
Supplementary information

An ATP γ S recycling strategy for practical biocatalytic thiophosphorylation

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An ATP γ S recycling strategy for practical biocatalytic thiophosphorylation

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SUPPLEMENTARY MATERIAL

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1. General Information

Unless otherwise noted, all chemicals and reagents for chemical reactions were purchased at the highest commercial quality and used without further purification. Reactions were monitored by thin layer chromatography (TLC) and liquid chromatography/mass spectrometry (LC/MS). TLC was performed with 0.25 mm E. Merck silica plates (60F-254) using short-wave UV light as the visualizing agent, and KMnO_4 , cerium ammonium molybdate (Hanessian's stain), ninhydrin, or *p*-anisaldehyde and heat as developing agents. LC/MS was performed on an Agilent Infinity 1290 II UHPLC system equipped with a ZORBAX SB-C18 column (2.1 $\times$ 50 mm, 1.8 micron). HRMS data were acquired using an Agilent Infinity 1290 II UHPLC coupled to an 6545XT AdvanceBio LC/Q-TOF. NMR spectra were recorded on a Bruker AVANCE AV600 (600 MHz for ^1H , 151 MHz for ^{13}C , 243 MHz for ^{31}P , 565 MHz for ^{19}F) and calibrated by using residual CHCl_3 (δH = 7.26 ppm, δC = 77.16 ppm), MeCN (δH = 1.94 ppm, δC = 118.26 ppm), and D_2O (δH = 4.79 ppm) as internal references. Biochemicals and media components were purchased from standard commercial sources. Purified enzymes were accessed via immobilized metal ion affinity chromatography with HisTrap HP column. Expression vectors were obtained via DNA synthesis from Twist Bioscience and were used directly to electrocompetent BL21(DE3). Electrocompetent *E. coli* BL21(DE3) strains were purchased from Lucigen. All *E. coli* strains generated in this work are stored as glycerol stocks at -80°C . Sonication was performed using a Qsonica Q500 sonicator. Purified enzymes were acquired via immobilized metal ion affinity chromatography with HisTrap HP columns (5 mL, GE Healthcare, Piscataway, NJ) coupled to a Cytiva ÄKTA Go FPLC. Protein concentrations were measured using a Thermo Scientific NanoDrop 2000c spectrophotometer.

Hexokinase from *Saccharomyces cerevisiae* was purchased from Sigma-Aldrich (H6380). T4 Polynucleotide Kinase was purchased from New England Biosciences. Peptides KK-15 was purchased from ProteoGenix.

Abbreviations: DMAP–4-dimethylaminopyridine, DMF–N, N–dimethylformamide, DCM–dichloromethane, dr–diastereomeric ratio, Et_2O –diethyl ether, Et_3N –triethylamine, EtOAc–ethyl acetate, EtOH–ethanol, MeCN–acetonitrile, MeOH–methanol, THF–tetrahydrofuran, TBDSCl–tert-butyldimethylsilyl chloride, TBDPSCl–tert-butyldiphenylsilyl chloride, H_2O –water.

2. Protein and DNA Sequences

Protein sequence of *Ta*-NK (from *Thermoplasma acidophilum*, Uniprot ID: Q9HJT3)

MRFLAYFGHLNIDVLISVDSIPREGSVNVKDLRPRFGGTAGNFAIVAQKFRIPFDLYSAV
GMKTHREYLAMIESMGINTGHVEKFEDESGPICYIATDGKKQVSFMHQGAMEKWKPQL
ADEYEYVHFSTGPNYLDMAKSIRSKIIFDPSQEIHKYSKDELKKFHEISYMSIFNDHEYRV
FREMTGLSSPKVTTIVTNGERGSSLFMDGKKYDFPAIPSSGDTVAGGDSFRAGLYLALY
NRRSIEKGMiyGTIIAHHVIDDGIENFSLNMEDLERETENYRRMFTKRS*

