Supporting Information

2'-*N*-Guanidino,4'-*C*-Ethylene Bridged Thymidine (GENA-T) modified Oligonucleotide Exhibits Triplex Formation with Excellent Enzymatic Stability

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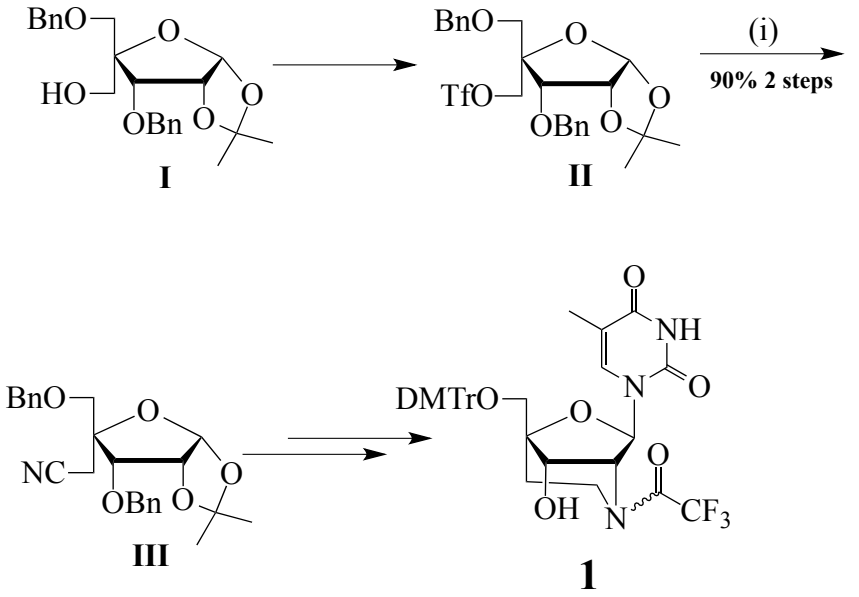
Materials and Methods

All chemicals were purchased from Sigma Aldrich, Sweden, unless otherwise stated. Chromatographic separations were performed on Merck G60 silica gel. Thin layer chromatography (TLC) was performed on Merck pre-coated silica gel 60 F_{254} glass-backed plates. ^{1}H NMR spectra were recorded on Jeol JNM-ECP Series FT NMR system at a magnetic field strength of 9.4 T, operating at 400 MHz for ^{1}H with TMS (0.0 ppm) as internal standards. ^{13}C NMR spectra were recorded at 100 MHz, using the central peak of $CDCl_3$ (76.9 ppm) as an internal standard. ^{31}P NMR spectra were recorded at 81 MHz using 85% phosphoric acid as external standard. Chemical shifts are reported in ppm (δ scale). Compound names for the bicyclic structures are given according to the von Baeyer nomenclature. MALDI-TOF mass spectra were recorded in positive ion mode for oligonucleotides and for other compounds as indicated. The mass spectrometer was externally calibrated with a peptide mixture using alpha-cyano-4-hydroxycinnamic acids as matrix. Thermal denaturation experiments were performed on a PC-computer interfaced UV/VIS spectrophotometer with Peltier PTP-1+1 and PCB 1500 temperature controller system.



Scheme S1. Synthetic route for compound **1**.

Reagents and conditions: (i) (a) Tf_2O, pyridine, CH_2Cl_2, 0 °C, 3 h; (b) acetone cyanohydrin, MeLi, LiH, DMF, r.t., 3 days. The synthetic procedure for other steps was identical with that of aza-ENA.

