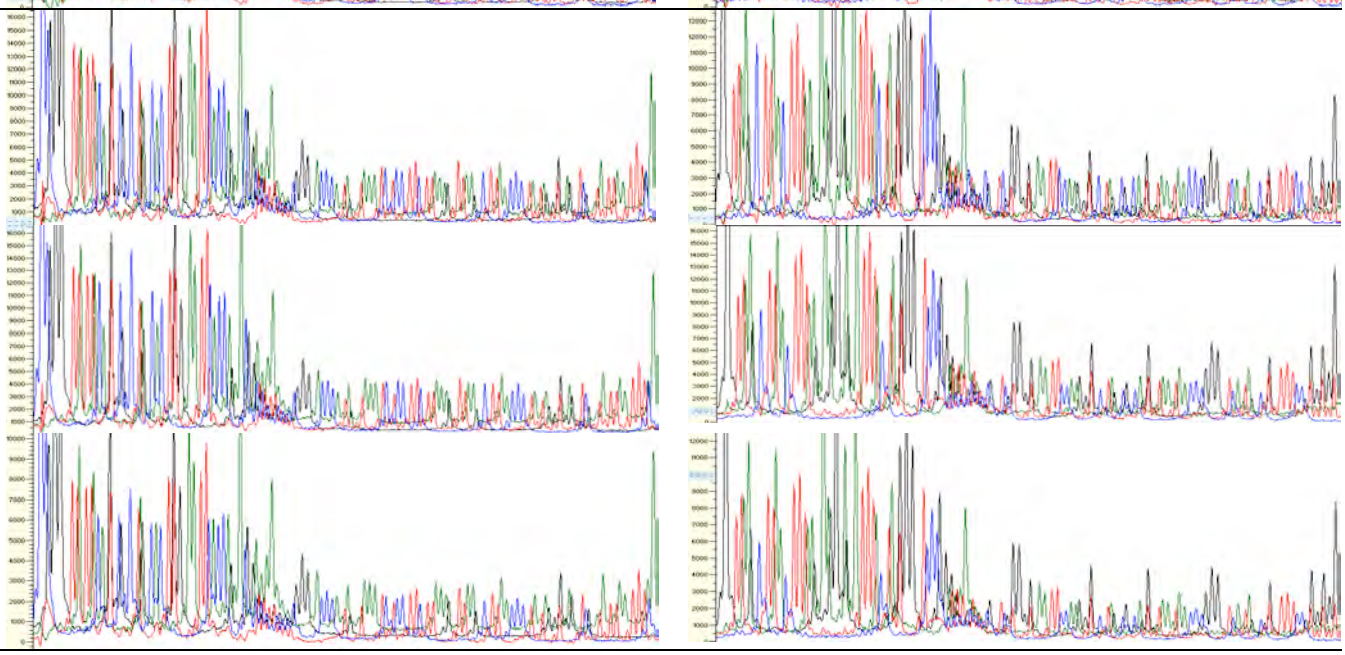

TfMO
VMO

ZMO	
QMO	

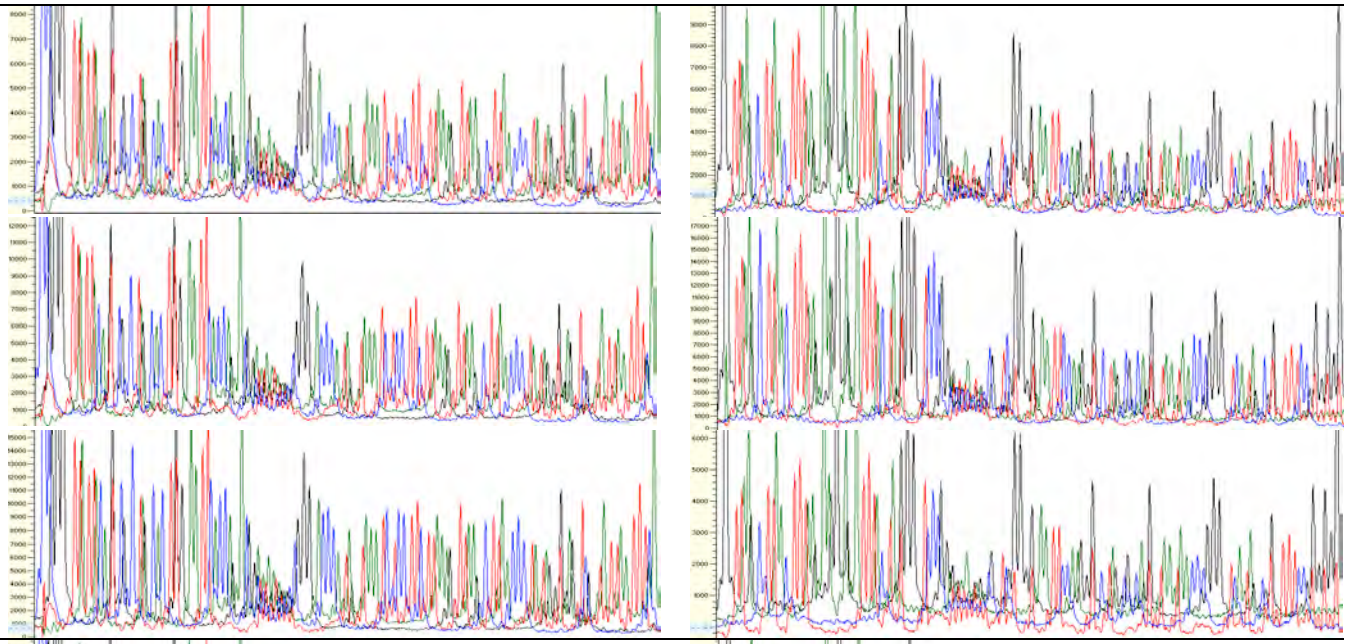
FIMO



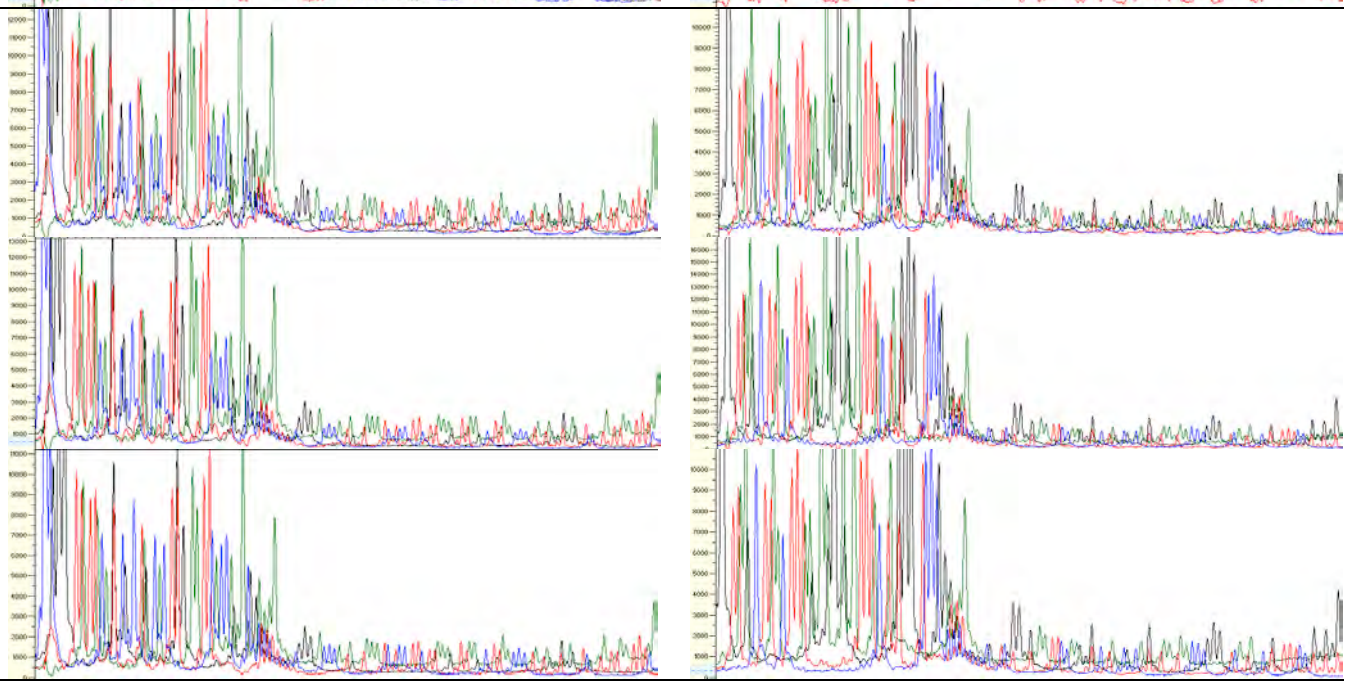
FEMO

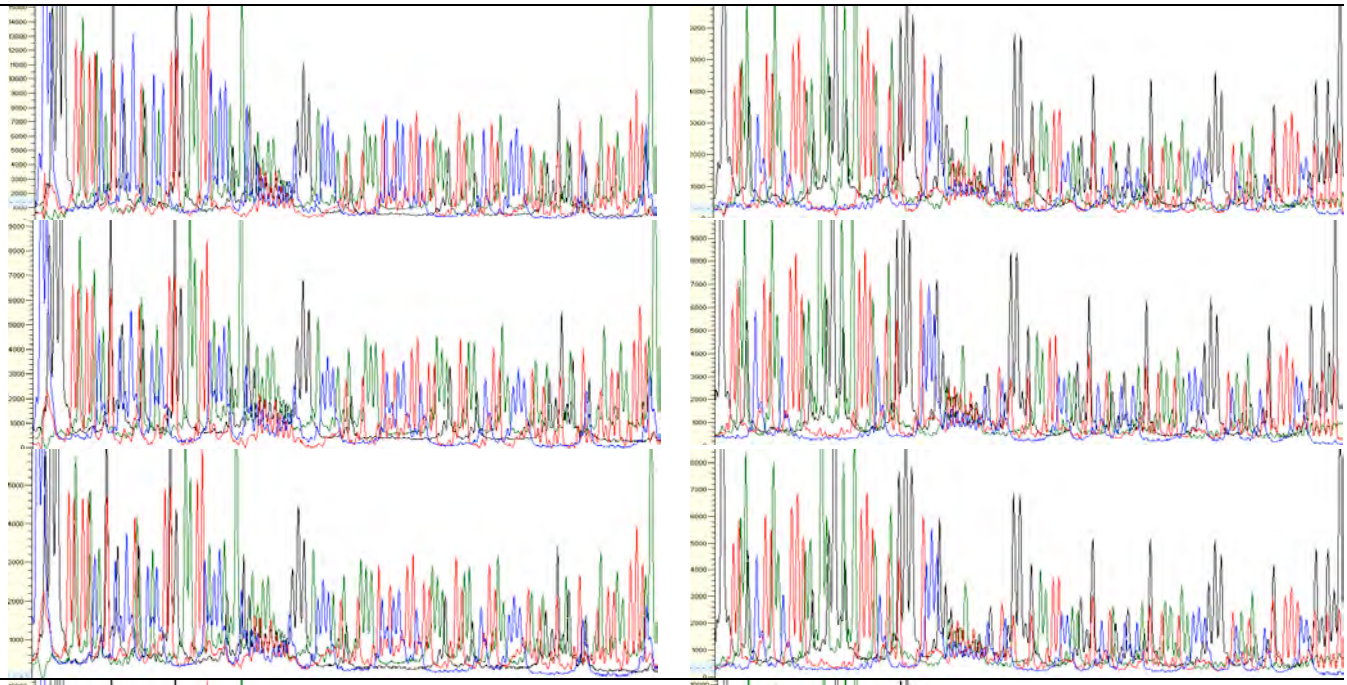
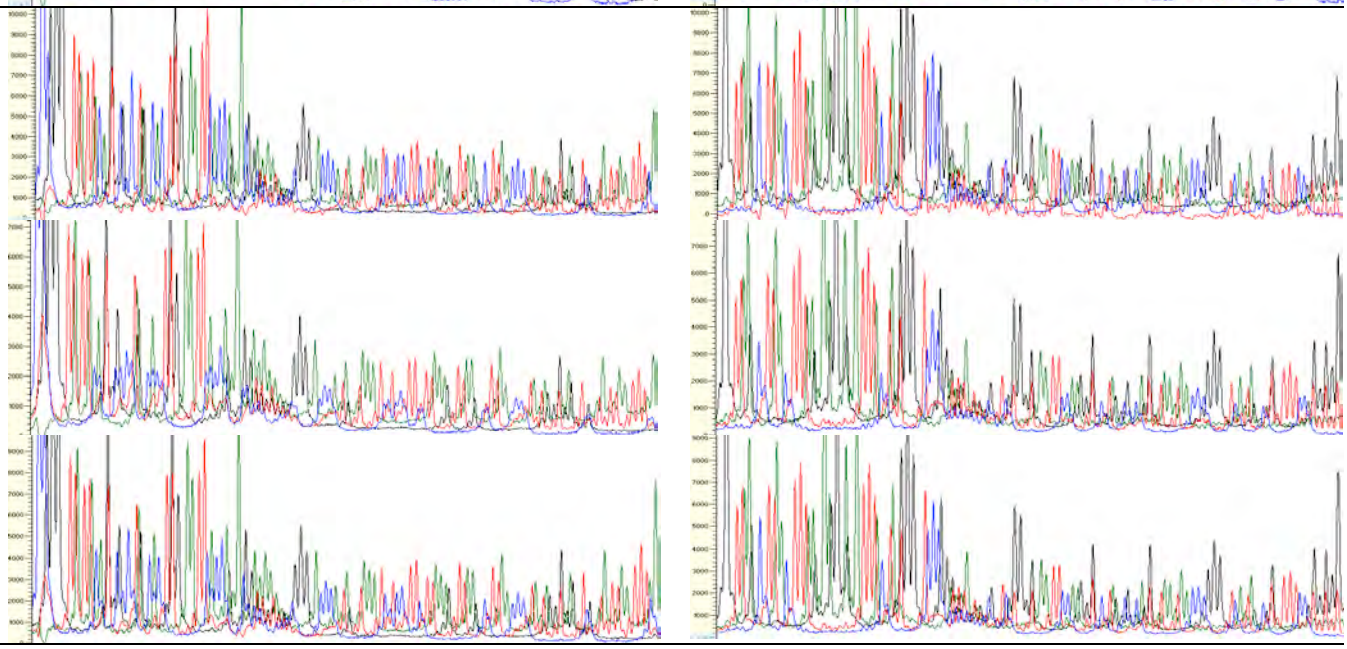