DNA sequence of *Ta*-NK

ATGCGGTTCTTAGCATACTTCGGGCACCTGAACATAGACGTTCTCATCAGCGTCGAC
TCTATCCCGCGAGAAGGGTCGGTAAACGTCAAGGACCTTCGCCC GCGGTTTCGGTGG
CACTGCTGGTAATTTTCGCAATAGTGGCACAAAAGTTTCGGATACCATTTGATTTATA
CTCAGCTGTAGGCATGAAAACACACCGTGAGTATCTTGCTATGATAGAGTCAATGGG
TATCAATACGGGTCATGTAGAGAAATTTGAGGACGAATCGGGTCCAATATGTTATAT
AGCAACTGATGGGAAGAAGCAGGTATCTTTCATGCATCAAGGAGCGATGGAGAAAGT
GGAAGCCACAACCTGGCAGATGAGTACGAGTATGTTCACTTTTCGACTGGACCAAAC
ACCTTGACATGGCCAAAAGCATTTCGATCTAAGATTATCTTCGATCCATCGCAAGAGA
TACACAAGTACTCAAAGGACGAGCTTAAGAAATTCACGAAATCTCCTACATGAGT
ATTTTCAACGATCACGAGTACCGTGTCTTTAGAGAGATGACGGGCCTTAGTTCTCCA
AAAGTCACCACCATTGTAACGAATGGCGAACGTGGTTCGTCTCTGTTTATGGACGGT
AAGAAGTACGACTTCCCCGCCATACCGAGTTCAGGGGACACTGTAGGAGCGGGCGA
TTCATTGAGAGCCGGACTGTATCTGGCACTGTACAATAGACGGAGCATTGAGAAAG
GGATGATCTACGGCACCATAATAGCGCACACGTCATCGATGACGGAATTGAAAAC
TTCAGTCTGAATATGGAAGACCTGGAGAGAGAACTGAAAACCTACAGACGCATGTT
TACAAAGCGTTCCTAA

Protein sequence of *Mt*-NK (from *Methanothermobacter thermautotrophicus*, Uniprot ID: O27587)

MSEDRDLLAVGHATAFDYIIHLDEFPEPNTSTAIKMRNLHGGAANVALVGSRLGLRTS
LVSAVGGDFEGSEYRELLESSGIDIESMILVADESTPTAFVMTSDHNQISYFYWGAARY
FKDAETPADAIKSARAVHLATGDPSFNCRCGEFARSLGKIISFDPGQDLHMYSRSLERA
VGVCDILFGNHHEIDRICSKLSVDIHGLREMGPGVVVKTYGKEGSIIYSDDVIKIDAI
PREAVDPTGAGDSYRAGFMRAYLRGADLKTGCRFASAVASFIVEDEGTQTNIPDTGEAVKR
FTAQWGYEPP*

DNA sequence of *Mt*-NK

ATGAGTGAAGATCGGGATTTATTGGCTGTTGGGCATACGGCTTTTGACTACATTATC
CACCTCGACGAGTTTCCAGAGCCGAACACATCAACAGCCATAAAGAGAATGAGAAA
TCTGCATGGTGGTGCTGCTGCGAACGTCGCTCTTGTCGGATCACGCTTAGGCCTGCG
GACATCCCTCGTCTCGGCAGTCGGCGGTGACTTTGAGGGTTCAGAGTACCGCGAATT
ACTGGAATCAAGCGGCATCGACATCGAAAGCATGATTCTGGTTGCCGACGAATCTA
CTCCTACAGCATTTGTTATGACAGATTCAGACCATAATCAAATAAGCTATTTCTACT
GGGGCGCAGCGCGTTACTTTAAGGACGCTGAAACCCAGCGGACGCTATCAAGTCT
GCCAGAGCAGTACACTTAGCCACCGGGGACCCATCCTTCAACTGTCGCTGTGGAGA
GTTTTCGCGGTCATTGGGAAAGATAATCAGTTTTGACCCCGGACAGGATCTTCACAT
GTATAGCCGAAGTCAACTGGAGCGGGCCGTTGGTGTCTGTGACATTCTTTTCGGTAA
CCATCACGAAATAGACAGAATATGTTCAAAATTAAGTGTAGATATTCATGGTTTGCG
CGAAATGGGGCCAGGTGTCGTTGTGAAGACATACGGGAAGGAAGGGTCCATCATAT
ATTCAGACGACGTTATCAAGATCGACGCCATACCTCGTGAGGCTGTTGACCCAACCG