Experimental Section

3,5-Di-O-benzyl-4-C-cyanomethyl-1,2-O-isopropylidene-R-D-ribofuranose (III). The sugar **I** (8.5 g, 21.25 mmol) was dissolved in an anhydrous dichloromethane/pyridine mixture (210 mL, 3:1, v/v) and cooled in an ice bath. To this solution, triflic anhydride (4.3 mL, 25.5 mmol) was added dropwise and stirred for 3 h under nitrogen atmosphere. The reaction was quenched with cold saturated aqueous $NaHCO_3$ and extracted with dichloromethane. The organic phase was dried over anhydrous $MgSO_4$ and evaporated under reduced pressure followed by co-evaporation with toluene (three times) and dichloromethane (three times) to give **II**. For preparing cyano sugar, we generated LiCN *in situ* by slighly modifying previously published article.[1] Briefly, MeLi in diethyl ether (1.6 M, 40 mL, 63.85 mmol) was added dropwise via syringe to an anhydrous hexane (50 mL) solution containing acetone cyanohydrin (8.75 mL, 95.86 mmol) at -10 °C under N_2. The

resulting white suspension was warmed to room temperature and stirred for additional 30 min. Rotary evaporation of the suspension afforded LiCN.acetone as a white solid (98% yield for a 1:1 complex). Sugar **II** was dissolved in anhydrous DMF (50 mL) and was slowly added to the LiCN.acetone complex suspension in anhydrous DMF (90 mL). The reaction was stirred for 3 days at room temperature. The solvent was evaporated in vacuo, and the residue was dissolved in dichloromethane. Saturated aqueous $NaHCO_3$ was added to this solution and extracted with dichloromethane (three times). The organic phase was collected and dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography (0-20% ethylacetate in cyclohexane, v/v), which afforded **III** (7.9 g, 19.31 mmol, 90% after two steps). R_f = 0.61 (60:40 cyclohexane/ ethylacetate (v/v)). All analytical data were identical to those previously reported.[2]

Synthesis of ***N,N'-di((2-cyanoethoxy)carbonyl)-S-methylisothiourea*** **(2)**: This reagent was synthesized according to the previous report.[3]

2-Cyanoethyl(((1R,5R,7R,8S)-5-(4,4'-dimethoxytrityloxymethyl)-8-hydroxy-7-(thymin-1-yl)-2-aza-6-oxabicyclo[3.2.1]octan-2-yl)(((2-cyanoethoxy)carbonyl)amino)methylene)carbamate **(3)**. Nucleoside 1 (100 mg, 0.14 mmol) was dissolved in 40% aqueous methylamine (0.15 mL) and stirred for 1.5 h, thereafter the solvent was evaporated, co-evaporated with anhydrous pyridine to remove traces of water and dissolved in 1.5 mL of dry CH_2Cl_2. To this 100 mg (0.36 mmol) of guanylating reagent and 0.08 mL (0.58 mmol) of triethylamine was added and stirred at r.t. for 4 days. The solvent was evaporated in vacuum and the crude product was purified by silica gel column chromatography (0-3% methanol in dichloromethane, v/v containing 1% pyridine) to afford 3 (58 mg, 48% in two steps). R_f = 0.56 (CH_2Cl_2/CH_3OH 94:6 v/v); MALDI-TOF *m/z* $[M + Na]^+$ found 821.5, calcd 821.3; ^{13}C NMR (100 MHz, $CDCl_3$ + DABCO): 164.2, 158.8, 155.0, 150.3, 144.4, 137.9, 134.4, 135.2, 135.0, 130.2, 129.1, 128.3, 128.1, 127.2, 125.3, 113.4, 110.4, 86.8, 86.4, 85.4, 65.7, 63.6, 61.2, 60.2, 55.3, 38.4, 29.7, 26.2, 21.5, 18.7, 18.4, 12.1.

2-Cyanoethyl(((1R,5R,7R,8S)-5-((4,4'-dimethoxytrityloxymethyl)-8-(((2-cyanoethoxy)(diisopropylamino)phosphino)oxy)-7-(thymin-1-yl)-2-aza-6-