FDMO



NaM



MMO2	
DMO	

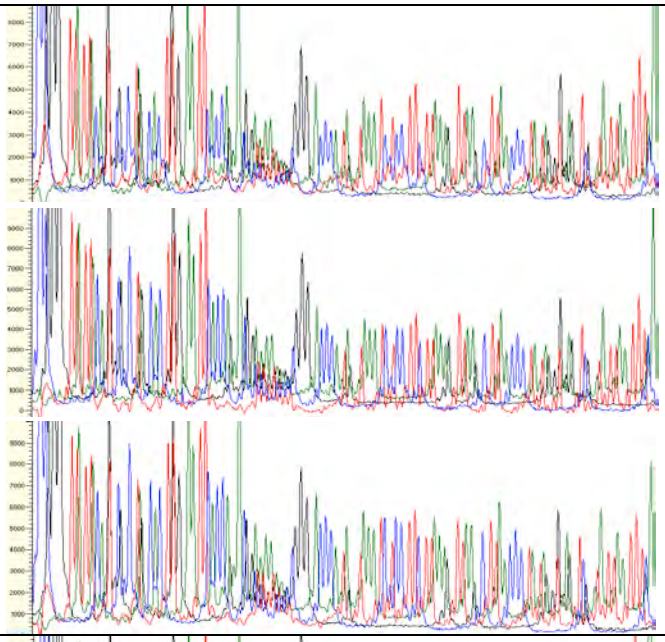
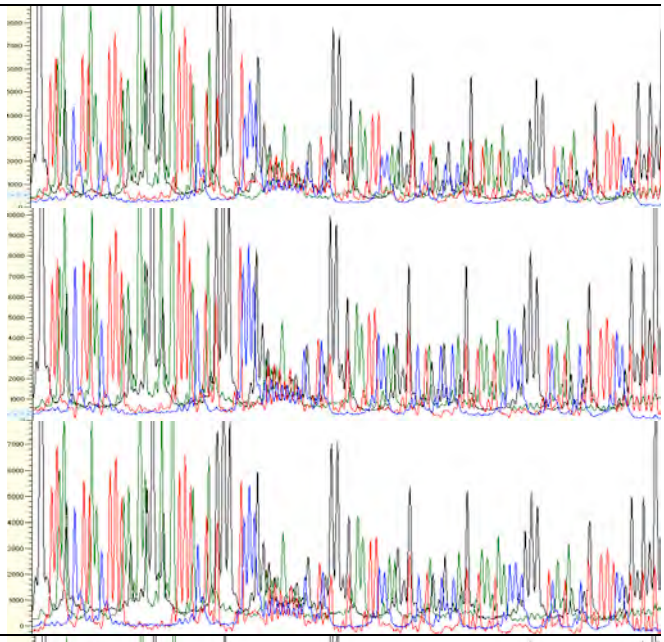
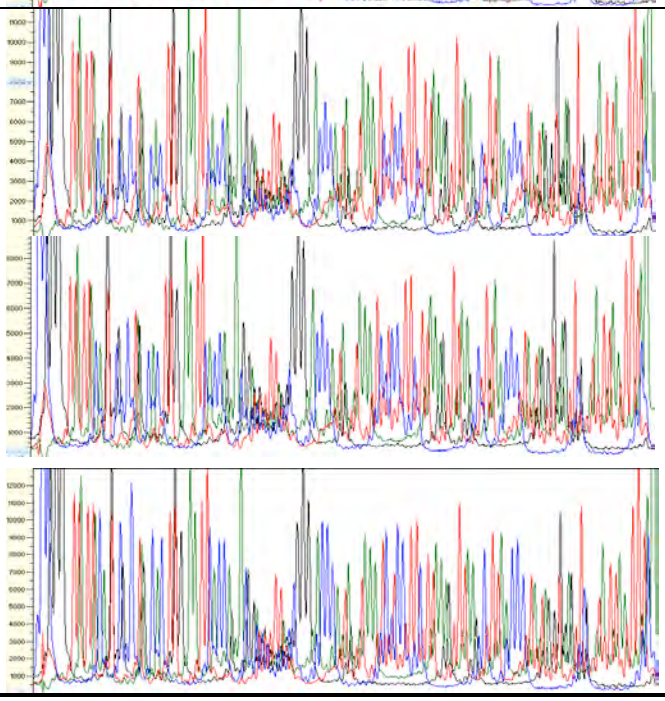
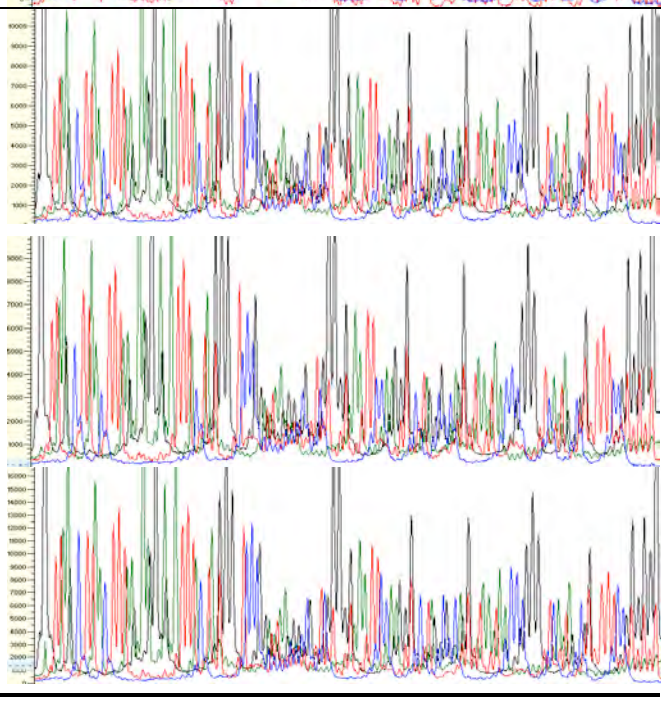
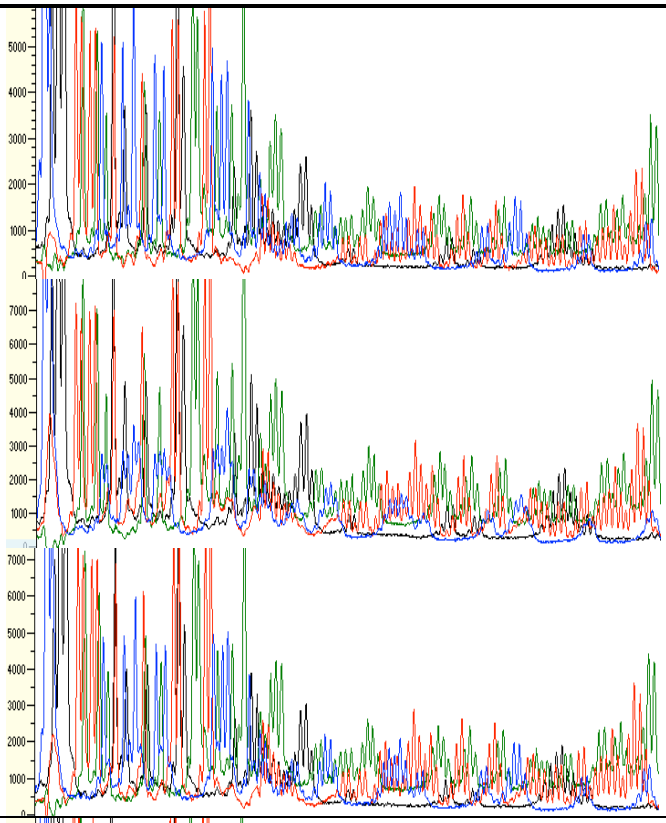
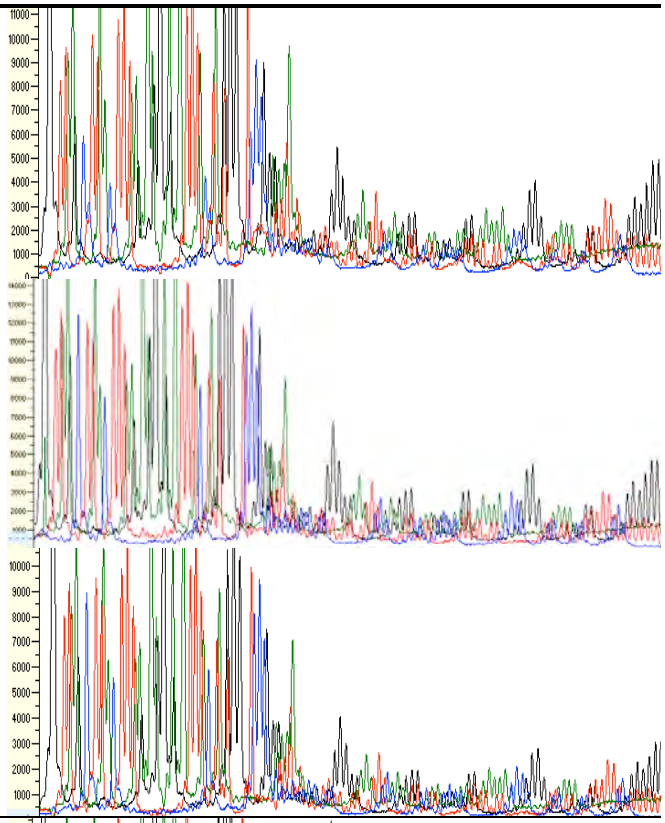
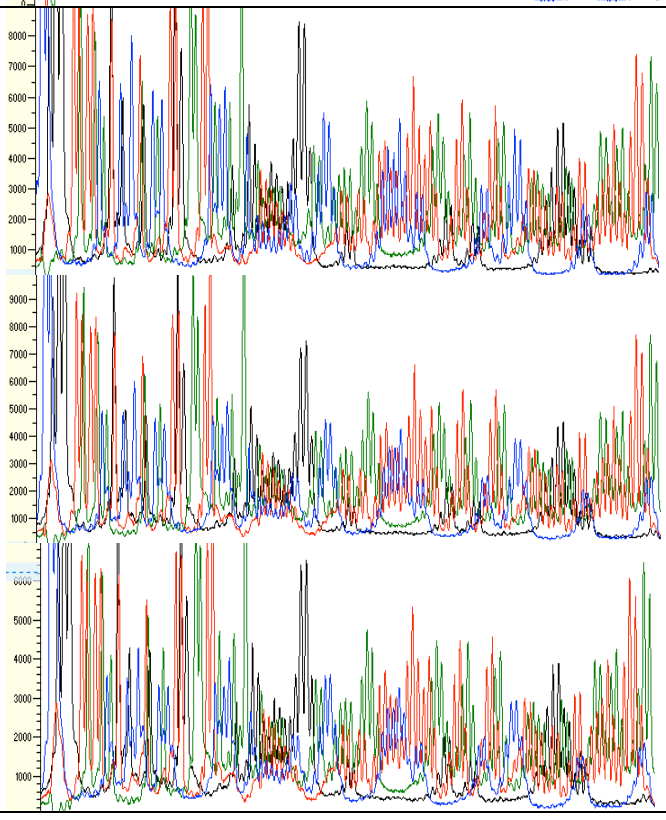
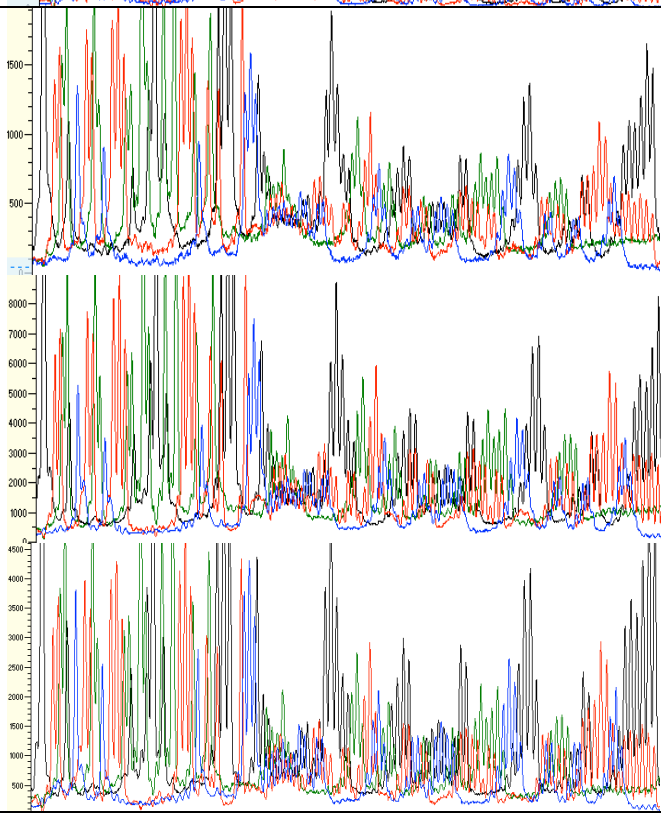
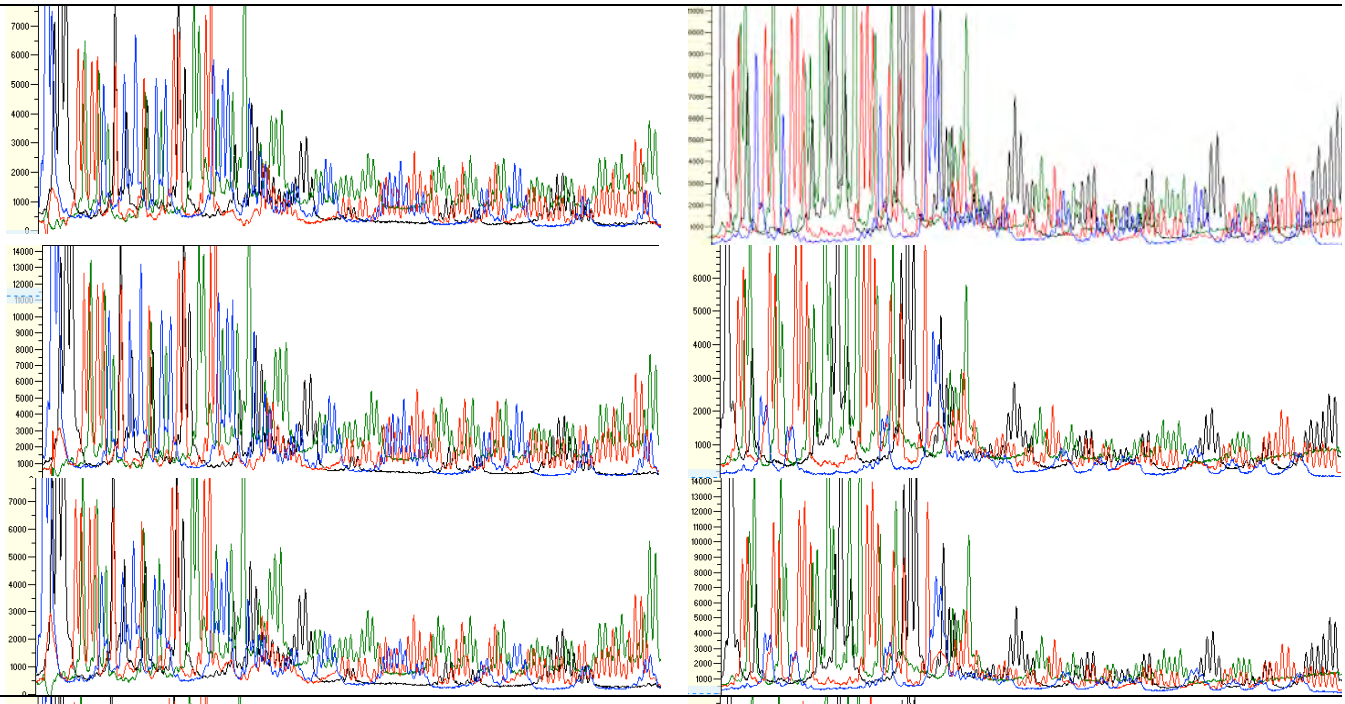
5FM		
Only natural dNTPs		