GGGCTGGGGACTCGTATCGCGCAGGATTCATGCGAGCATATCTGCGTGGGGCTGAC
CTGAAGACTTGTGGGCGGTTTCGCTAGTGCTGTGGCATCATTTCATCGTAGAAGACGAA
GGGACACAAACAAATATACCAGACACTGGGGAAGCTGTCAAACGATTTACCGCTCA
ATGGGGCTACGAGCCGCCTATTTGA

Protein sequence of *Mj*-NK (from *Methanocaldococcus jannaschii*, Uniprot ID: Q57849)

MGGKMEKITCVGHTALDYIFNVEKFPEPNTSIQIPSARKYYGGAAANTAVGIKKLGVNS
ELLSCVGYDFKNSGYERYLKNLDINISKLYYSEEEETPKAWIFTDKDNQITFFLWGA
HYKELNPPNFNTEIVHIATGDPEFNLKCAKKAYGNNLVSFDPGQDLPQYSKEMLLEIIEH
TNFLFMNKHEFERASNLLNFEIDDYLERVDALIVTKGSKGSVIYTKDKKIEIPCIKAGKVI
DPTGAGDSYRAGFLSAYVKGYDLEKCGLIGAATASFVVEAKGCQTNLPTWDKVVERLE
KHRI*

DNA sequence of *Mj*-NK

ATGGGCGGCAAAATGGAAAAGATTACCTGTGTGCGGTCACACAGCCTTAGACTACAT
ATTTAATGTGGAGAAGTTTCCGGAACCTAATACGTCAATTCAAATCCCTAGTGCACG
TAAATACTACGGTGGGGCGGCCCAACACAGCTGTAGGTATAAAGAAGCTCGGTG
TAAATTCTGAGCTTCTGTCTTGCGTGGGCTATGATTTTAAGAATAGCGGCTACGAGC
GGTACTTGAAGAATTTAGACATAAATATTAGTAAGCTTTATTACAGCGAGGAAGAG
GAGACGCCGAAAGCTTGGATCTTTACAGATAAGGATAACAACCAAATCACTTTCTTC
CTTTGGGGTGCCGCCAAGCACTACAAAGAGTTAAATCCGCCTAACTTTAATACGGAG
ATAGTGCACATAGCGACAGGAGACCCCGAGTTCAACCTTAAGTGTGCAAAGAAGGC
GTACGGAAACAACCTTGGTCAGTTTCGACCCAGGTCAAGACTTACCCCAATATAGTAA
GGAAATGCTTCTTGAAATTATCGAGCATACCAATTTCTCTTTATGAACAAGCACGA
GTTCGAACGTGCATCTAATCTCCTGAATTTTGAGATCGACGACTACCTTGAACGTGT
CGACGCCCTTATTGTGACTAAGGGATCGAAGGGGTCCGTCATCTACACTAAGGACA
AGAAGATTGAGATACCATGCATTAAGGCCGGAAGATCATTGATCCTACTGGAGCT
GGAGACAGTTACCGTGCGGGCTTTCTCTCAGCATACGTAAAGGTTACGACTTAGAA
AAGTGTGGGCTCATTGGCGCGGCGACCGCTTCTTTCGTCGTGGAAGCAAAGGTTGT
CAAACCAATTTGCCGACCTGGGATAAGGTTGTTGAACGCCTGGAGAAGCACCGGAT
ATAA

Protein sequence of *Hb*-CK (from *Homo sapiens*, Uniprot ID: P12277)

MPFSNSHNALKLRFPAEDEFPDLSAHNNHMAKVLTPELYAELRAKSTPSGFTLDDVIQT
GVDNPGHPYIMTVGCVAGDEESYEVFKDLFDPIEDRHGGYKPSDEHKTDLNPDNLQGG
DDLDPNYVLSSRVRTGRSIRGFCLPPHCSRGERRAIEKLAVEALSSLDGDLAGRYYALKS
MTEAEQQQLIDDHFLFDKPVSPLLASGMARDWPDARGIWHNDNKTFVLVWVNEEDHL
RVISMQKGGNMKEVFTRFCTGLTQIETLFKSKDYEFMWNPHLGYILTCPSNLGTGLRAG
VHIKLPNLGKHEKFSEVLKRLRLQKRGTGGVDTAAVGGVFDVSNADRLGFSEVELVQM
VVDGVKLLIEMEQRLEQQAIDDLMPAQK*

