Supporting Information

for

Incorporation of Phosphorylated Tyrosine into Proteins: *In Vitro* Translation and Study of Phosphorylated IκB-α and its Interaction with NF-κB

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Table S1. Mass Values of the Peptides NTQ<u>Y</u>EQLFK

Amino acid residue	Type b ion (NH_2CHRCO^+)	Type y ion ($^+NH_3CHRCOOH$)
Asn	-	-
Thr	216	1057
Gln	344	955
Tyr	507 [587]	827 [907]
Glu	636 [716]	664
Gln	764 [844]	535
Leu	877 [957]	407
Phe	1025	294
Lys	-	-

Values in brackets are for the corresponding phosphorylated peptide derivatives.

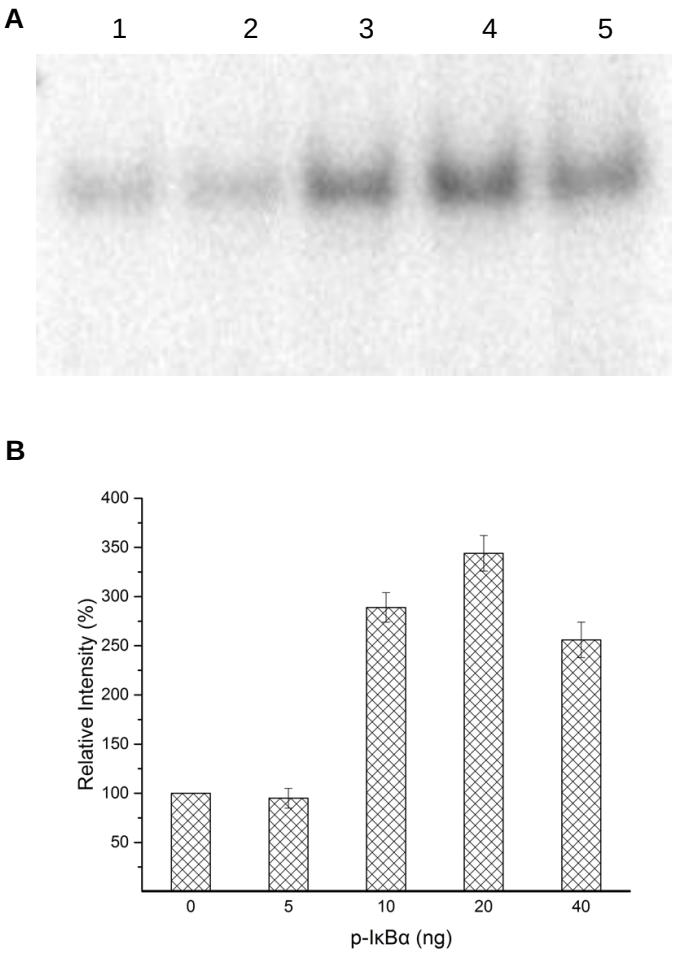
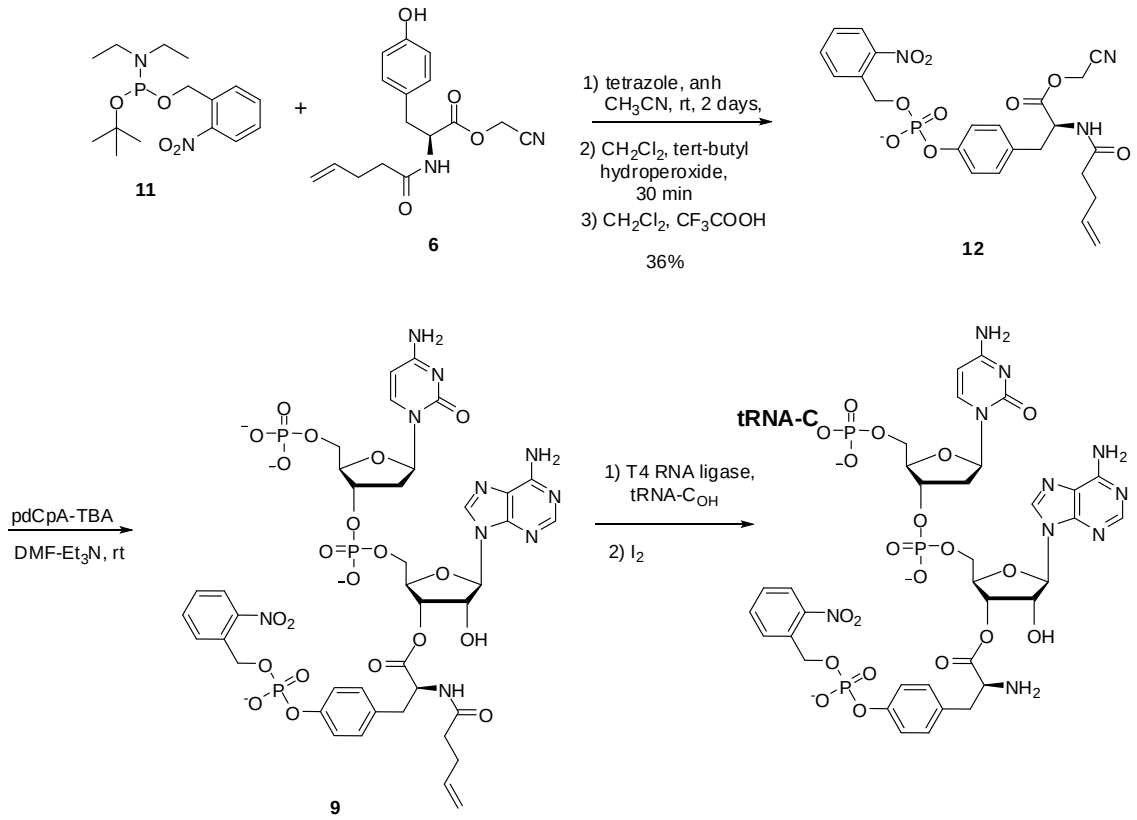
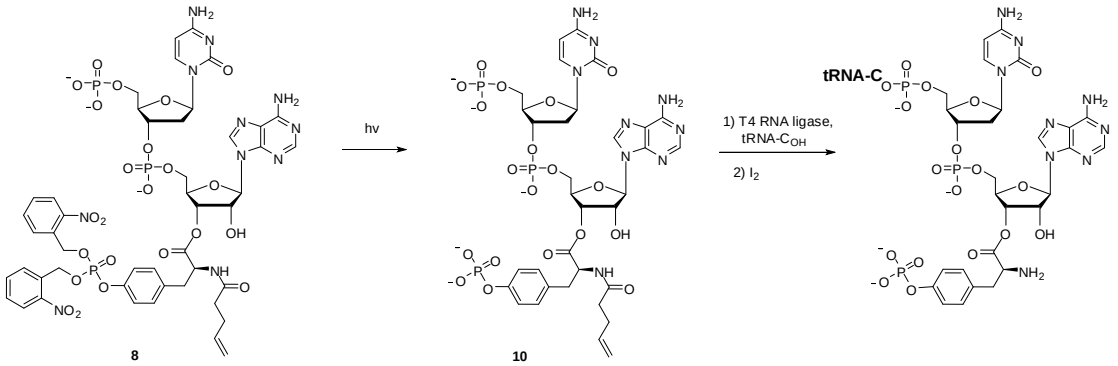


Figure S1. Concentration-dependent binding of NF-κB and DNA. (A) Binding of NF-κB (3 μL) and DNA (2 pmol) in the presence of phosphorylated IκB-α (0 – 40 ng) at 37 ^{o}C for 5 min. Samples were analyzed by 6% native polyacrylamide gel electrophoresis and quantified using a phosphorimager. Lane 1, without phosphorylated IκB-α; lane 2, 5 ng phosphorylated IκB-α; lane 3, 10 ng phosphorylated IκB-α; lane 4, 20 ng phosphorylated IκB-α; lane 5, 40 ng phosphorylated IκB-α. (B) Relative intensity of NF-κB–DNA complexes in the 6% native polyacrylamide gel. The intensity of the NF-κB–DNA complex in the reaction of NF-κB and DNA without phosphorylated IκB-α was defined as 100%.