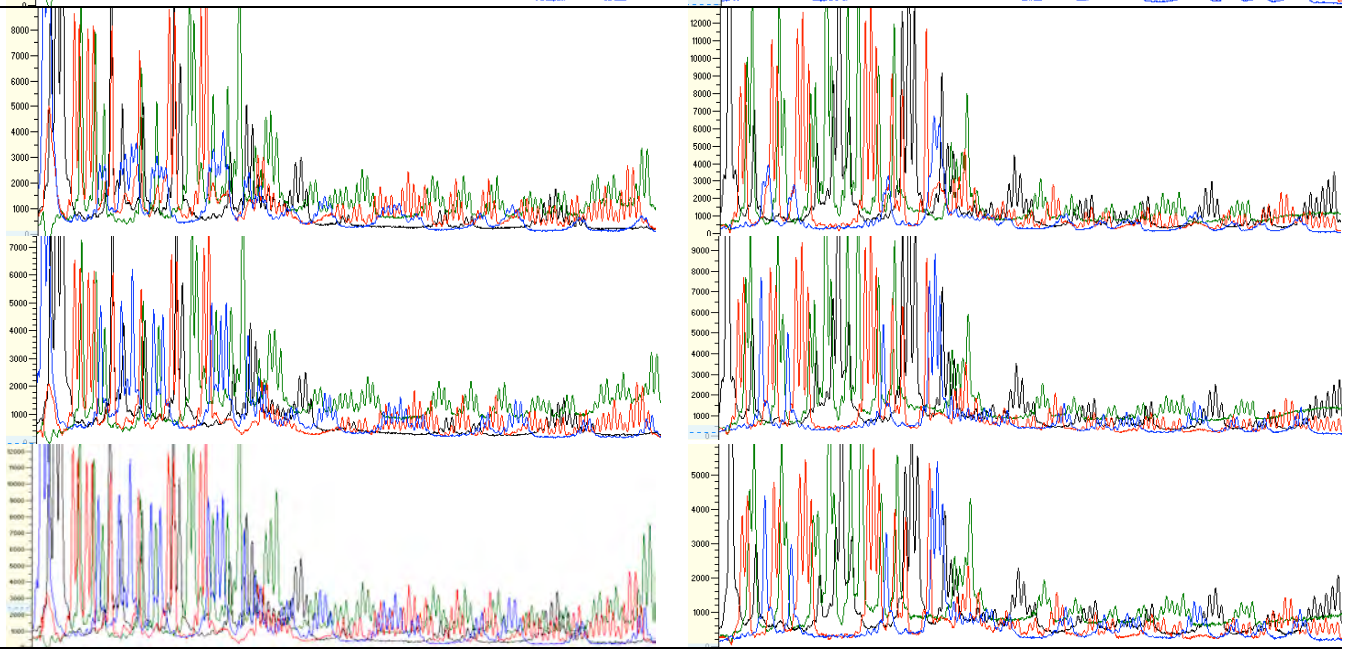
Figure S77. Raw sequencing data after PCR amplification by KOD polymerase in the presence of d**5SICS**TP and d**X**TP. Triplicate data shown.