DNA sequence of *Hb*-CK

ATGCCGTTTTCTAATAGCCACAATGCACTTAAGTTACGTTTCCCCGCCGAGGATGAG
TTCCCCGGACCTGTCCGCACACAACAACCACATGGCGAAAGTCTTAACCTCCCGAGTTA
TACGCAGAGCTTCGTGCAAAGTCGACCCCTCGGGTTTCACCCTTGATGATGTAATC
CAGACTGGGGTAGATAATCCAGGCCACCCATATATTATGACCGTGGGGTGTGTAGC
GGGCGATGAGGAGAGCTACGAGGTTTTCAAGGACCTTTTCGATCCAATTATTGAAGA
CCGTCACGGTGGCTATAAGCCCTCGGATGAGCATAAGACAGACCTGAATCCTGACA

ACCTTCAGGGTGGAGACGATCTGGACCCGAATTATGTTTTAAGCTCAAGAGTGCGCA
CTGGAAGATCAATCCGTGGGTTTTGCCTTCCACCTCACTGCTCCCGCGGTGAACGTA
GAGCAATCGAGAAGCTTGCCGTTGAAGCTTTATCATCATTGGACGGCGACTTGGCAG
GTCGCTACTATGCCCTGAAGAGCATGACCGAGGCGGAGCAACAACAGTTAATAGAC
GATCACTTCTTGTTTCGACAAACCAGTTTCACCACTTCTCCTTGCATCTGGTATGGCCC
GCGACTGGCCTGACGCACGTGGCATCTGGCACAATGACAATAAGACTTTTCTGGTCT
GGGTTAATGAAGAGGACCATCTGCGGGTGATTTTCGATGCAAAAAGGGTGGAAATATG
AAGGAAGTGTTTACTAGATTCTGCACGGGTCTCACTCAAATCGAACTTTGTTCAAG
TCAAAGGATTATGAGTTCATGTGGAATCCACACCTCGGATACATCTTAACGTGTCCT
AGCAACTTAGGCACGGGGCTGCGTGACGGCGTTCATATCAAGTTACCGAACTTAGG
AAAACACGAGAAGTTCTCCGAGGTGTTGAAGCGGCTCCGTTTACAGAAGCGTGGCA
CCGGCGGGGTTGACACAGCGGCAGTAGGCGGTGTCTTTGACGTGTCTAATGCGGAC
CGGCTGGGATTTTCCGAGGTTGAGCTGGTTCAAATGGTTGTAGACGGCGTAAAGCTG
CTGATTGAGATGGAGCAGCGGCTTGAGCAAGGGCAAGCAATCGACGATCTCATGCC
AGCCCCAAAAGTGA

Protein sequence of *Hm-CK* (from *Homo sapiens*, Uniprot ID: P06732)

MPFGNTHNKFKNLYKPEEEYPDL SKHNNHMAKVL TLELYKKLRDKETPSGFTVDDVIQ
TGVDNPGHPFIMTVGCVAGDEESYEVFKELFDPIISDRHGGYKPTDKHK TDLNHENLKG
GDDLDPNYVLSSRVRTGRSIKGYTLPPHCSRGERRAVEKLSVEALNSLTGEFKGKYYP
KSMTEKEQQQLIDDHFLFDKPVSPLLL ASGMARDWPDARGIWHNDNKSFLVWVNEED
HLRVISMEKGGNMKEVFRRFCVGLQKIEEIFKKAGHPFMWNQHLGYVLTCPSNLGTGL
RGGVHVKL AHL SKHPKFEEILTRLRLQKRGTGGVDTAAVGSVFDVSNADRLGSSEVEQ
VQLVVDGVKLMVEMEKKLEKGQSIDDMIPAQK*