Scheme S1. Synthesis of the pdCpA Ester of *o*-Nitrobenzylphosphotyrosine and *o*-Nitrobenzylphosphotyrosyl-tRNA$_{CUA}$



Scheme S2. Synthesis of the pdCpA Ester of Phosphotyrosine and Phosphotyrosyl-tRNA$_{CUA}$



Experimental Procedures

Chemical Syntheses

General Methods

All experiments requiring anhydrous conditions were conducted in flame-dried glassware fitted with a rubber septum under a positive pressure of dry nitrogen or dry argon. Reactions were performed at room temperature unless otherwise indicated. Analytical thin layer chromatography was performed using glass plates pre-coated with silica gel (0.25 mm, 60 Å pore size, 230–400 mesh, Silicycle) impregnated with a fluorescent indicator. TLC plates were visualized by exposure to ultraviolet (UV) light (254 nm). Flash column chromatography was performed employing silica gel (60 Å pore size, 40–63 μm, standard grade, Silicycle).

^{1}H NMR and ^{13}C NMR spectra were recorded on Varian INOVA 400 (400 MHz) and Varian INOVA 500 (500 MHz) spectrometers at 25 °C. Proton chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to residual protium in the NMR solvent ($CDCl_3$, DMSO-d_6 or CD_3OD). Splitting patterns are designated as follows: s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High resolution mass spectra were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Facility or the Michigan State University Mass Spectrometry Facility. HPLC purification was performed with a Waters 600 pump coupled with a Varian ProStar 340 detector and a Grace Econosil C_{18} column (250 × 10 mm, 5 μm).

Synthesis of the pdCpA Esters of Bis-(*o*-nitrobenzyl)phosphotyrosine (8), *o*-Nitrobenzylphosphotyrosine (9) and Phosphotyrosine (10)

***N*-4-Pentenoyl-*S*-tyrosine Cyanomethyl Ester (6).** To a solution containing 1.00 g (5.52 mmol) of *S*-tyrosine and 1.50 g (11.0 mmol) of K_2CO_3 in 15 mL of H_2O (to which a few drops of 6 N NaOH were added for solubility) was added 1.30 g (6.62 mmol) of 4-pentenoyloxy succinimide ester. The reaction mixture was stirred at room temperature for 16 h. Eighty mL of 1 N $NaHSO_4$ was then added to the reaction mixture. The aqueous layer was extracted with three 125-mL portions of ethyl acetate. The combined organic extract was then dried over anhydrous Na_2SO_4 and concentrated under diminished pressure. The crude product was dissolved in 10 mL of acetonitrile. To this solution was added 4.60 mL (33.1 mmol) of Et_3N and 2.10 mL (33.1 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 20 h, then diluted with 150 mL of ethyl acetate. The organic layer was washed with three 75-mL portions of sat. $NaHCO_3$. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under diminished pressure. The residue was purified on a silica gel column (15 × 5 cm); elution with 1:1 ethyl acetate−hexanes afforded *N*-4-pentenoyl-L-tyrosine cyanomethyl ester (**6**) as a colorless solid: yield 1.03 g (62% over two steps); mp 52-53 ^{o}C; R_f 0.45 (1:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 2.22-2.32 (m, 4H), 2.90-3.05 (m, 2H), 4.64 (ABq, 2H, J = 15.7 Hz), 4.76-4.82 (m, 1H), 4.90-5.00 (m, 2H), 5.63-5.75 (m, 1H), 6.74 (d, 2H, J = 8.5 Hz), 6.93 (d, 2H, J = 8.5 Hz) and 7.73 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 29.2, 35.2, 36.6, 49.0, 53.3, 114.1, 115.77, 115.83, 126.2, 130.2, 136.5, 156.0, 170.5 and 173.3; mass spectrum (ESI), *m/z* 325.1169 $(M+Na)^+$ ($C_{16}H_{18}N_2O_4Na$ requires *m/z* 325.1159).

***N*-4-Pentenoyl-bis-(*o*-nitrobenzyl)phospho-*S*-tyrosine Cyanomethyl Ester (7).** To a stirred solution of 416 mg (0.95 mmol) of bis-*o*-dinitrobenzyl *N*,*N*-diisopropyl phosphoramidite (**5**)[1] and 92.0 mg (1.31 mmol) of tetrazole in 5 mL of anhydrous acetonitrile was added 107 mg (0.35 mmol) of *N*-pentenoyl-*S*-tyrosine cyanomethyl ester (**6**). The reaction mixture was stirred at room temperature for 30 min, and then a 5 M solution of *tert*-butylhydroperoxide in decane (262 μL, 1.31 mmol) was added. The reaction mixture was stirred for 10 minutes, and then diluted with 50 mL of ethyl acetate and washed with three 30-mL portions of sat $NaHCO_3$. The organic layer was then dried (anh $MgSO_4$) and concentrated under diminished pressure. The residue was purified by silica gel chromatography (15 cm × 5 cm); elution with 1:1 ethyl acetate−hexane afforded **7** as a yellow oil: yield 138 mg (60%); ^{1}H NMR δ 2.28-2.35 (m, 4H), 3.07-3.18 (m, 2H), 4.74 (ABq, 2H, *J* =12 Hz), 4.88-4.93 (m, 1H), 4.97-5.05 (m, 2H), 5.61-5.63 (m, 4H), 5.72-5.82 (m, 1H), 6.04 (d, 1H, *J* = 8 Hz), 7.11 (d, 2H, *J* = 8 Hz), 7.18 (d, 2H, *J* = 12 Hz), 7.51 (t, 2H, *J* = 8 Hz), 7.66-7.74 (m, 4H) and 8.13 (d, 2H, *J* = 8 Hz); ^{13}C NMR δ 29.3, 35.3, 36.8, 49.0, 53.2, 66.6, 66.7, 114.0, 115.88, 115.90, 125.2, 126.2, 128.6, 129.3, 130.3, 131.7, 131.8, 134.3, 136.6, 146.8, 156.0, 170.5 and 172.9; mass spectrum (MALDI), *m/z* 675.7 $(M+Na)^+$ (theoretical *m/z* 675.5).

Bis-(*o*-nitrobenzyl)phosphotyrosine pdCpA Ester (8). To a stirred solution containing 10.0 mg (7.40 μmol) of pdCpA[2] tetrabutylammonium salt (pdCpA-TBA) in 100 μL of 9:1 anhydrous DMF–triethylamine was added 24.0 mg (36.7 μmol) of cyanomethyl ester **7**. The reaction mixture was sonicated at room temperature for 8 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aqueous ammonium acetate, pH 4.5–acetonitrile over a period of 45 min. The retention time of the desired product was ~ 30 min. The fractions containing the product were lyophilized to afford **8** as a light yellow solid: yield 2.0 mg (22%); mass spectrum (ESI), *m/z* 1230.2360 $(M-H)^-$ ($C_{47}H_{51}N_{11}O_{23}P_3$ requires *m/z* 1230.2372). The tetra-*n*-butylammonium (TBA) salt of pdCpA was prepared using Dowex 50W X8, 200–400 mesh, activated in its TBA form.

***o*-Nitrobenzyl *O*-*tert*-Butyl *N*,*N*-Diethyl phosphoramidite (11).** To a solution containing 4.37 mL (50.0 mmol) of PCl_3 in 150 mL of diethyl ether at 0 °C was added 34 mL (300 mmol) of diethylamine and the reaction mixture was stirred for 8 h. The reaction mixture was filtered and the filtrate was concentrated under diminished pressure. The residue was redissolved in 100 mL of THF and the solution was treated with 3.34 g (949 mmol) of tetrazole followed by 4.75 mL (50.0 mmol) of *tert*-butanol. The reaction mixture was stirred at room temperature for 5 h under argon. Nitrobenzyl alcohol (1 eq) was added to the reaction mixture, which was stirred overnight under argon. The solvent was concentrated under diminished pressure and the residue was purified by chromatography on a silica gel flash column (12 × 3 cm). Elution with 97:3 hexane−triethylamine afforded **11** as a yellow oil: yield 5.1g (31%); ^{1}H NMR δ 1.06-1.10 (m, 6H), 1.38 (s, 9H), 2.05-3.17 (m, 4H), 5.01-5.05 (m, 2H), 7.41 (t, 1H), 7.64 (t, 1H), 7.90 (d, 1H, *J* = 7.6 Hz) and 8.08 (d, 1H, *J* = 8 Hz); ^{13}C NMR δ 15.2, 31.0, 37.9, 61.6, 75.4, 124.7, 127.7, 129.0, 136.8 and 147.0; mass spectrum (MALDI), *m/z* 328.6 $(M+H)^+$ (theoretical *m/z* 328.4).