X	Primer 1	Primer 2
EMO		
IMO		

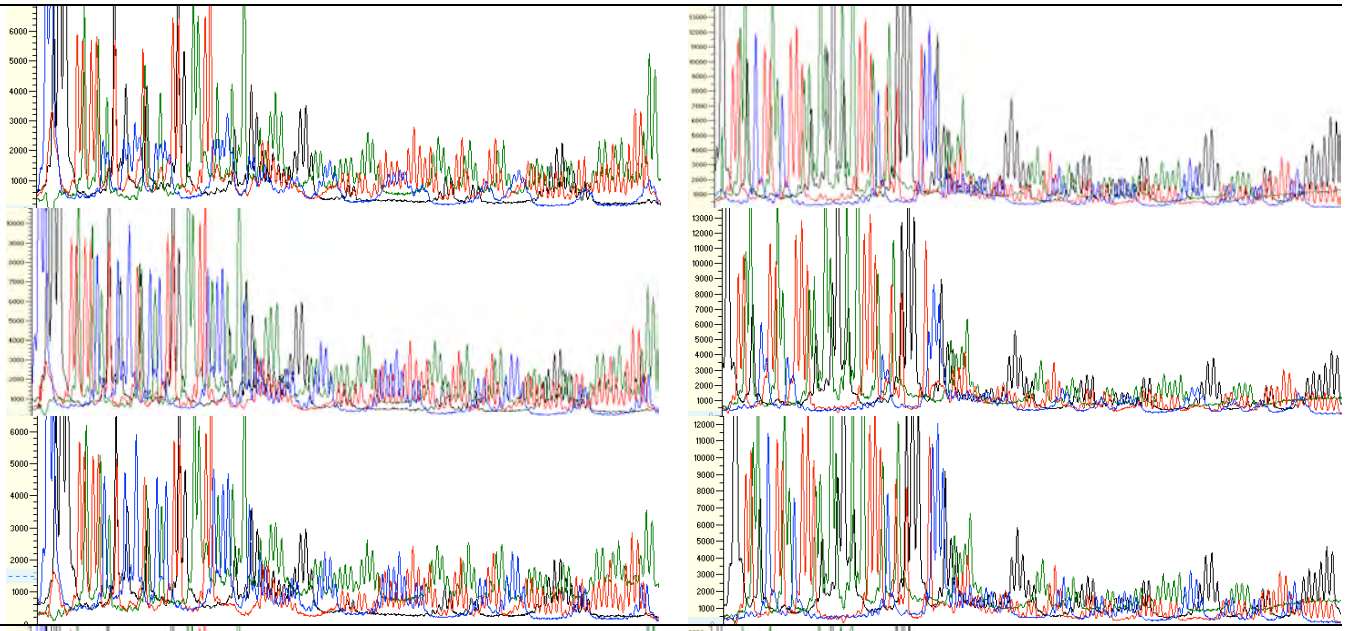
ClMO



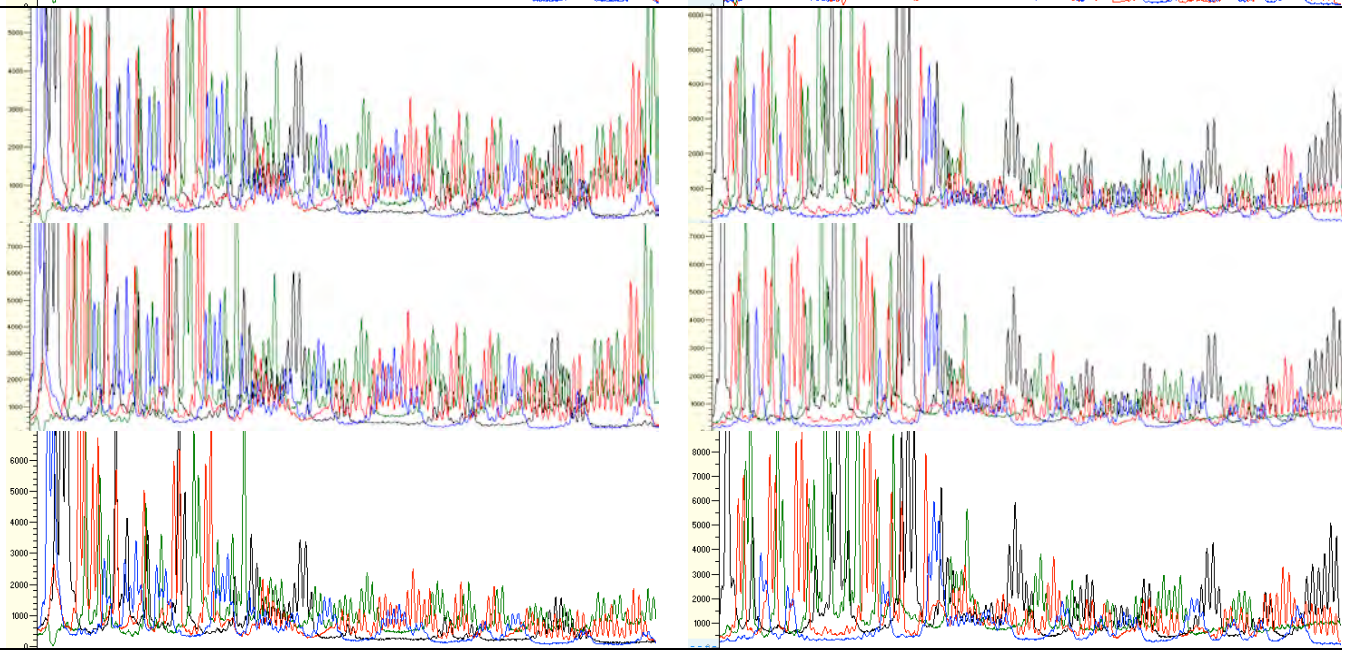
CNMO



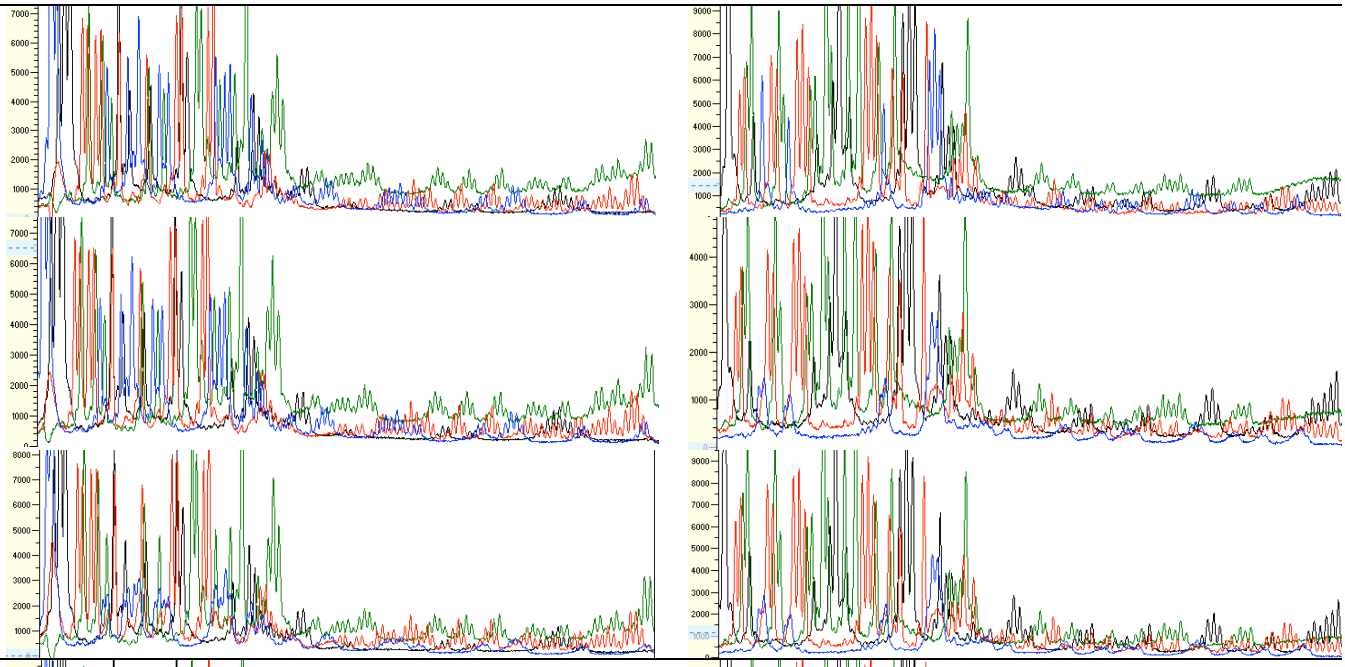
ZMO