***oxabicyclo[3.2.1]octan-2-yl)(((2-cyanoethoxy)carbonyl)amino)methylene)carbamate* (4)**. Compound 3 (100 mg, 0.12 mmol) was dissolved in 1.2 mL of dry CH_2Cl_2, diisopropylethylamine (0.12 mL, 0.66 mmol) was added at 0 °C followed by 2-cyanoethyl *N, N*-diisopropylphosphoramido chloridite (0.04 mL, 0.2 mmol). After 30 min. reaction was warmed to room temperature and stirred overnight. MeOH (0.5 mL) was added and stirring was continued for 5 min. thereafter saturated aqueous $NaHCO_3$ was added and extracted with freshly distilled CH_2Cl_2 (3 × 20 mL). The organic phase was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (40-100% CH_2Cl_2 in cyclohexane containing 1% Et_3N) afforded 4 (645 mg, 0.73 mmol, 61%) as a mixture of four isomers. R_f = 0.7 (CH_2Cl_2/CH_3OH 94:6 v/v); MALDI-TOF *m/z* $[M + Na]^+$ found 1044.6, calcd 1044.4; ^{31}P NMR (109.4 MHz, $CDCl_3$): 150.7, 150.3, 149.3, 148.8.

Synthesis, deprotection and purification of ONs

All oligonucleotides were synthesized using BioAutomation MerMade 4 DNA/RNA Synthesizer. For modified ONs containing aza-ENA-T units, fast deprotecting phosphoramidites (nucleobases were protected using the following groups: Ac for C, ^{i}Pr-PAC for G, and PAC for A) were used. These ONs were de-protected at room temperature by aqueous NH_3 treatment for 24 h. For GENA containing ONs, 50% piperidine in water was used to deprotect ONs from solid support, for 24 h at rt. The solid support was filtered, washed with water and evaporated. The crude material is further treated with aqueous NH_3 for 24 h. All ONs and the target RNA and DNA were purified by 20% polyacrylamide/7M urea) PAGE, extracted with 0.3 M NaOAc, desalted with C18-reverse phase cartridges and their purity (greater than 95%) was confirmed by PAGE.

^{32}P Labelling of ONs

The oligoribonucleotide, oligodeoxyribonucleotides were 5'-end labeled with ^{32}P using T4 polynucleotide kinase and [γ-^{32}P] ATP by standard procedure. Labeled ONs and RNA were purified by 20% denaturing PAGE and specific activities were measured using Beckman LS 3801 counter.

3'-Exonuclease degradation studies

Stability of the ONs toward 3'-exonucleases was tested using snake venom phosphodiesterase from Crotalus adamanteus. All reactions were performed at 3 μM DNA concentration (5'-end ^{32}P labeled with specific activity 50,000 cpm) in 56 mM Tris-HCl (pH 7.9) and 4.4 mM $MgCl_2$ at 21 °C. Exonuclease concentration of 17 ng/μL was used for digestion of oligonucleotides. Total reaction volume was 14 μL. Aliquots (3 μL) were taken at 0, 2, 4, 9 and 24 h and quenched by addition to 7 μL volume of 50 mM EDTA in 80% formamide. Reaction progress was monitored by 20% denaturing (7 M urea) PAGE and autoradiography.

Stability studies in human serum

ONs (6 μL) at 2 μM concentration (5'-end ^{32}P labeled with specific activity 90 000 cpm) were incubated in 26 μL of human serum (male AB, Sigma Aldrich) at 21 °C (total reaction volume was 36 μL). Aliquots (3 μL) were taken at 0, 2, 4, 10, 24, 33 and 48 h, and quenched with 7 μL quenching solution containing 50 mM EDTA in 80% formamide, resolved in 20% polyacrylamide denaturing (7 M urea) gel electrophoresis and visualized by autoradiography.

UV melting experiments

Determination of the T_m of the ON/RNA hybrids was carried out in the following buffer: 60 mM Tris-HCl at pH 7.5, 60 mM KCl, 0.8 mM $MgCl_2$, and 2 mM DTT. Absorbance was monitored at 260 nm in the temperature range from 20 °C to 70 °C using UV spectrophotometer equipped with PTP-1+1 Peltier system with the heating rate of 1 °C per minute. Prior to measurements, samples (1 μM of ON and 1 μM RNA mixture) were pre-annealed by heating to 80 °C for 5 min followed by slow cooling to 4 °C and 30 min equilibration at this temperature. T_m was determined as the temperature for half dissociation of the formed duplexes, which was determined by the first derivative of the melting curve.