***N*-4-Pentenoyl-*o*-nitrobenzylphospho-*S*-tyrosine Cyanomethyl Ester (12).** To a stirred solution containing 272 mg (0.83 mmol) of *o*-nitrobenzyl-*O*-*tert*-butyl-*N*,*N*-diethyl phosphoramidite (**11**) and 70.0 mg (0.99 mmol) of tetrazole in 2 mL of anhydrous CH_3CN was added 100 mg (0.33 mmol) of *N*-4-pentenoyl-*S*-tyrosine

cyanomethyl ester (**6**). The reaction mixture was stirred for 48 h at room temperature and then concentrated under diminished pressure and redissolved in 10 mL of CH_2Cl_2. This solution was washed with two 4-mL portions of saturated $NaHCO_3$, dried (anh $MgSO_4$) and concentrated under diminished pressure. The phosphite was then dissolved in 2 mL of anhydrous CH_2Cl_2 and 200 μL (5M solution in decane) of *tert*-BuOOH was added dropwise. The reaction mixture was stirred at room temperature for 30 min, then diluted with 3 mL of CH_2Cl_2 and washed with two 3-mL portions of saturated $NaHCO_3$. The organic layer was dried (anh $MgSO_4$) and concentrated under diminished pressure. The crude phosphate was then dissolved in 2 mL of anhydrous CH_2Cl_2 followed by addition of 300 μL of CF_3COOH. The solution was stirred for 30 min, then concentrated and the crude mixture was purified via preparative C_{18} reversed phase HPLC (linear gradient of 93:7 → 0:100 water–acetonitrile containing 0.1% CF_3COOH over a period of 35 min, retention time 24.3 min) to afford **12** as a brown oil: yield 62.0 mg (36%); ^{1}H NMR δ 2.30 (s, 4H), 3.01-3.13 (m, 2H), 4.72 (ABq, 2H, J = 16 Hz), 4.85 (br s, 1H), 4.95-5.03 (m, 2H), 5.51-5.55 (m, 2H), 5.68-5.80 (m, 1H), 6.35 (br s, 1H), 7.07 (d, 2H, J = 8 Hz), 7.13 (d, 2H, J = 8 Hz), 7.47 (t, 1H, J = 16 Hz), 7.65 (t, 1H, 16 Hz), 7.76 (d, 1H, J = 8 Hz) and 8.11 (d, 1H, J = 4 Hz); ^{13}C NMR δ 29.1, 35.0, 36.7, 48.9, 52.9, 66.2, 66.3, 113.7, 115.9, 120.3, 120.4, 124.9, 128.2, 128.7, 130.5, 132.5, 134.2, 136.3, 170.0 and 173.0; mass spectrum (ESI), *m/z* 518.1328 $(M+H)^+$ ($C_{23}H_{25}N_3O_9P$ requires 518.1329).

***o*-Nitrobenzylphosphotyrosine pdCpA Ester (9).** The reaction was carried out as described above for **8** starting from 5.0 mg (3.7 μmol) of pdCpA-TBA and 9.5 mg (18.4 μmol) of cyanomethyl ester **12** to afford **9** as a solid, resolvable into the (2′- and 3′-*O*) regioisomers using the C_{18} reversed phase HPLC system described for **8**; the retention times of the products were ~ 18 and 19 min; mass spectrum (ESI) *m/z* 1095.2013 $(M-H)^-$ ($C_{40}H_{46}N_{10}O_{21}P_3$ requires *m/z* 1095.2057).

Phosphotyrosine pdCpA Ester (10). A solution containing 1.30 mg (1.19 μmol) of pdCpA ester **8** in 100 μL DMSO was irradiated using a 500 W mercury-xenon lamp at 0 °C for 5 min (spectral distribution 185 – 2000 nm). Following irradiation, C_{18} reversed phase HPLC analysis using the mobile phase described above showed that all of the starting material had been consumed, and that there was a new peak (retention time ~ 13 min) corresponding to the desired product **10**; mass spectrum (ESI) *m/z* 960.1712 $(M-H)^-$ ($C_{33}H_{41}N_9O_{19}P_3$ requires *m/z* 960.1737). This material was used directly for T4 RNA ligase-mediated attachment to the abbreviated tRNA transcript (tRNA-C_{OH}).

Phosphopuromycin. To a slurry containing 25.0 mg (50.0 μmol) of Fmoc-phosphotyrosine and 12.0 mg (0.10 mmol) of *N*-hydroxysuccininide (NHS) in 1 mL of DMF was added 12.0 mg (50 μmol) of dicyclohexylcarbodiimide (DCC). The reaction mixture was stirred at room temperature for 2 h and the solvent was then concentrated under diminished pressure. The residue was triturated with 10 mL of ether and dissolved in 1 mL of anhydrous DMF. To this solution were added 14.0 μL (10.0 mg, 0.10 mmol) of Et_3N and 15.0 mg (50.0 μmol) of puromycin aminonucleoside. The resulting solution was sonicated in a water bath at room temperature for 2 h and then 0.1 mL of piperidine was added. The reaction mixture was stirred at room temperature for 1 h and then diluted with 1 mL of 1:1 acetonitrile–water. Purification was carried out in several batches by C_{18} reversed phase HPLC using a gradient of 1%→40% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 30 min. The fractions eluting

at 9.4 min were collected, combined and lyophilized to afford phosphopuromycin as a colorless oil: yield 5.0 mg (18%); mass spectrum (ESI), *m/z* 536.1665 $(M-H)^-$ ($C_{21}H_{27}N_7O_8P$ requires *m/z* 536.1664).

Biochemical Procedures

General Methods

Ni-NTA agarose was obtained from Qiagen Inc. DNA oligonucleotides were purchased from Integrated DNA Technologies. DEAE-Sepharose, ammonium persulfate, acrylamide, *N*, *N´*-methylene-bis-acrylamide, acetic acid, potassium glutamate, ammonium acetate, dithiothreitol, magnesium acetate, phospho(enol)pyruvate, *Escherichia coli* tRNA, isopropyl β-D-thiogalactopyranoside (IPTG), ATP, GTP, CTP, UTP, cAMP, amino acids, rifampicin, formamide, Tween 20, sodium pyruvate, glutamine, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187 and phenylmethanesulphonyl fluoride (PMSF) were obtained from Sigma-Aldrich. Tris and SDS were obtained from Bio-Rad Laboratories. [^{35}S]-methionine (1000 Ci/mmol, 10 μCi/μL) and [γ-^{32}P]-ATP (10Ci/mmol, 2 μCi/μL) were purchased from PerkinElmer Inc. Protease inhibitor (complete, EDTA-free) was obtained from Boehringer Mannheim Corp. T4 RNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs Inc. SuperSignal West Pico Chemiluminescent Substrate was obtained from Thermo Fisher Scientific. RPMI 1640 medium, penicillin, streptomycin, and fetal bovine serum were obtained from American Type Culture Collection (ATCC).

Human leukemic Jurkat T cell line was purchased from American Type Culture Collection (ATCC). Anti-phosphorylated IκB-α (pTyr42) rabbit polyclonal antibody and goat anti-rabbit-HRP antibody were obtained from Santa Cruz Biotechnology. NF-κB (p50) rabbit polyclonal antibody was obtained from MBL International Corp.

Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.2 software from Molecular Dynamics. UV spectral measurements were made using a Perkin-Elmer Lamdba 20 UV/vis spectrometer. MS/MS analysis was performed using an AB SCIEX TOF/TOF 4800 Plus MALDI. Chemiluminescent immunoassays were scanned using a BioRad Chemi-Doc Imaging System.