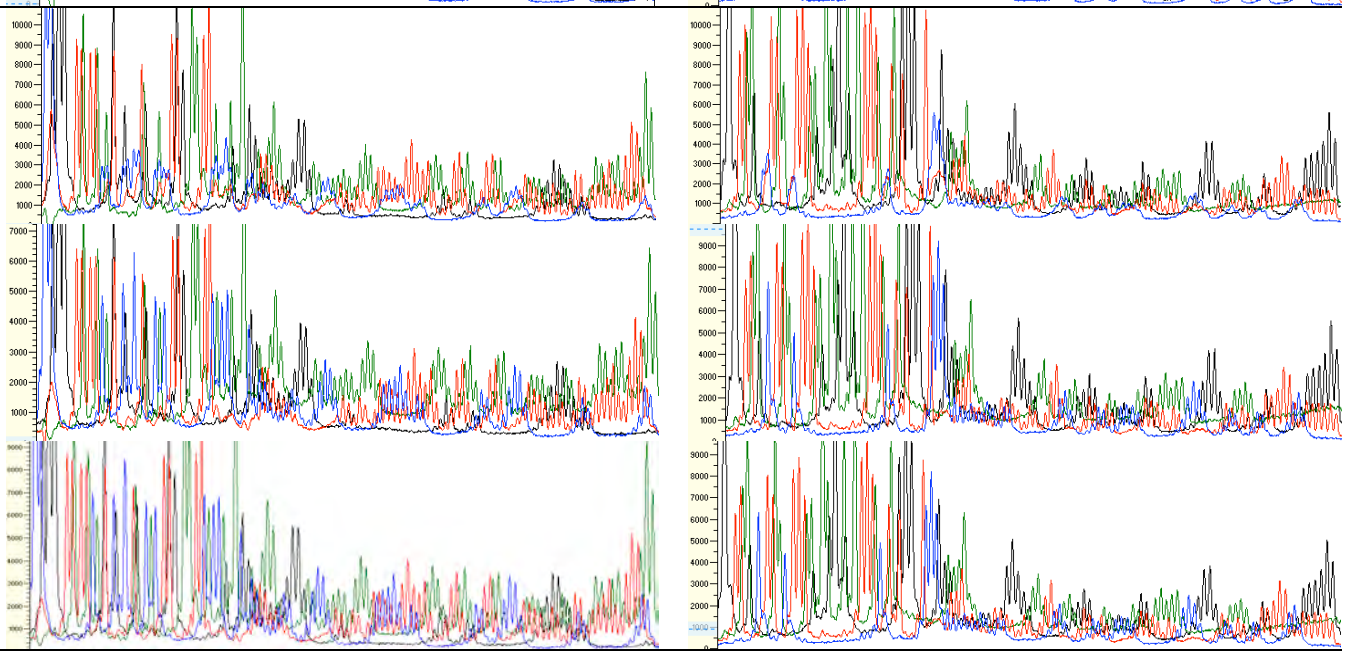
FIMO



FEMO



NaM



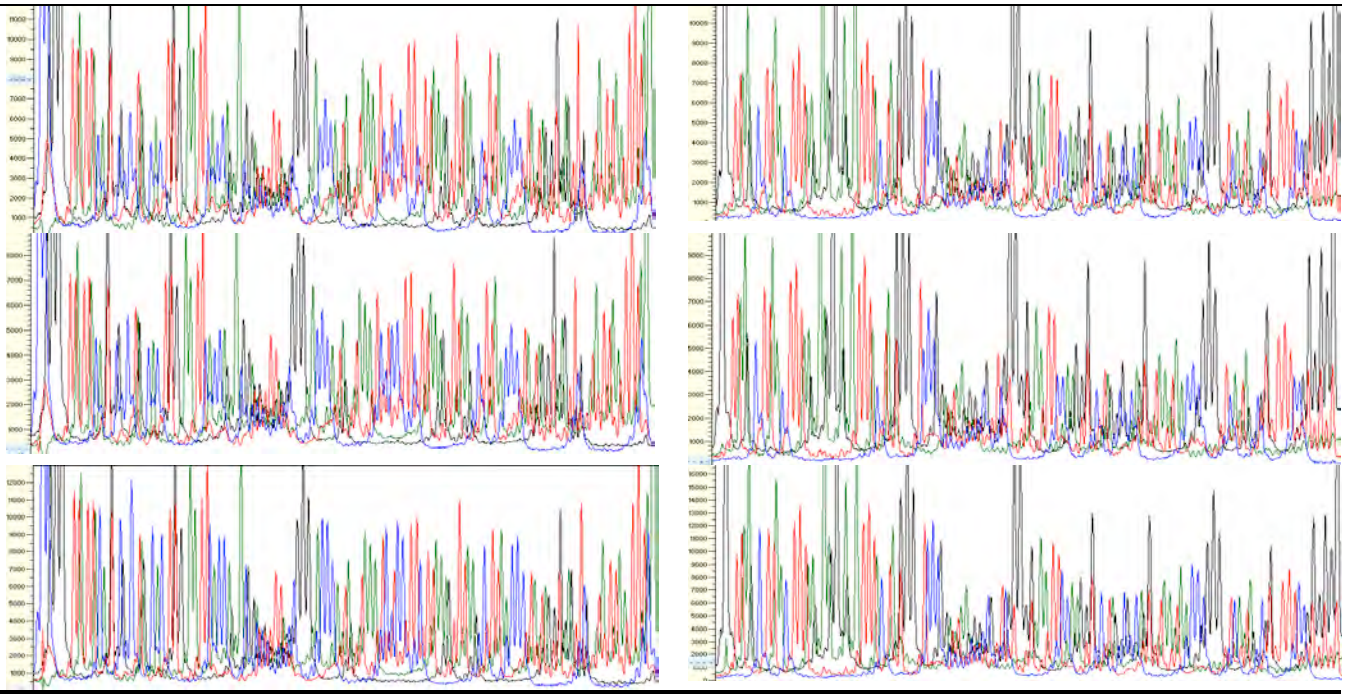
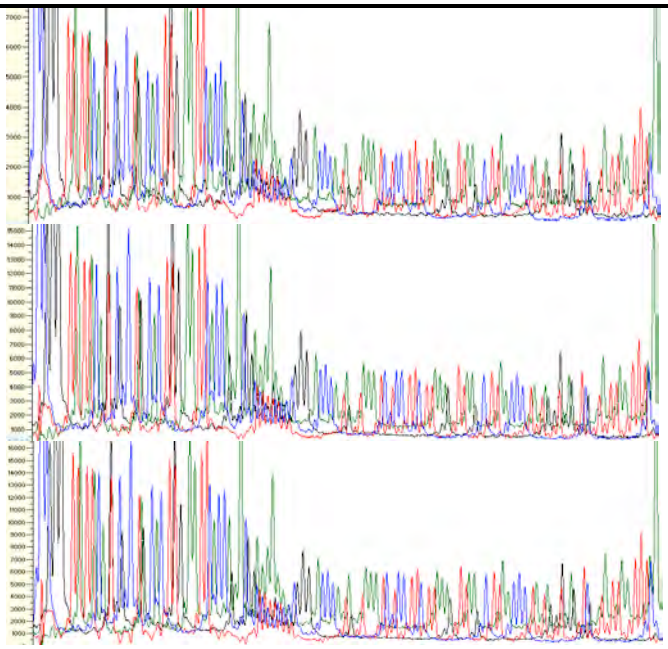
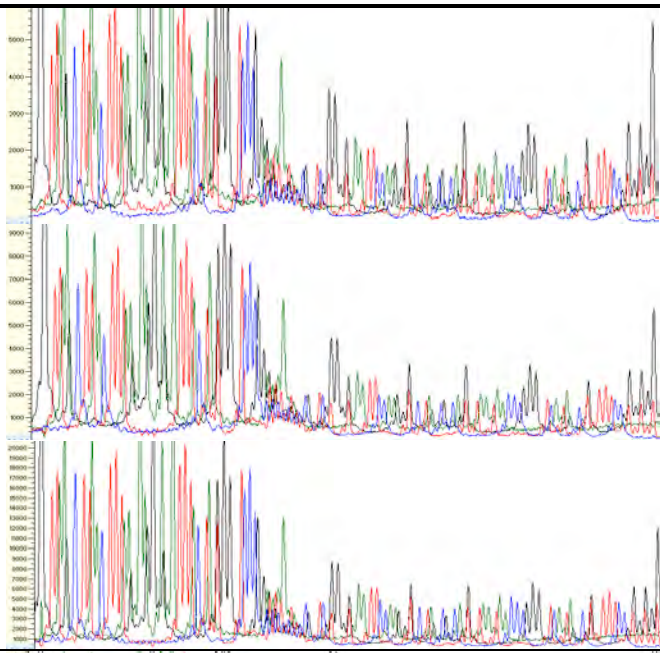