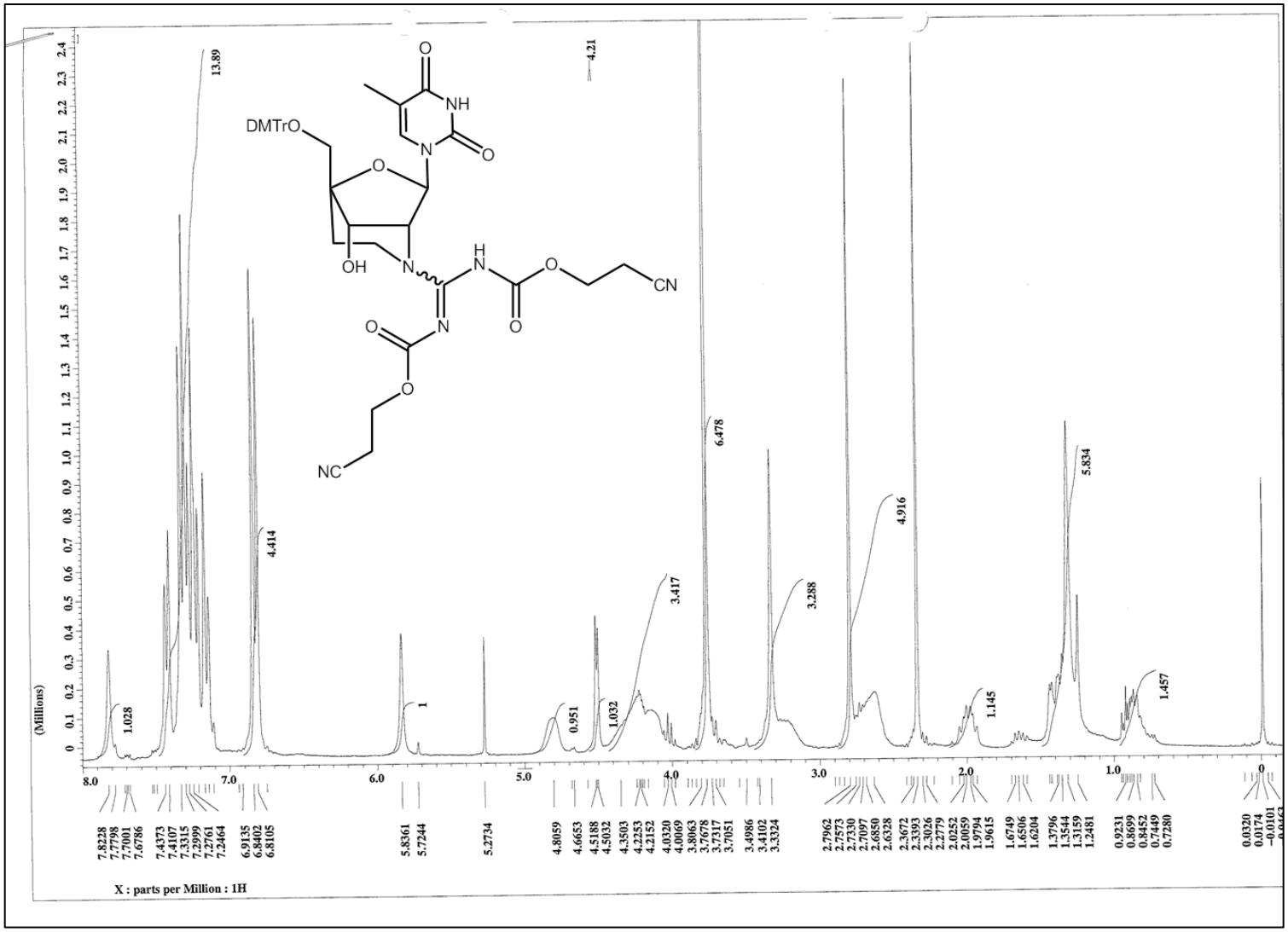
To determine the T_m for triplexes, 7.0 mM Na_2HPO_4 buffer pH 7, containing 140 mM KCl was used to give the total strand concentration of 1 μM. The strands were annealed by heating the samples at 90 °C for 5 minutes followed by slow cooling to room temperature for 1 h. Absorbance was monitored at 260 nm in the temperature range from 20 °C to 85 °C using UV spectrophotometer equipped with PTP-1+1

Peltier system with the heating rate of 0.5 °C per minute. T_m was determined as mentioned above.