DNA sequence of *Hm-CK*

ATGCCATTTGGTAATACCCACAATAAGTTCAAGCTTAACCTACAAACCTGAGGAAGA
GTACCCGGACCTTTCAAAGCACACAACCACATGGCCAAAGTTCTGACCCTTGAGTT
GTACAAGAAGCTCCGTGATAAGGAACTCCTAGCGGATTCACGGTGGATGATGTTA
TCCAGACAGGAGTCGATAATCCCGGGCACCTTTTATTATGACGGTAGGTTGTGTGCG
CCGGAGATGAGGAGTCGTACGAGGTCTTTAAGGAATTGTTGATCCAATTATTAGTG
ACCGGCACGGCGGGTATAAGCCGACAGATAAGCATAAGACGGACCTGAATCACGA
GAACCTTAAGGGTGGGGACGATTTGGACCCTAATTATGTATTGTCGTCACGTGTGAG
AACGGGGAGATCAATCAAAGGCTATACGCTCCACCTCACTGCTCACGTGGCGAAC
GTCGCGCCGTCGAGAAGTTGTCGGTTGAAGCGTTAAACAGCCTCACAGGTGAGTTTA
AGGGTAAGTACTATCCGTTGAAGTCTATGACCGAGAAGGAGCAACAACAGCTGATT
GACGATCACTTTCTCTTCGACAAACCTGTTAGTCCTCTCCTTCTTGCCTCTGGTATGG
CACGTGACTGGCCTGACGCTCGTGGCATTGTCGACAATGACAATAAGTCGTTCTGG
TCTGGGTAAATGAAGAGGACCATCTGAGAGTGATTAGCATGGAAAAGGGTGGCAAT
ATGAAGGAAGTATTTTCGTCGTTTCTGCGTGGGATTGCAGAAAATCGAAGAAATATTC
AAGAAGGCGGGTCATCCGTTTCATGTGGAATCAACACTTAGGCTACGTATTAACCTGT
CCTAGTAACTTAGGTACGGGCCTGCGCGGCGGCGTTCATGTAAAGTTGGCGCACTTA
TCAAAACACCCTAAGTTCGAAGAGATATTGACGCGGCTGCGCTTGCAGAAGCGTGG
TACCGGTGGGGTTGACACAGCTGCTGTAGGATCAGTGTTTGACGTTAGTAATGCCGA
CCGCTTGGGATCTAGTGAGGTGGAGCAAGTACAATTAGTTGTTGACGGCGTTAAGTT
GATGGTCGAGATGGAGAAGAAGTTGGAGAAAGGCCAAAGCATCGACGATATGATA
CCCGCCCCAAAAGTGA

Protein sequence of *Rm*-CK (from *Oryctolagus cuniculus*, Uniprot ID: P00563)

MPFGNTHNKYKLNKSEEEYPDL SKHNNHMAKVLPDL YKKLRDKETPSGFTLDDVIQ
TGVDNPGHPFIMTVGCVAGDEESYTVFKDLFDPIIQDRHGGFKPTDKHKTDLNHENLKG
GDDLDPHYVLSSRVRTGRS IKGYTLPPHCSRGERRAVEKLSVEALNSLTGEFKGKYYP
KSMTEQEQQLIDDHFLFDKPVSPLLL ASGMARDWPDARGIWHNDNKSFLVWVNEED
HLRVISMEKGGNMKEVFRRFCVGLQKIEEIFKKAGHPFMWNEHLGYVLTCP SNLGTGL
RGGVHVKL AHL SKHPKFEEILTRLRLQKRGTGGVDTAAVGSVFDISNADRLGSSEVEQV
QLVVDGVKLMVEMEKKLEKGQSIDDMIPAQK*