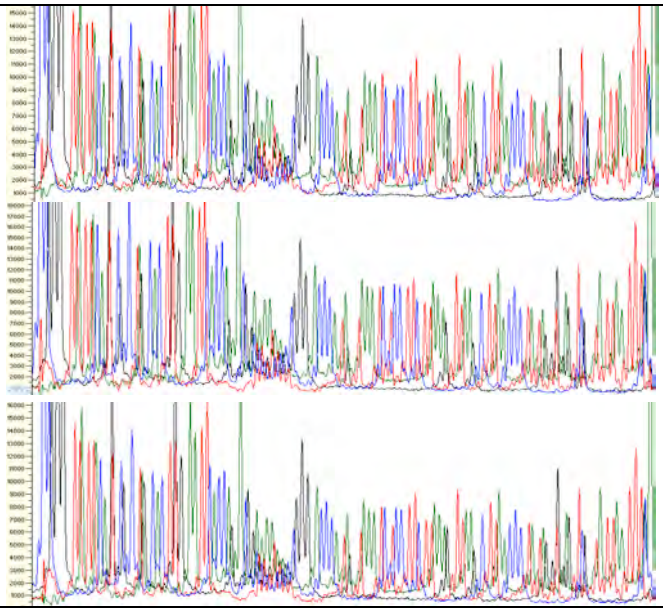
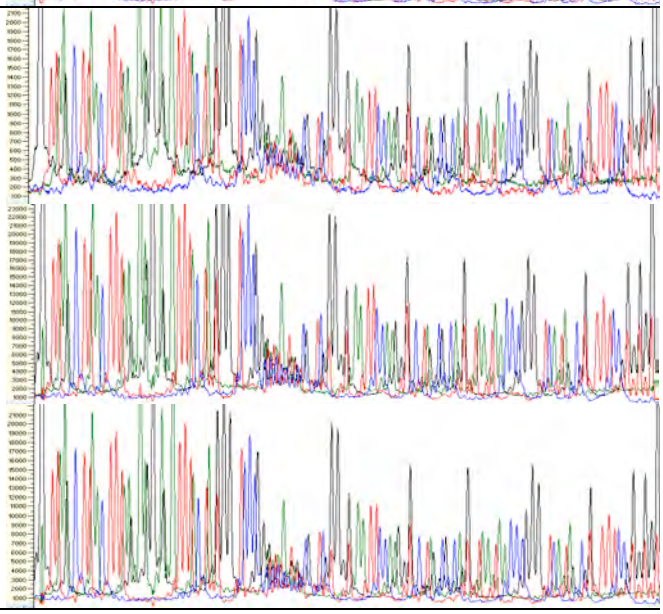
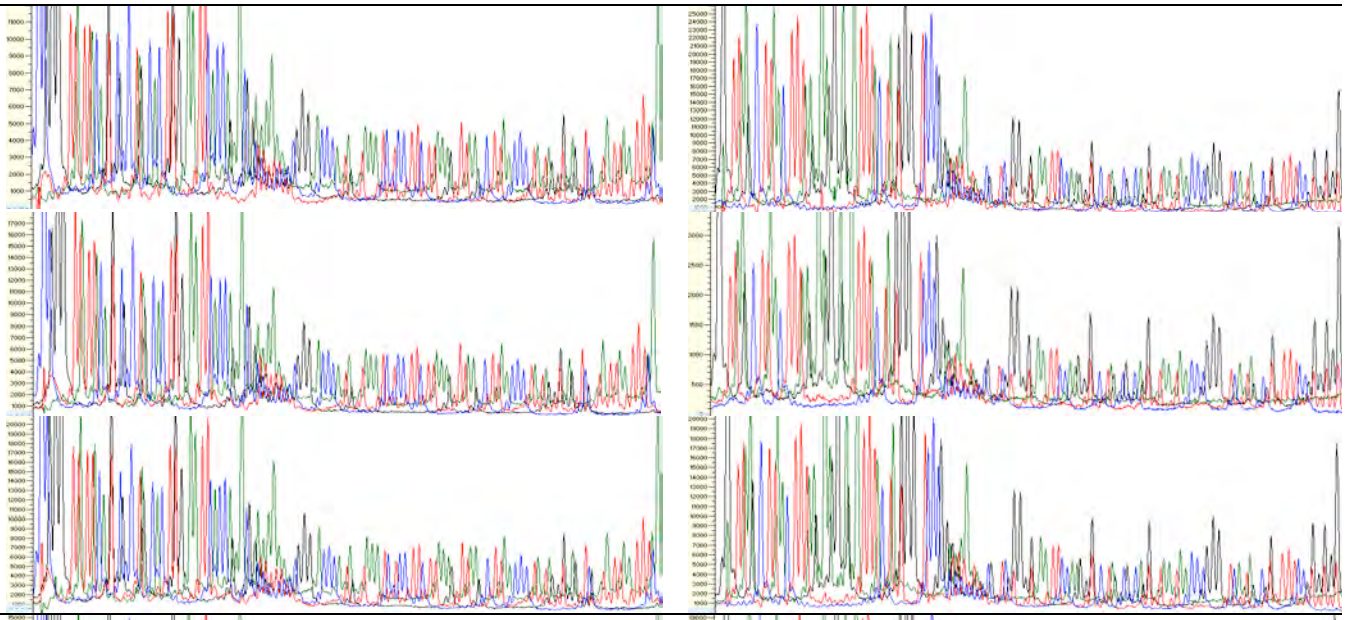
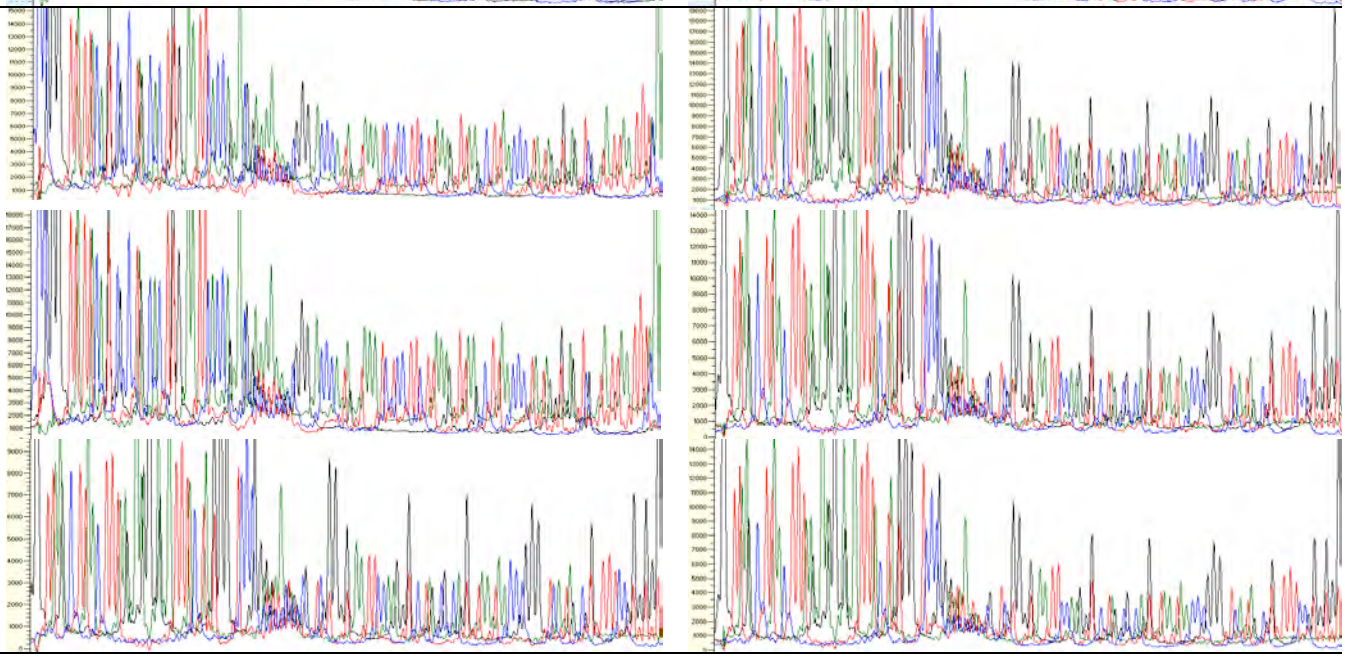