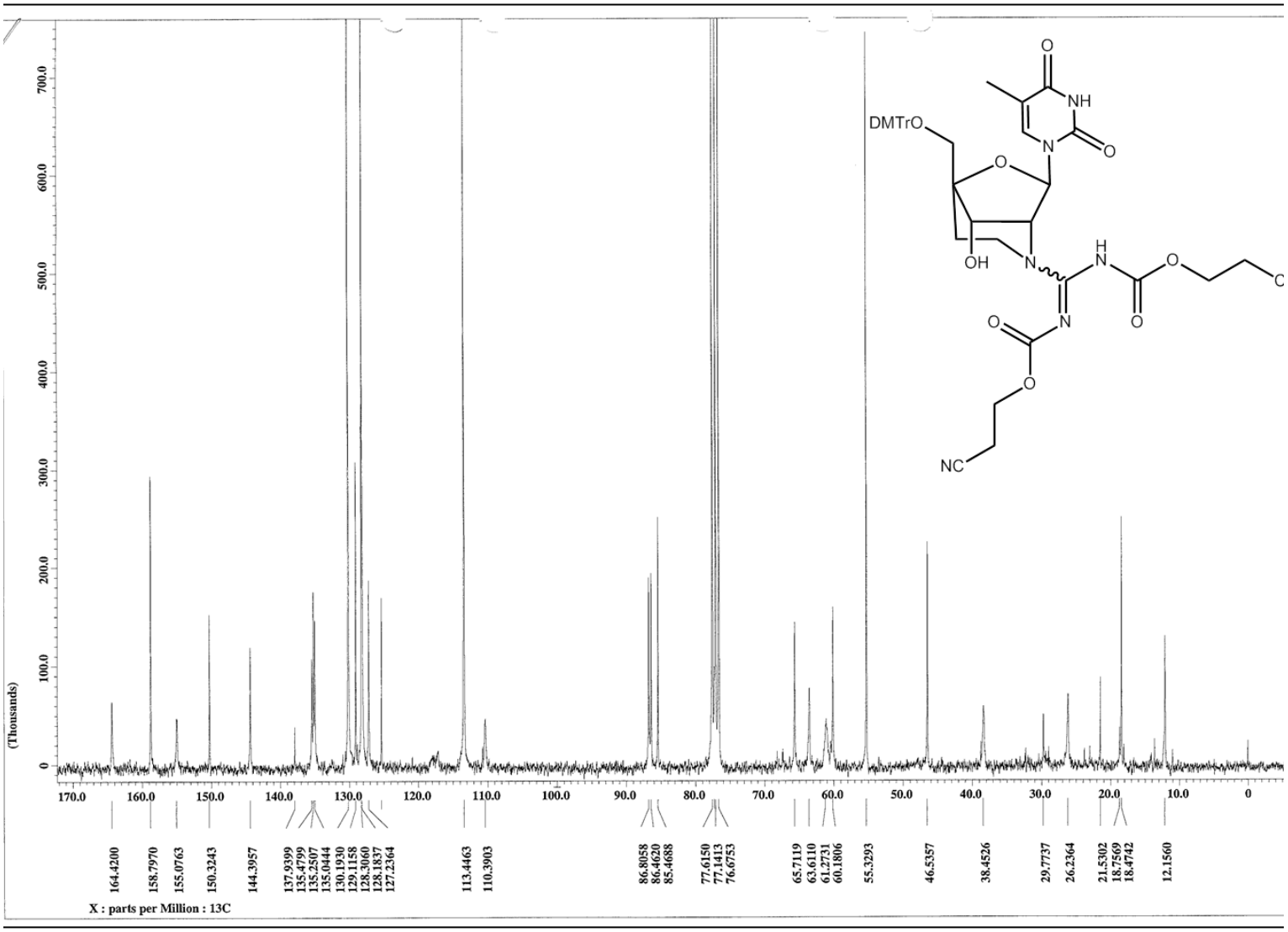
Reference

1. J. A. Ciaccio, M. Smrtka, William A. Maio and D. Rucando, *Tetrahedron Lett.*, 2004, **45**, 7201–7204.
2. O. P. Varghese, J. Barman, W. Pathmasiri, O. Plashkevych, D. Honcharenko and J. Chattopadhyaya, *J. Am. Chem. Soc*., 2006, **128**, 15173–15187.
3. T. P. Prakash, A. Pu1schl, E. Lesnik, V. Mohan, V. Tereshko, M. Egli and M. Manoharan, *Org. Lett*., 2004, **6**, 1971–1974.