Preparation of Aminoacyl-tRNA$_{CUA}$s. The activation of suppressor tRNA$_{CUA}$s was carried out as described previously.[3] Briefly, the reaction was carried out in 100 μL reaction mixture (total volume) of 100 mM Na Hepes buffer, pH 7.5, containing 1.0 mM ATP, 15 mM $MgCl_2$, 100 μg of suppressor tRNA$_{CUA}$-C$_{OH}$, 0.5 A_{260} unit of *N*-pentenoyl-protected aminoacyl-pdCpA, 15% DMSO, and 100 units of T4 RNA ligase. The reaction mixture was incubated at 37 °C for 1.5 h and quenched by the addition of 0.1 vol of 3 M NaOAc, pH 5.2. The *N*-protected aminoacylated tRNA was precipitated with 3 vol of cold absolute ethanol, and recovered following centrifugation. The efficiency of ligation was estimated by 8% polyacrylamide–7 M urea gel electrophoresis (pH 5.0).[4] The *N*-pentenoyl-protected aminoacyl-tRNA$_{CUA}$s were deprotected by treatment with 5 mM aqeuous iodine for 20 min at room temperature.[3] The solution was centrifuged, and the supernatant was adjusted to 0.3 M NaOAc and treated with 3 vol of cold ethanol to precipitate the aminoacylated tRNA. The tRNA pellet was collected by centrifugation, washed with 70% aq ethanol, air dried and dissolved in 20 μL of RNase-free water.

Site-directed Mutagenesis of the rrnB Operon and Preparation of Library. We screened 611 clones from a library described previously,[5] In addition, 361 new clones were screened from a new library produced using eight PCR reactions were carried out to prepare a set of cells having mutations in two additional regions of the 23S rRNA (Table 2). Four different plasmids, having different erythromycin resistant mutations (2058-2063 region of 23S rRNA (01-04[5])) and two oligonucleotides 5′-GTTTAGAACGTCGTGAG**BDBHVV**CGGTCCCTAT CTGCC-3′ (04) and 5′-AACGTCGTGAGACAGTT **DHHVDD**CTATCTGCCGTGGGC-3′ (05) for positions 2600-2605 and 2606-2611, respectively (B = C+G+T; H = C+A+T; V = A+G+C; D = A+G+T) were used as templates and primers for the polymerase chain reactions (PCR). The PCR reactions were carried out using a modified Quik-ChangeTN site-directed mutagenesis protocol.[6]

Oligonucleotide primer phosphorylation was performed in reaction mixtures (20 μL total volume) containing 100 pmol of primer, 1 mM ATP, 70 mM Tris buffer, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT and 1 unit of T4 polynucleotide kinase were incubated at 37 ºC for 1 h and then chilled on ice. The solution was diluted with aq NH_4OAc and the product was precipitated with cold ethanol. The product was isolated by centrifugation and the pellet was washed with 70% ethanol, air-dried and dissolved in 50 μL of RNase-free water.

PCR was carried out in 50 μL (total volume) of 35 mM Tris-HCl, pH 8.0, containing 300 ng of template, 14 pmol of primer, 10 nmol of dNTPs, 12 mM KOAc, 5 mM DTT, 0.05 % Triton X-100, 0.05 mM EDTA, 2.5 U of Pfu polymerase and 20 U of Taq DNA ligase. The terminal cycler was programmed as follows: pre-incubation at 95 °C for 2 min, 18 cycles at 95 °C for 1 min, 50 °C for 1 min and 65 °C for 24 min, then final extension for 7 min at 72 °C and cooling to room temperature. One μL of restriction endonuclease *Dpn*I was added and the reaction mixture was incubated at 37 °C for 1 h. Then the samples were subjected to denaturation at 95 °C for 1 min, followed by 2 cycles at 95 °C for 1 min, 50 °C for 1 min and 70 °C for 24 min. The samples were then precipitated by the successive additions of NaOAc, pH 5.2, to a concentration of 0.1 M and 3 vol of cold ethanol. After incubation for 20 min at –20 °C, the samples were centrifuged, and the pellets were washed with 70% ethanol, air-dried and dissolved in 10 μL of purified water. DH5α high efficiency competent cells (> 10^7cfu/μg) were transformed using 5 μL of the PCR products per 50 μL of cell suspension and the transformants were selected on LB agar with 100 μg/mL ampicillin and 0.5 mM IPTG and incubated at 37 ºC for 18-24 h.

Each single colony from the corresponding agar plate was transferred into 0.5 mL of LB medium supplemented with ampicillin, grown at 37 ºC for 5 h and mixed with 0.5 mL of 30% glycerol. A library containing "master plates" was organized in 96 well format (200 μL in each well; one clone per well). A culture having pUC-18 plasmid without rrnB operon was placed in three wells of each plate as a control.

Dual Selection of Ribosomal Clones with Phosphopuromycin and Erythromycin. A library having 972 different clones with mutations in two regions of 23 S rRNA (four different variants of first 2057-2063 region (01-02)[5] and four different variants of second region, 2496-2501 (02); 2502-2507 (03); 2600-2605(04); or 2606-2611(05)) were present in 11 "master plates". Three new plates were prepared by transferring 2 μL of culture from each well of the "master plates" to the corresponding well of the new plate. Then 98 μL of one of three assay solutions was added. Assay 1 contained LB medium, pH 8.4, containing 100 μg/mL of ampicillin and 1 mM IPTG, and was used for checking the rate of cell growth in the library (assay). Assay 2 contained the same components as assay1 except that phosphopuromycin was added to a

concentration of 100 μg/mL to select the cells having sensitivity to this puromycin analogue. Assay 3 also contained the same components as assay 1 except that erythromycin was added to a concentration of 3 μg/mL to select clones with the highest resistance to this antibiotic. All plates were incubated at 37 ºC for 16-18 h in a thermostated shaker and the extent of cell growth was estimated by measuring the optical density at 600 nm.

Inhibition of cell growth by both antibiotics was estimated for each well as [100-(A_{600}exp/A_{600}cont) x 100]. The selection cutoff criteria were 40% and 70% for phosphopuromycin and erythromycin assays, respectively. The absence of inhibition (<5 %) in the phosphopuromycin assay and full inhibition (>99%) in the erythromycin assay were observed in control cultures.

Plasmids from clones exhibiting phosphorpuromycin sensitivity and having some resistance to erythromycin were isolated and characterized by restriction analysis (*Eco*RI) and sequenced in the mutagenized regions. The erythromycin sensitivity for all of the clones having modified 23S rRNAs was in the range 3.1 – 12.6 μg/mL as a consequence of our selection for this property.[5]

Preparation of Cell Free Extracts (S-30) from *E. coli* Cultures, Harboring Plasmids with Modified rrnB Operons. Plasmids, isolated from clones that had been selected using phosphorpuromycin were transformed into BL-21 (DE-3) competent cells; at least 5 single clones from each transformation were transferred into 0.5 mL LB medium, supplemented with 100 μg/mL ampicillin, and incubated at 37 ºC for 5 h, then glycerol was added to a final concentration of 15% to prepare glycerol stocks. Each of the glycerol stocks was tested in the erythromycin assay and cultures with highest resistance to this antibiotic (MIC > 6.25 μg/mL) were used for S-30 preparation. BL-21 (DE-3)-pUCrrnBmut cells (from glycerol stocks) were first grown in 3 mL of LB supplemented with 100 μg/mL ampicillin and 0.5 mM IPTG until the OD_{600} was ~0.3, then diluted to OD_{600} 0.01 by the use of the same medium supplemented with 2.5 μg/mL of erythromycin and grown 12-16 h at 37 ºC. The optimal concentration of the final cultures had OD_{600} 0.5-1.0. Cells were harvested by centrifugation (5000 × g, 4 ºC, 10 min), washed three times with S-30 buffer (1 mM Tris-OAc, pH 8.2, containing 1.4 mM $Mg(OAc)_2$, 6 mM KOAc and 0.1 mM DTT) supplemented with 0.5 mL/L β-mercaptoethanol, and once with S-30 buffer having 0.05 mL/L β-mercaptoethanol.