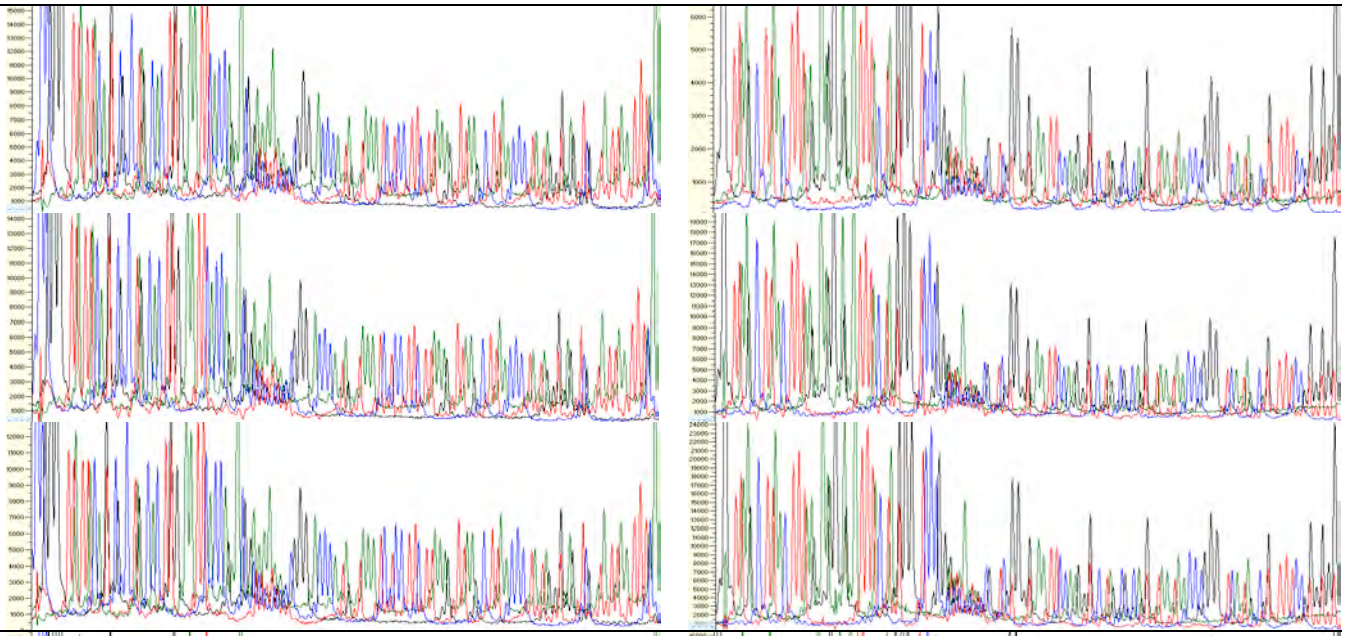
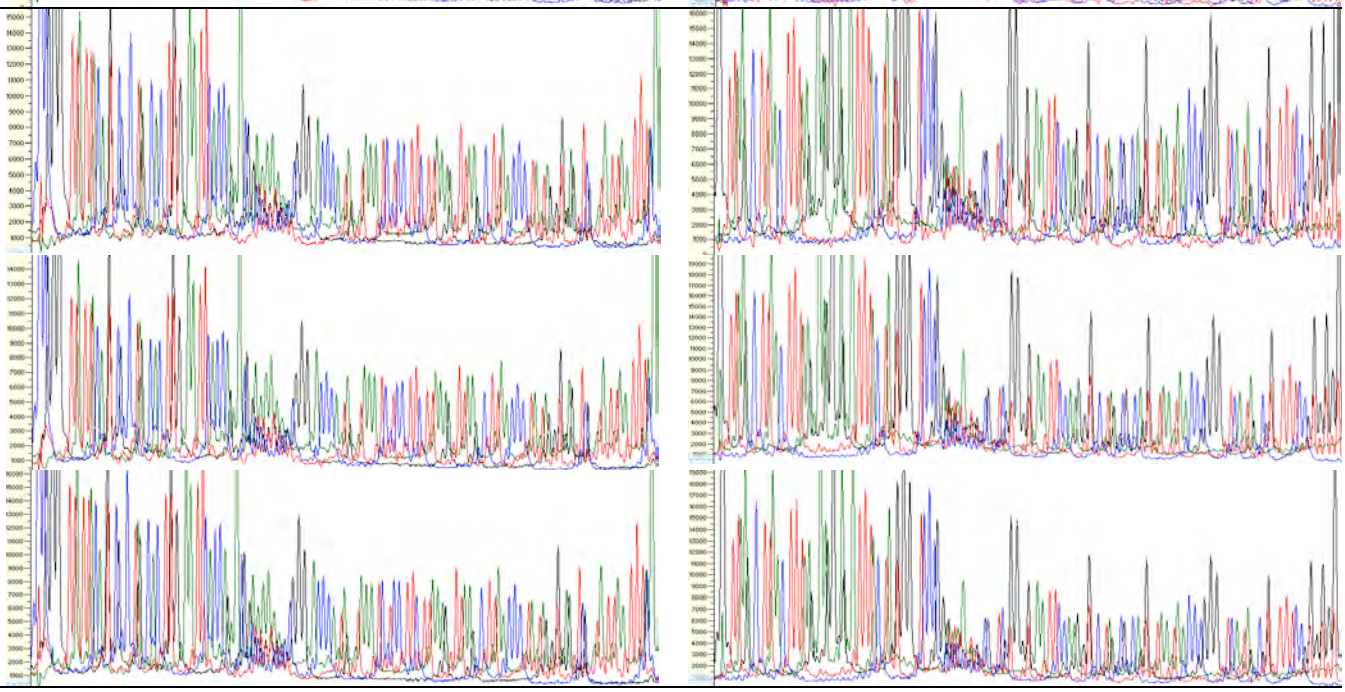
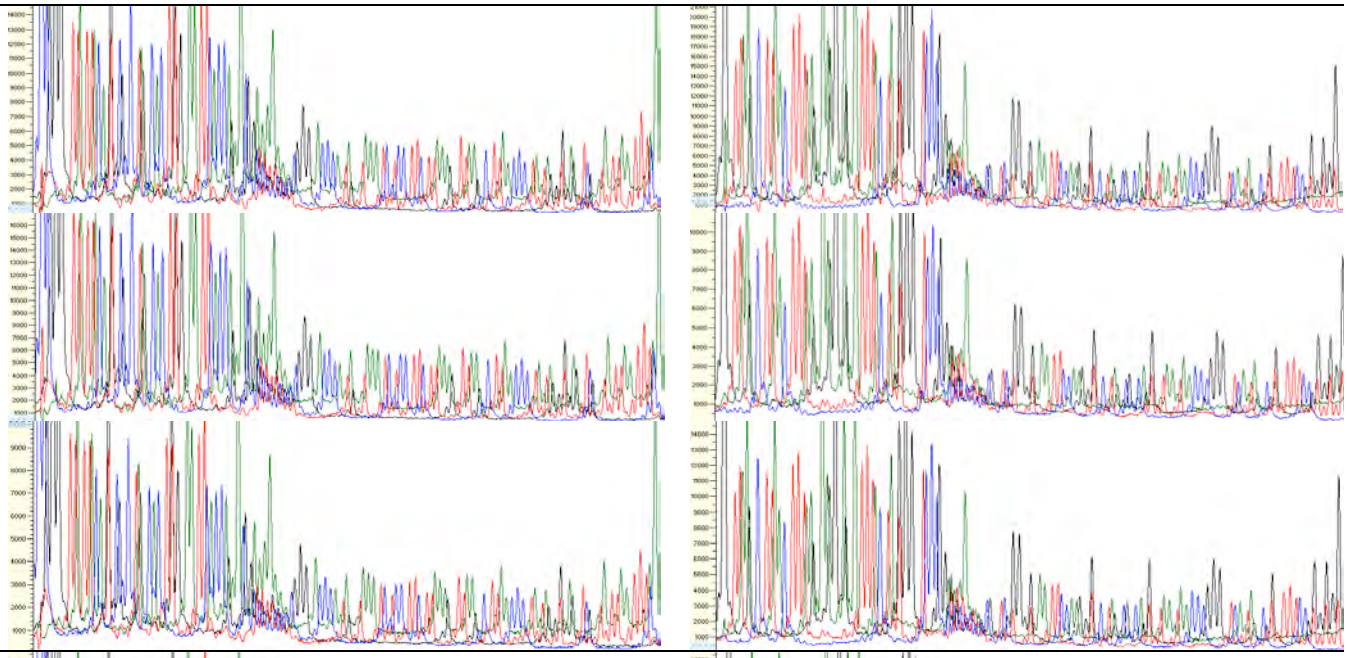
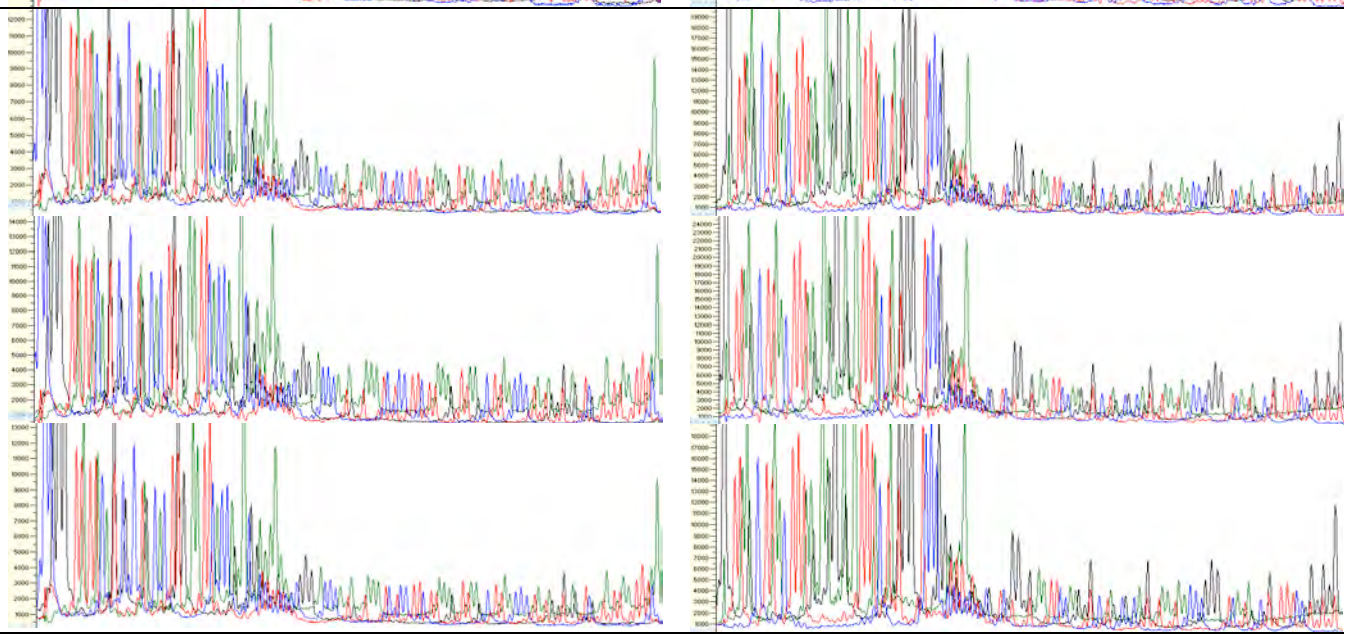
Only natural dNTPs	