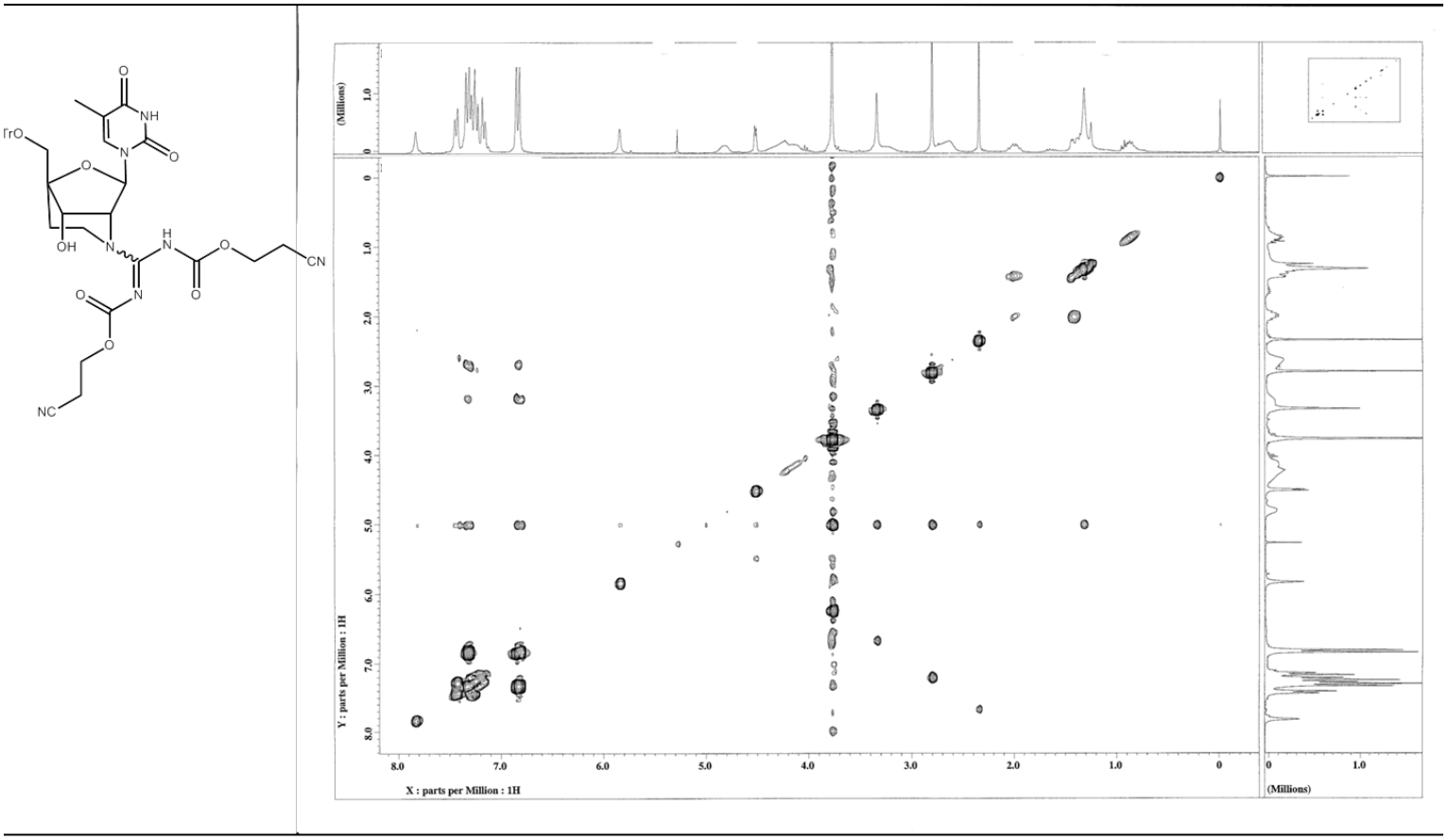
1) ^{1}HNMR spectra of **3**



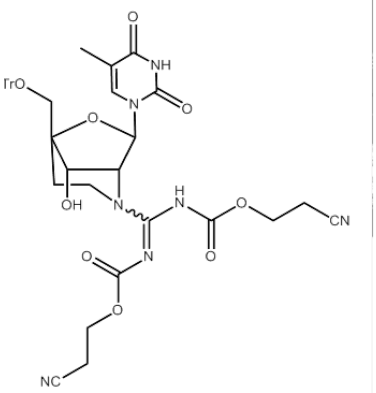