The weight of the wet pellet was estimated and 1.27 mL of S-30 buffer was added to suspend each 1 g of cells. The volume of the suspension was measured and used for estimating the amount of the other components. Pre-incubation mixture (0.3 mL) (0.29 M Tris, pH 8.2, containing 9 mM $Mg(OAc)_2$, 13 mM ATP, 84 mM phosphoenol pyruvate, 4.4 mM DTT and 5 μM amino acids mixture), 15 units of pyruvate kinase and 10 μg of lysozyme were added per 1 mL of cell suspension and the resulting mixture was incubated at 37 ºC for 30 min. The incubation mixture was then frozen at – 80 ºC (~30 min), melted (37 ºC, 30 min), and again frozen and melted at room temperature (~30 min). Ethylene glycol tetraacetic acid (EGTA) was then added to 2.5 mM final concentration and the cells were incubated at 37 ºC for 30 min. The same molar concentration of $CaCl_2$ was added, mixed well and frozen (–80 ºC, 30 min). The frozen mixture was centrifuged (15,000 × g, 4 ºC, 1 h) and the supernatant was stored in aliquots at –80 ºC.

***In Vitro* Protein Translation.** Protein translation reactions were carried out in 15-2000 μL of incubation mixture containing 0.3 μL/μL of S-30 system, 170 ng/μL of plasmid, 35 mM Tris acetate, pH 7.4, 190 mM potassium glutamate, 30 mM ammonium acetate, 4 mM magnesium

acetate, 2 mM DTT, 0.2 mg/mL total *E. coli* tRNA, 3.5% PEG 6000, 20 μg/mL folinic acid, 20 mM ATP and GTP, 5 mM CTP and UTP, 100 mM amino acids mixture, 0.5 μCi/μL of ^{35}S-methionine (for 15-μL reactions) and 1 μg/mL rifampicin. In the case of plasmids having a gene with a TAG codon, an activated suppressor $tRNA_{CUA}$ was added to a concentration of 1.5 μg/μL. Reactions were carried out at 37 ºC for 1 h (for 15-μL reaction mixtures) to 1.5 h (for 500-2000 μL reaction mixtures) and terminated by chilling on ice. Aliquots from *in vitro* translation mixtures were analyzed by SDS-PAGE followed by quantification of the radioactive bands by phosphorimager analysis (for 15-μL reaction mixtures). Preparative translation mixtures were analyzed after purification and detected by Coomassie R-250 staining.

Site-directed Mutation to Obtain the Modified IκB-α (42TAG). The wild-type pET15b-His-IκB-α (FL) plasmid was a gift from Gourisankar Ghosh (Addgene plasmid # 44738). The antisense primer for modification with 42TAG was 5′-GAC TCC ATG AAA GAC GAG GAG TAG GAG CAG ATG GTC AAG GAG CTG-3′. A reaction mixture (25 μL total volume) contained 200 pmol of primer, 1 mM ATP, 30 units of T4 polynucleotide kinase and 2.5 μL of PNK buffer was incubated at 37 ^{o}C for 60 min.

A PCR reaction was carried out in a 50-μL incubation mixture containing 200 ng template plasmid DNA, 16 pmol of primer, 10 nmol of dNTPs, 5 units of *Pfu* DNA polymerase, 2.5 μL of *Pfu* buffer, 40 units of *Taq* DNA ligase and 2.5 μL of *Taq* buffer. The thermal cycle was programmed as follows: pre-incubation at 65 ^{o}C for 5 min to allow the ligase to repair any nicks in the template; initial denaturation at 95 ^{o}C for 2 min;18 cycles at 95 ^{o}C for 1 min, 51 ^{o}C for 1 min, 65 ^{o}C for 15 min, post-incubation at 75 ^{o}C for 7 min. The restriction enzyme *Dpn*I (1 μL) was added to the sample and incubated at 37 ^{o}C for 60 min to eliminate the methylated and hemi-methylated wild-type DNA template. Then the sample was denatured at 95 ^{o}C for 30 s, followed by 2 cycles at 95 ^{o}C for 30 s, 51 ^{o}C for 1 min and 70 ^{o}C for 7 min. Ten μL of the final sample was transformed into 100 μL of *E. coli* competent cell DH5α. The sample was plated on an ampicillin plate, and incubated at 37 ^{o}C overnight.

Purification of IκB-α with Ni-NTA Under Native Conditions. The *in vitro* expression mixture (100-1000 μL) was diluted three-fold with 50 mM Tris-HCl, pH 8.0, and combined with 30-100 μL of Ni-NTA beads. After incubation at 4 ^{o}C for 5 min, the beads were washed with 10 volumes of 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 20 mM imidazole. Finally, the IκB-α protein was eluted three times with 30-100 μL of 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 150 mM imidazole. Aliquots of each fraction were analyzed by 10% SDS–PAGE. Fractions containing full length protein were collected, desalted and concentrated using an Amicon-Ultra-05 centrifugal filter device.

Western Blot Analysis of the IκB-α Protein Phosphorylated at Tyr42 . The protein samples were loaded on a 4-12% glycine gradient SDS–PAGE gel. The gel was run at 150 V for 1 h. The proteins were transferred to a nitrocellulose membrane in NuPAGE® transfer buffer with 10% methanol at 30 V for 1 h. The nitrocellulose membrane was blocked with blocking buffer containing 3% BSA at room temperature for 1 h. After being washed three times with 1× PBS buffer containing 0.05% Tween 20, the nitrocellulose membrane was incubated in 10 mL 1× PBS buffer containing 0.05% Tween 20 and 50 μL of Tyr42-p-IκB-α Ab at room temperature for 1 h. After being washed three times with 1× PBS buffer containing 0.05% Tween 20, the nitrocellulose membrane was incubated in 10 mL 1× PBS buffer containing 0.05% Tween 20

and 5 μL of goat anti-rabbit-HRP Ab at room temperature for 1 h. After being washed three times with 1× PBS buffer containing 0.05% Tween 20, the nitrocellulose membrane was treated with SuperSignal West Pico Chemiluminescent Substrate at room temperature for 2 min, and the signals were scanned using a BioRad Chemi-Doc Imaging System.

Mass Spectrometric Analysis. The IκB-α protein bands were cut from Coomassie blue stained SDS–polyacrylamide gel and transferred into a 1.5 mL tube. The gel slides were washed with 500 μL of 0.1 M NH_4HCO_3 at room temperature for 1 h. The slides were then treated with 150 μL 0.1 M NH_4HCO_3 and 10 μL of 45 mM DTT. The mixture was heated at 60 ^oC for 30 min. The cooled mixture was treated with 10 μL 0.1 M of iodoacetamide and incubated at room temperature for 30 min in the dark. The gel slides were washed again with 500 μL of 50% acetonitrile–0.1 M NH_4HCO_3 at room temperature for 60 min, and were then treated with 50 μL of acetonitrile at room temperature for 20 min. The gel slides were dried in a rotatory evaporator, then treated with 30 μL of 0.02 mg/mL trypsin in 0.025 M of NH_4HCO_3 and incubated at 37 ^oC overnight. The gel slides were extracted with 50 μL of 60% acetonitrile–0.1%TFA. The solution was used to run MALDI mass spectrum using 2,5-dihydroxybenzoic acid (DHB) as the matrix.