Figure S78. Raw sequencing data after 10^{13} fold PCR amplification by OneTaq polymerase in the presence of d**5SICS**TP and d**X**TP. Triplicate data shown.

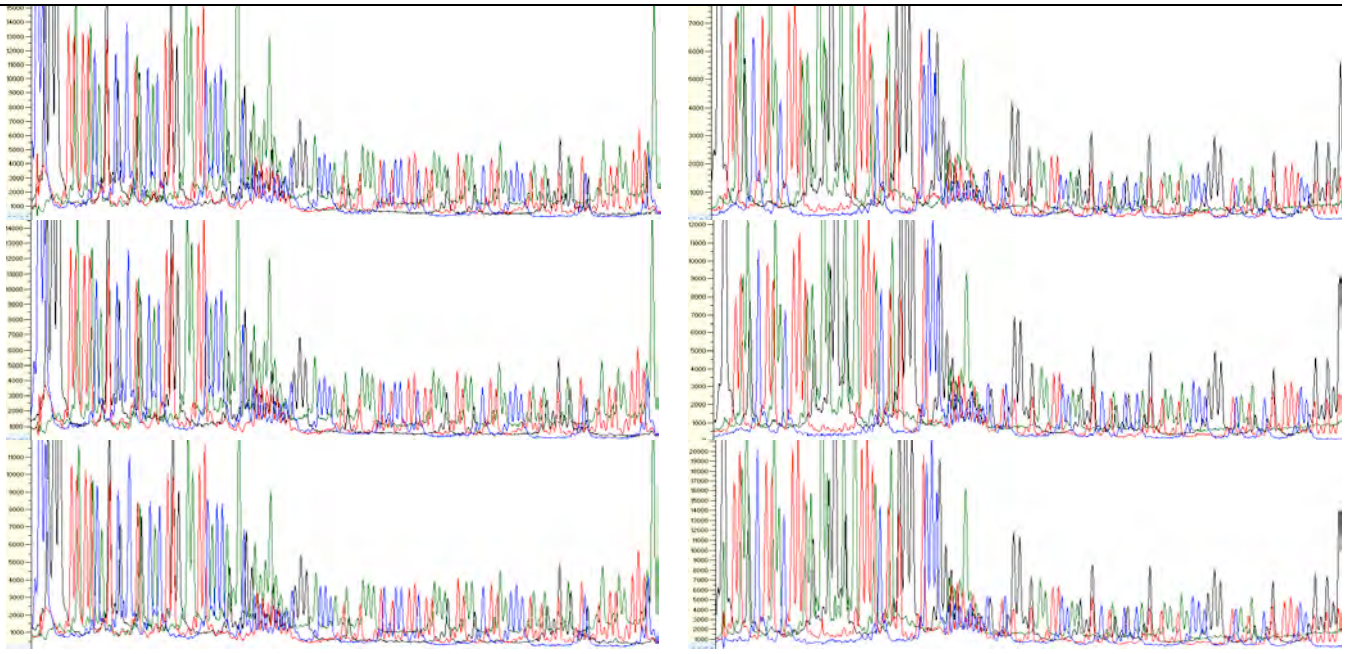
X	Primer 1	Primer 2
EMO		
NMO1		

IMO	
ClMO	

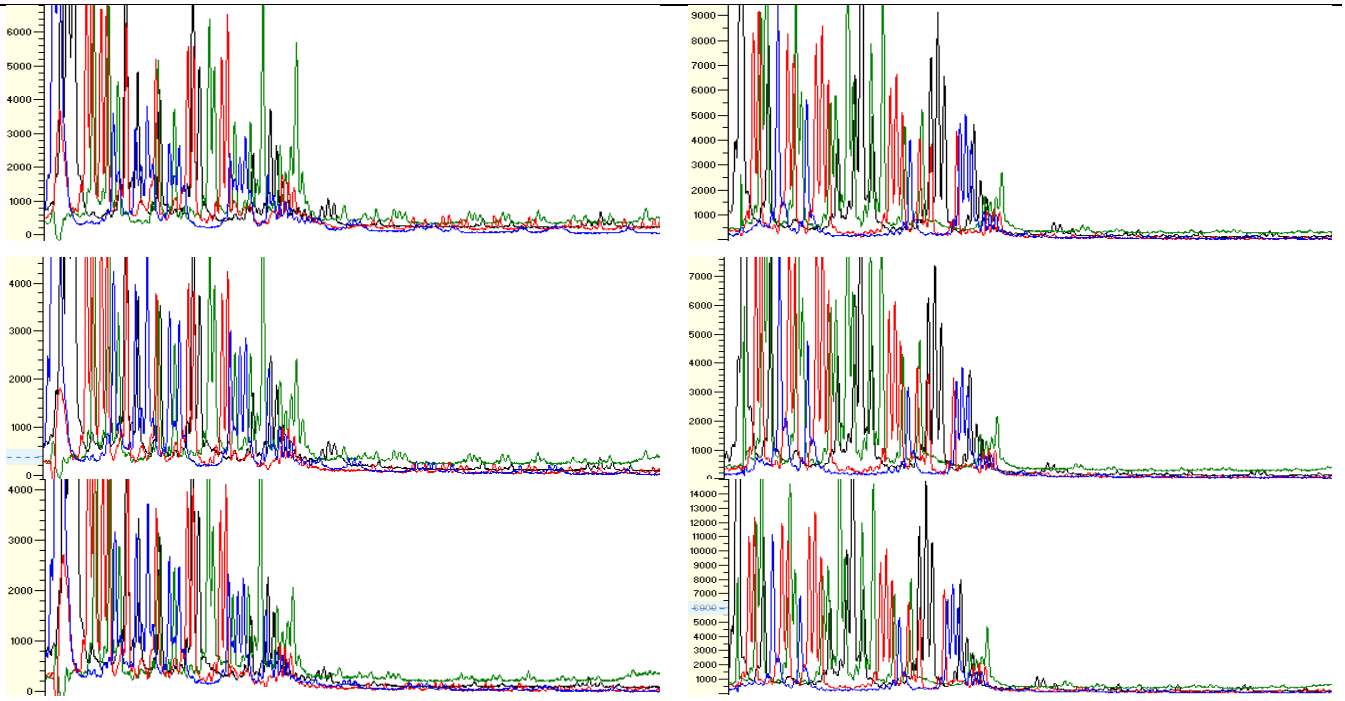
CNMO	
VMO	

ZMO	
FIMO	

FEMO



NaM



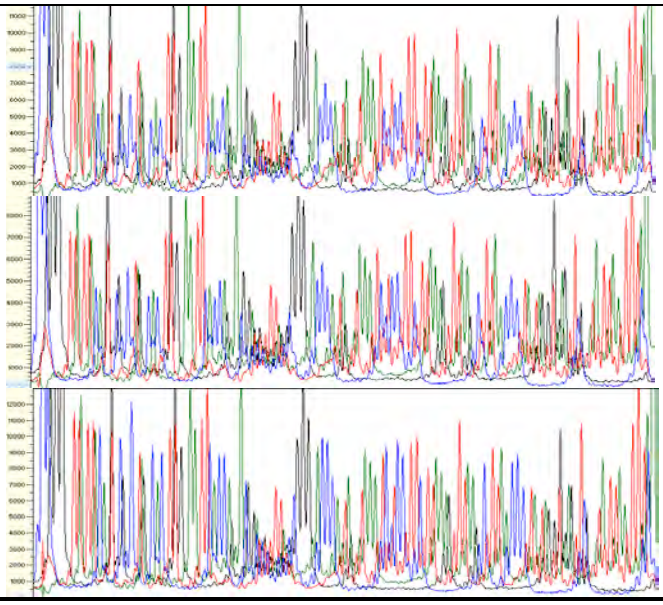
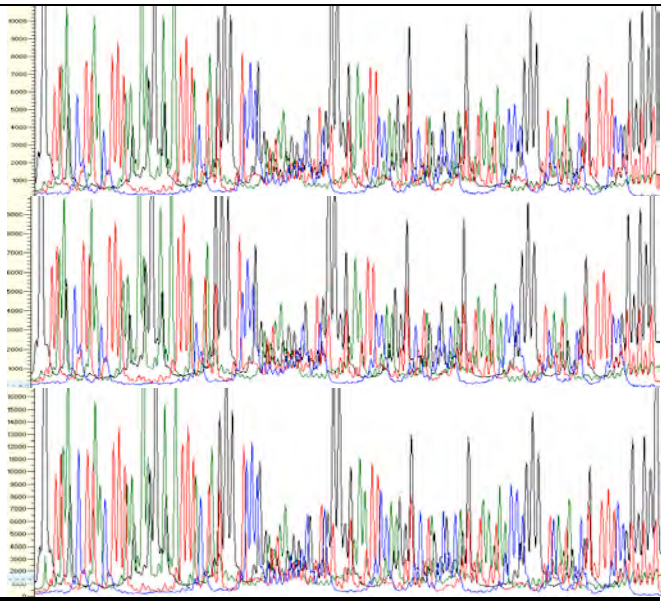
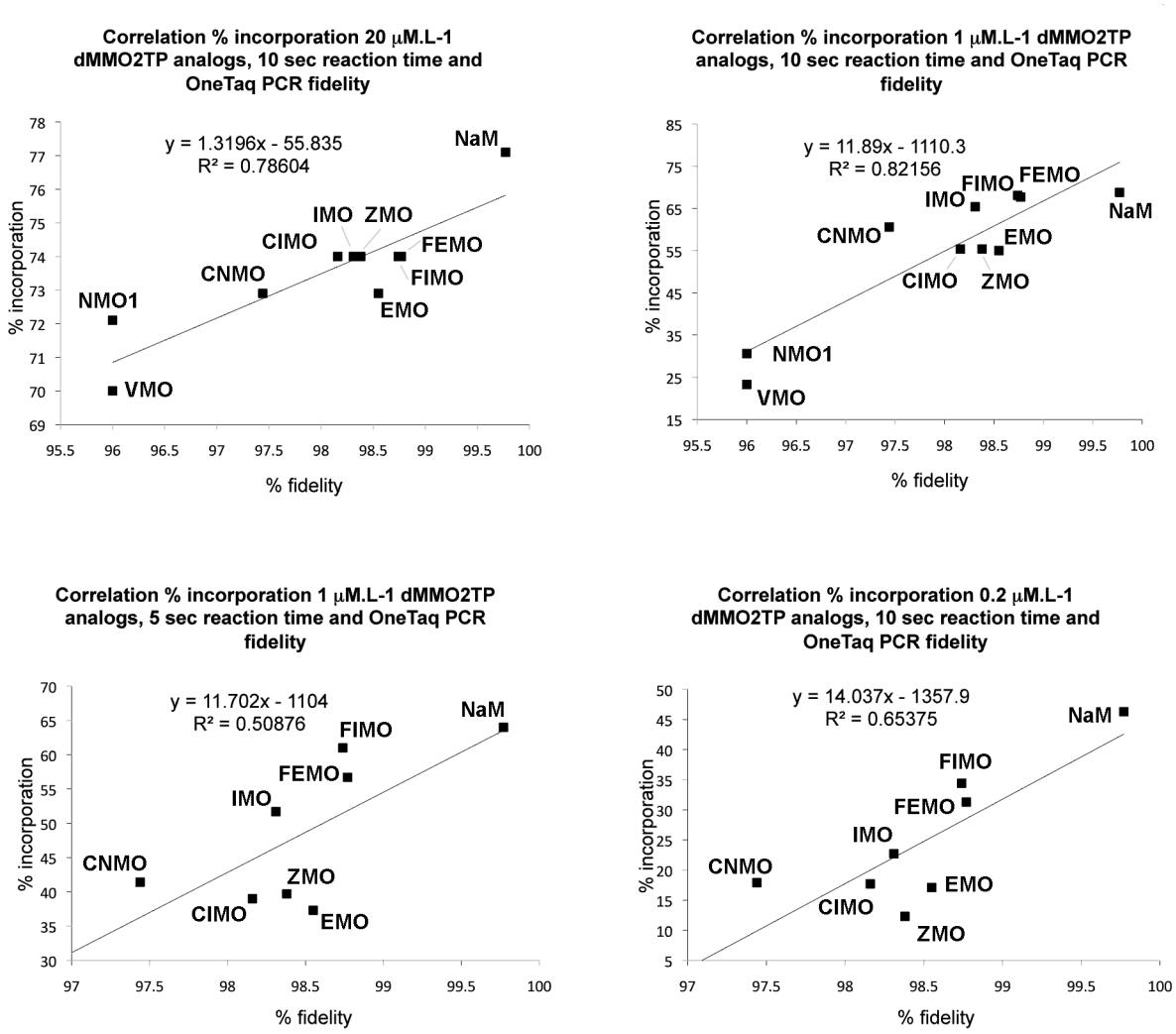
Only natural dNTPs		

Figure S79. Correlation between %incorporation and PCR fidelity



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