2) ^{13}C spectra of **3**

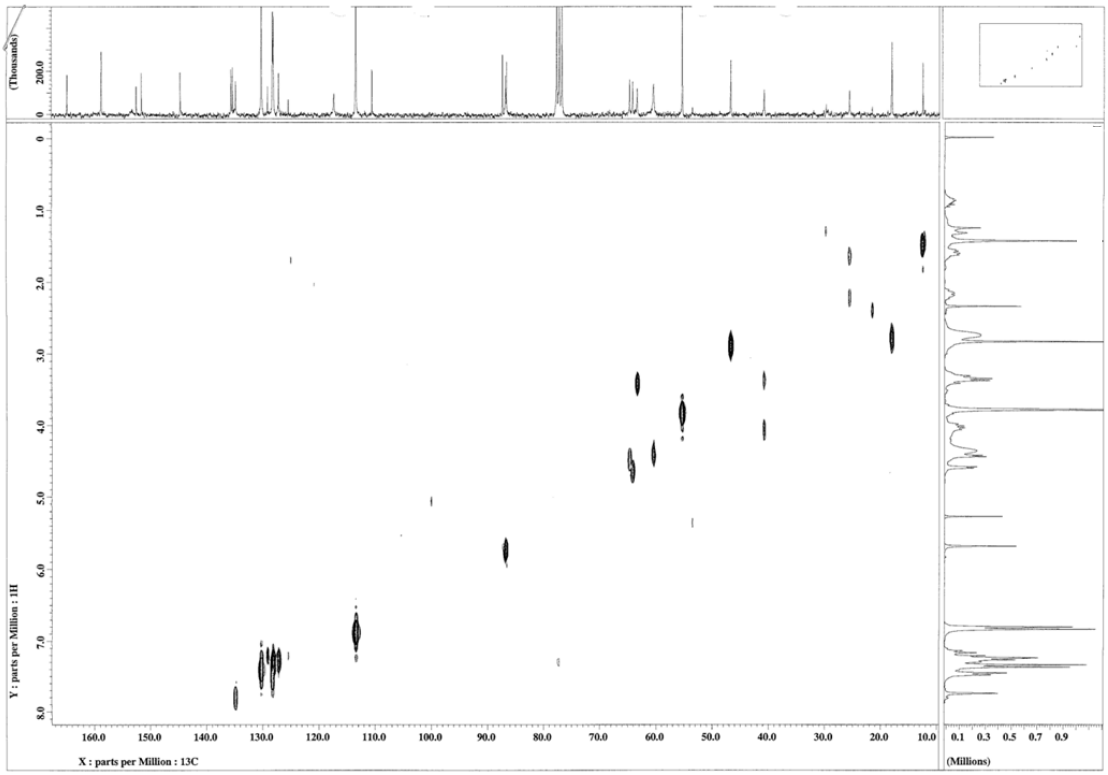


3) COSY spectra of **3**