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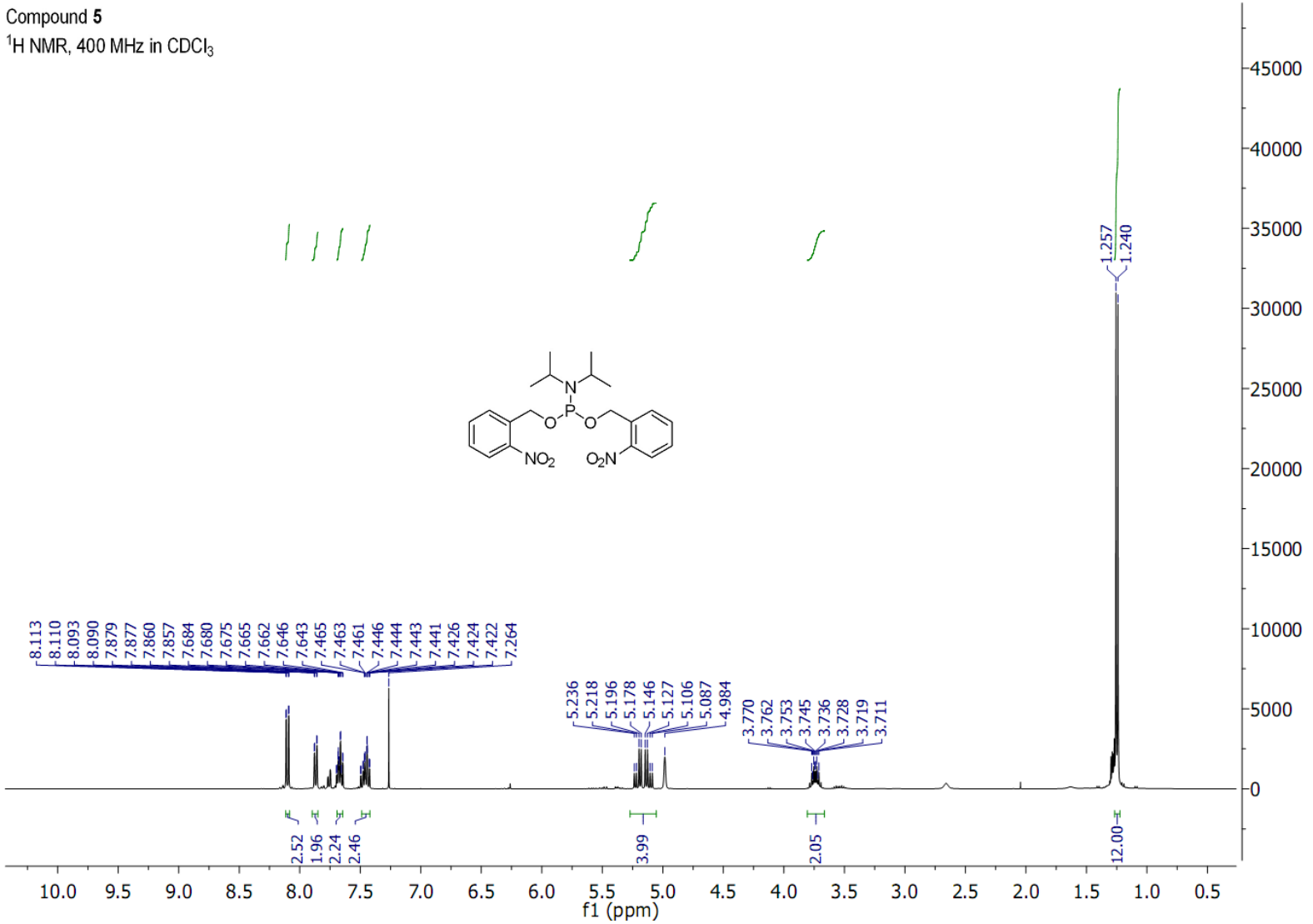
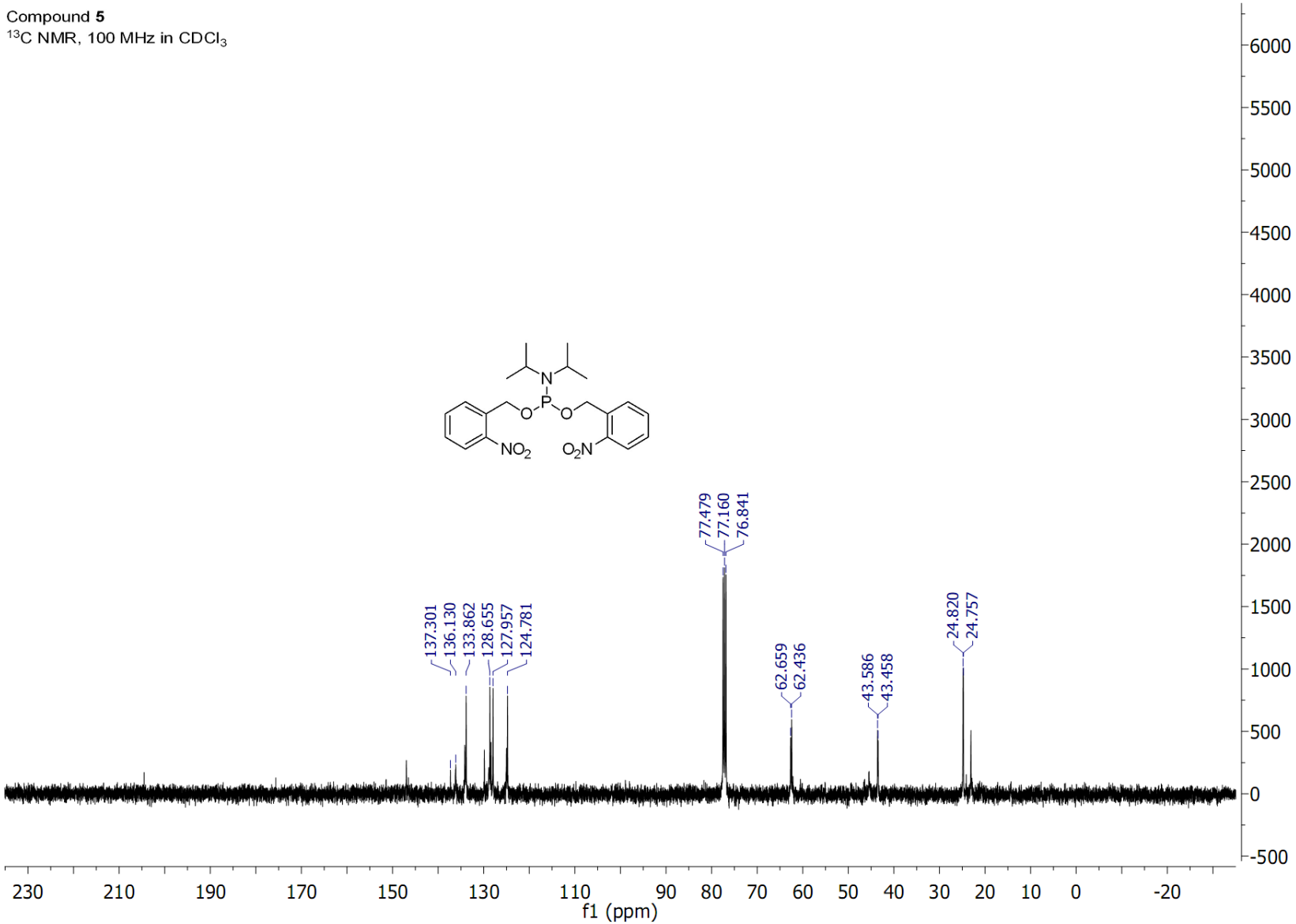
^{1}H NMR, ^{13}C NMR, MS and HRMS Spectra of the synthesized compounds