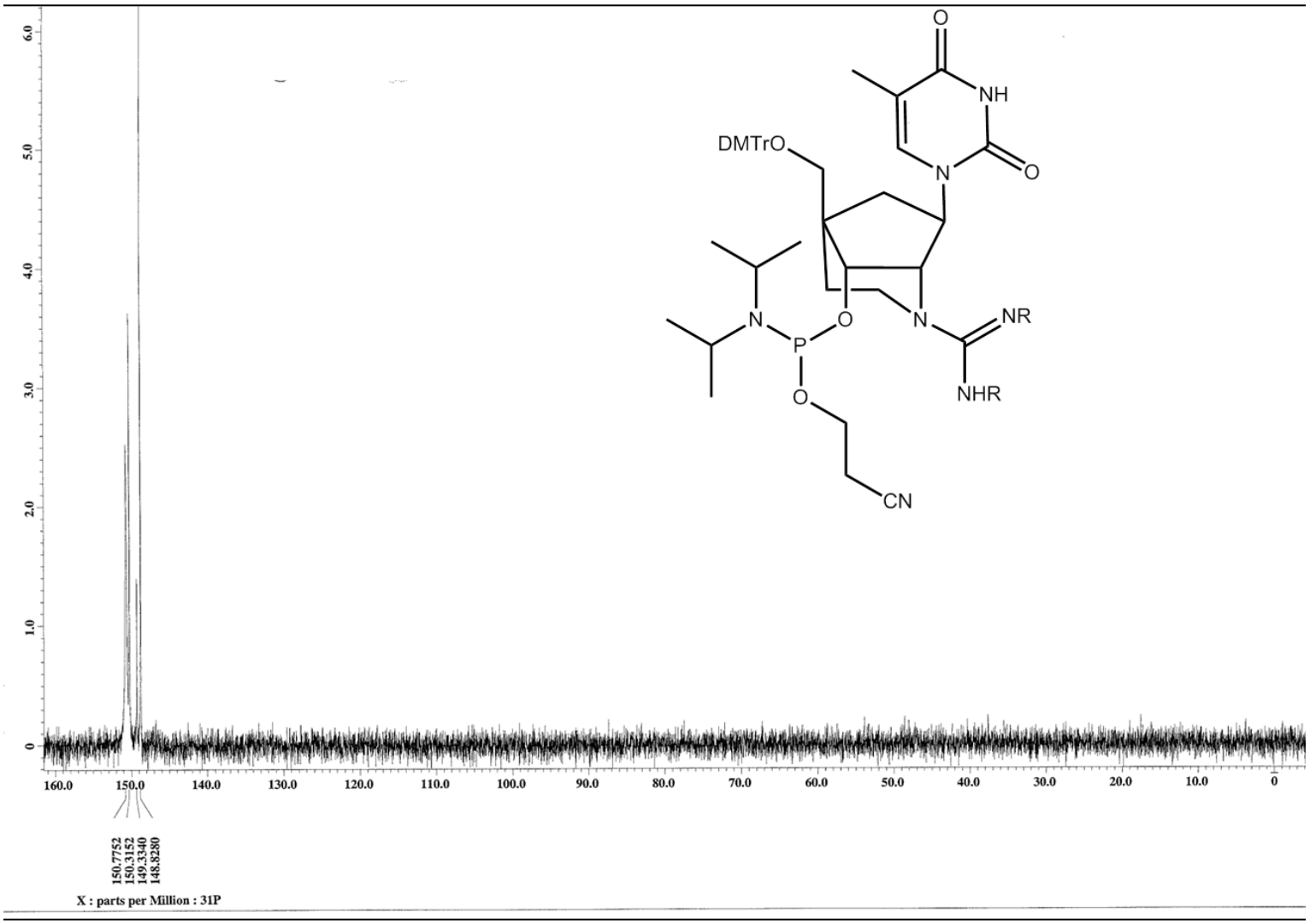


4) CHSHF spectra of **3**





5) ^{31}P NMR spectra of **4**



6) Mass spectra of **4**