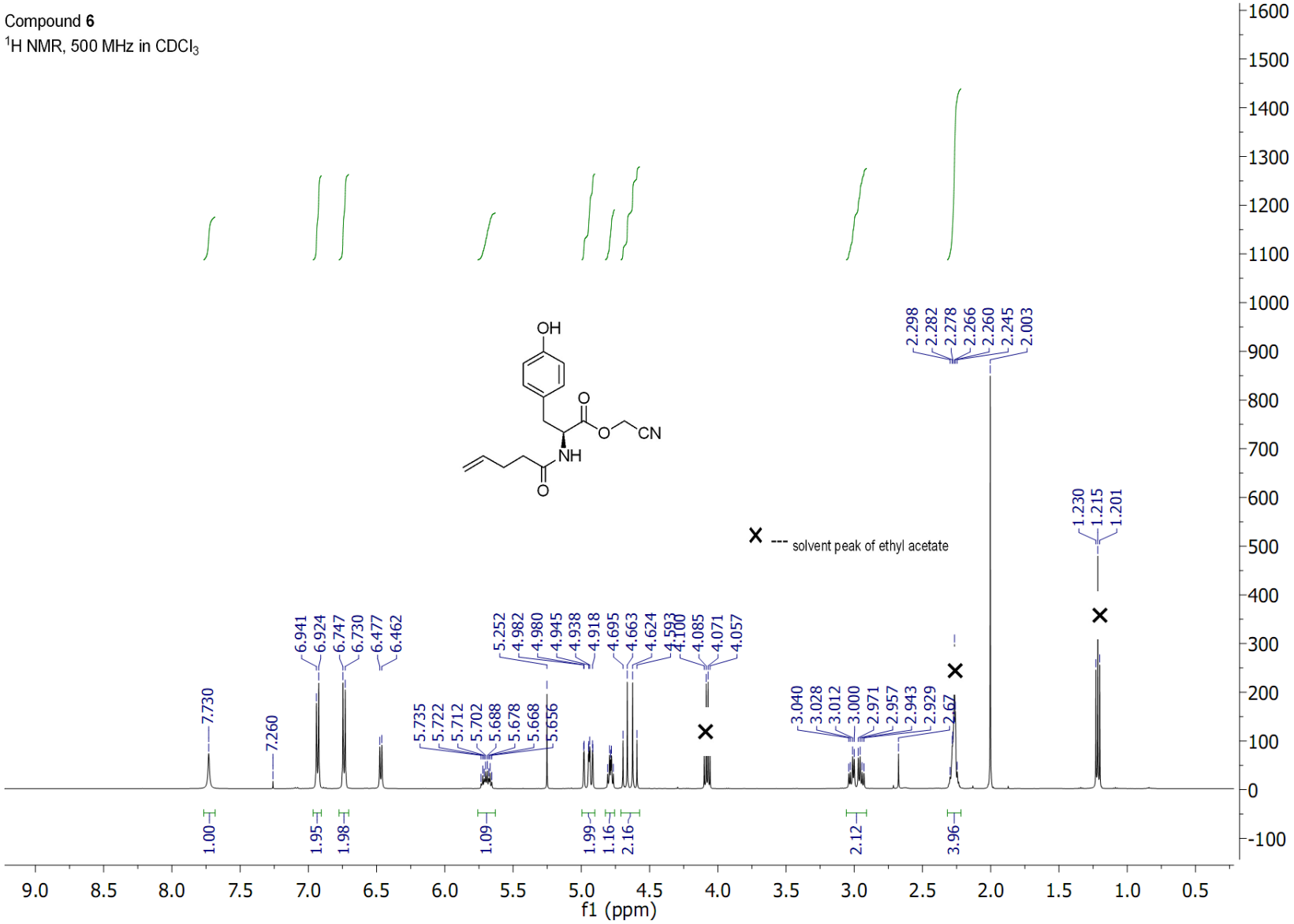
Compound **5**

^{13}C NMR, 100 MHz in $CDCl_3$



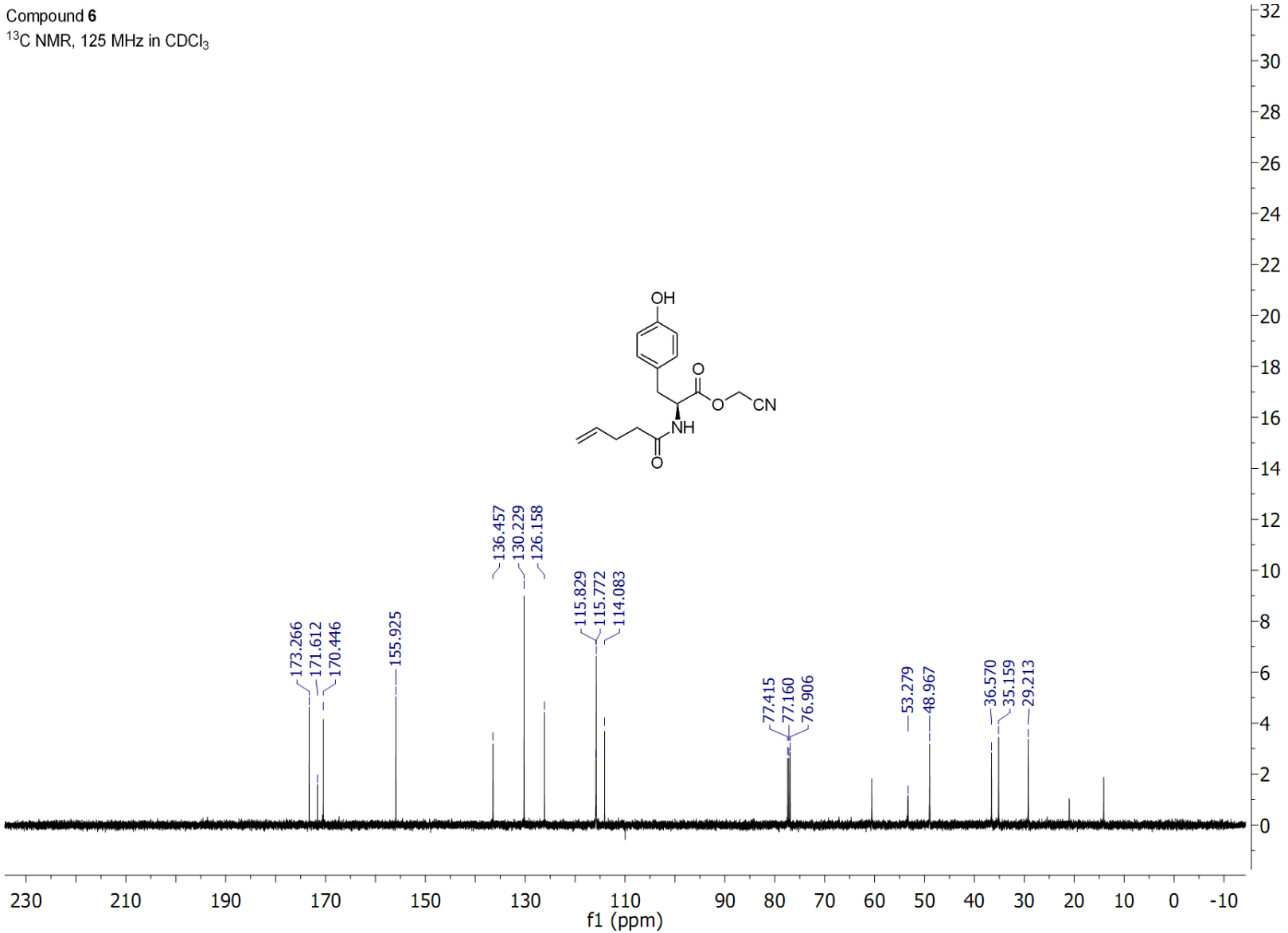
Compound **6**

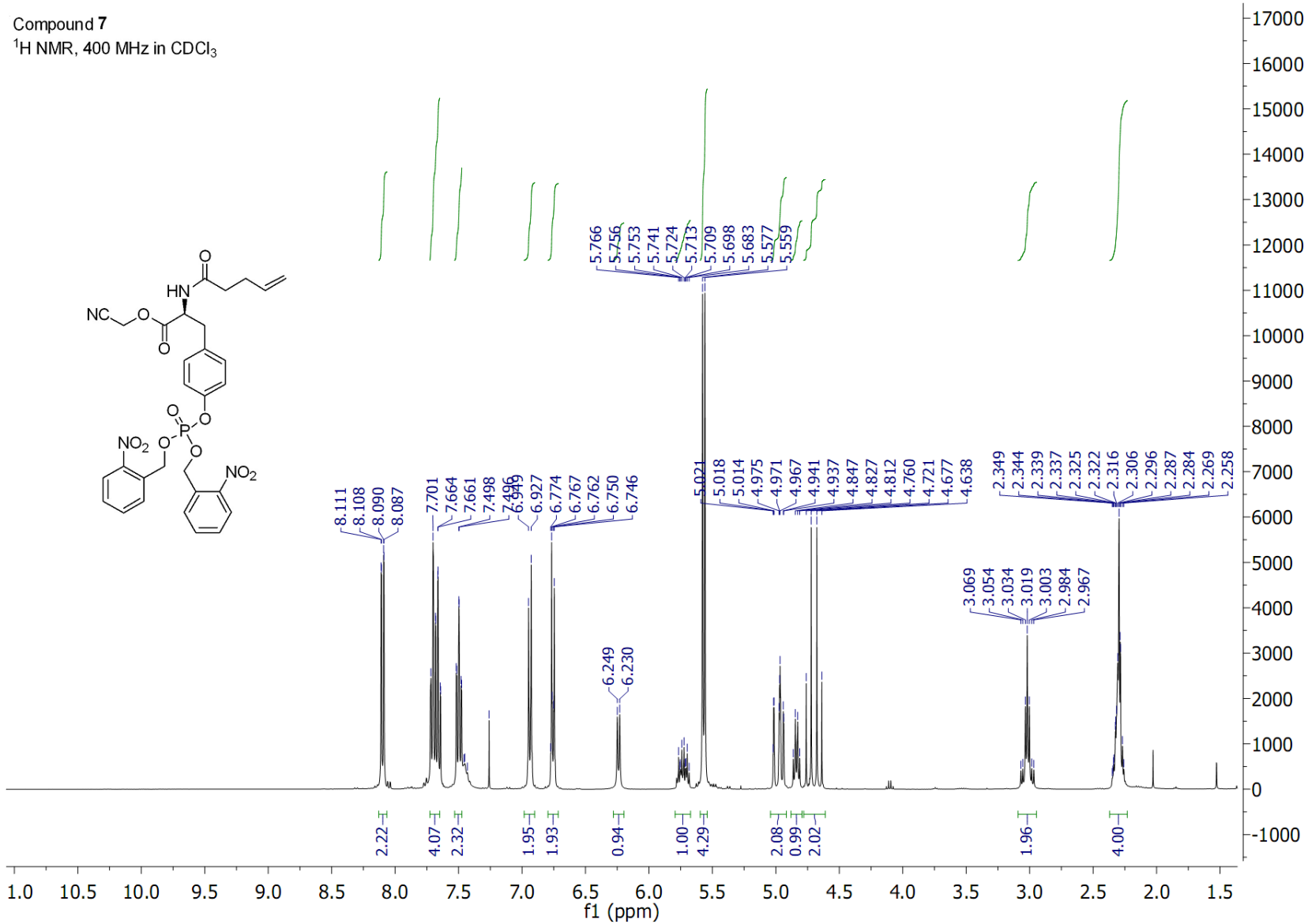
^{1}H NMR, 500 MHz in $CDCl_3$



Compound **6**

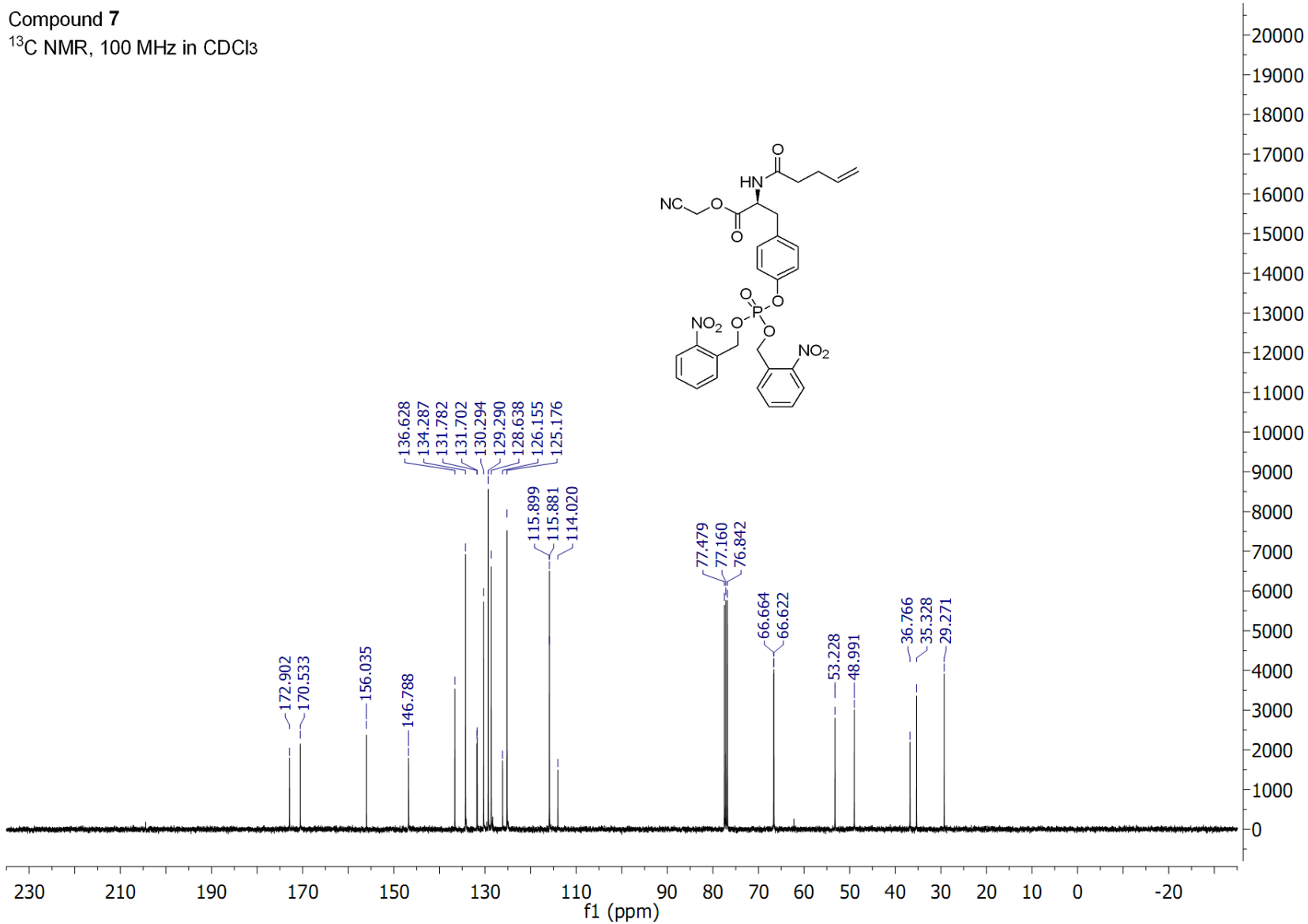
^{13}C NMR, 125 MHz in $CDCl_3$



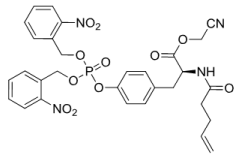

Compound 7
1H NMR, 400 MHz in CDCl3
f1 (ppm)

Compound **7**

^{13}C NMR, 100 MHz in $CDCl_3$

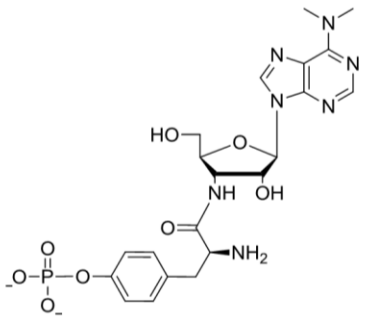


Compound 7
mass spectrum (MALDI-TOF-MS), m/z 674.7 and 675.7 (M+Na)+ (theoretical m/z 675.5)



Chemical Formula: $C_{30}H_{29}N_4O_{11}P$
Molecular Weight: 652.5

Accelerating voltage:	20000 V
Grid voltage:	68%
Mirror voltage ratio:	1.12
Guide wire 0:	0.002%
Extraction delay time:	325 nsec
Acquisition mass range:	250 -- 750 Da
Number of laser shots:	50/spectrum
Laser intensity:	2000
Laser Rep Rate:	20.0 Hz
Calibration type:	Default
Calibration matrix:	Sinapinic acid
Low mass gate:	Off
Timed ion selector:	Off
Digitizer start time:	23.2755
Bin size:	0.5 nsec
Number of data points:	33867
Vertical scale 0:	500 mV
Vertical offset:	0.5%
Input bandwidth 0:	500 MHz
Sample well:	F1_a
Plate ID:	96 WELL X2
Serial number:	4225
Instrument name:	Voyager-DE STR
Plate type filename:	C:\VOYAGER\96 well X2 plate.pl
Lab name:	PE Biosystems
Absolute x-position:	12877.6
Absolute y-position:	19521.6
Relative x-position:	825.311
Relative y-posititon:	-172.935
Shots in spectrum:	50
Source pressure:	1.188e-006
Mirror pressure:	3.537e-008
TC2 pressure:	0.00742
TIS gate width:	30
TIS flight length:	1167



Phosphopuromycin

High resolution ESI-MS: m/z 536.1665 $(M-H)^-$= ($C_{21}H_{27}N_7O_8P$ requires m/z 536.1664). Error: 0.2ppm.