DNA sequence of *Rm*-CK

ATGCCATTCGGAATACCCACAACAAGTACAAGTTGAATTACAAGTCAGAAGAAGA
ATACCCAGATTTGTGCGAAACATAATAATCACATGGCCAAGGCTTAACCCCCGATCT
TTATAAGAAGTTACGAGACAAGGAGACCCCTCGGGTTTTACATTAGACGACGTGAT
CCAAACCGGCGTTGACAATCCGGGCCACCCATT CATCATGACAGTTGGGTGCGTTGC
TGGAGACGAAGAGTCCTACACTGTATTCAAGGATCTGTTTCGATCCTATAATACAAGA
TCGGCATGGTGGTTTTCAAGCCAACCGACAAGCACAAGACGGACTTAAATCACGAGA
ACCTTAAGGGCGGGGATGACCTGGACCCGCATTATGTTTTATCGAGCCGCGTACGCA
CAGGCCGTTCAATCAAGGGCTACACATTGCCGCCACACTGCAGTCGCGGGGAACGG
CGTGCGGTAGAGAACTGAGCGTCGAGGCTTTGAATTCCTTAACCGGCGAGTTCAA
GGGCAAGTACTACCCGCTGAAAAGTATGACAGAGCAGGAACAGCAGCAGCTGATTG
ACGATCATTTTCTGTTTCGACAAACCCGTTAGTCCCCTGCTCCTTGCGTCCGGCATGGC
ACGTGACTGGCCTGATGCCAGAGGAATCTGGCACAACGATAACAAGAGTTTCTTAG
TATGGGTGAATGAGGAAGATCACCTGCGCGTGATCTCAATGGAGAAAGGCGGAAAT
ATGAAGGAAGTCTTTTCGACGGTTTTGCGTGGGTTTGCAAAAGATTGAGGAGATCTTC
AAGAAGGCCGGTCATCCGTT CATGTGGAACGAACATCTGGGGTATGTTTTAACATGT
CCCTCGAACCTTGGCACCCGGGCTGCGGGGTGGAGTTCACGTAAAGTTAGCCCATTTA
TCAAAGCACCCCAAGTTCGAGGAGATTTTGACGCGTCTGCGCTTG CAGAAGCGTGGT
ACCGGCGGCGTGGACACCGCTGCCGTGGGATCTGTGTTTCGATATATCAAACGCAGA
CAGATTGGGGAGCTCCGAAGTTGAGCAGGTGCAACTTGTTGTTGACGGCGTTAAGTT
GATGGTGGAGATGGAAAAGAAATTAGAGAAGGGCCAGAGTATAGACGATATGATA
CCAGCGCAAAAGTGA

Protein sequence of *Ag*-dNK (from *Anopheles gambiae*, Uniprot ID: Q86LB8)

MPPIASEKLGASGKKPFTV FVEGNIGSGKTTFLNHFQKFNDICLLTEPVEKWRNCGGVNL
LDLMYKESHRWAMPFQTYVTLTMLDMHTCQTDKSVKLMERSLFSARNCFVESMLASG
SLHQGMYNVLQEWYDFICCNIIHQADLIVYLQTSPEVVYERMKQRARSEESCVPLEYLK
ELHELHENWLIHGASPRPAPVLVLNADLDLNTIGA EYERSET SILKPILIENTNQHAILTSP
AKRAKTDF*

DNA sequence of *Ag*-dNK

ATGCCACCCATTGCAAGTGAGAACTCGGAGCTTCAGGGAAGAAGCCGTT CACAGT
ATTTGTTGAAGGGAACATCGGCAGCGGGAAGACTACCTTCTTGAACCACTTCCAAAA
GTTCAACGACATCTGCTTGTTGACTGAGCCCGTGGAGAAGTGGCGGAATTGCGGTGG
CGTGAACCTTACTTGACCTTATGTACAAGGAGTCACATCGTTGGGCTATGCCTTTCCA
GACATACGTTACCTTGACAATGTTAGACATGCACACGTGTCAAACAGATAAGTCAGT
TAAATTAATGGAACGCAGCCTGTTTT CAGCACGGAAGTGTTCGTCGAGAGTATGTT
GGCGTCAGGGAGCTTGCATCAAGGAATGTACAACGTACTTCAAGAGTGGTACGATT

TCATTTGTTGCAATATACATATACAAGCGGATCTTATCGTATACCTTCAAACGTCCCC
GGAAGTTGTTTACGAACGGATGAAGCAGCGCGCCCGTAGTGAGGAGTCATGCGTAC
CATTAGAGTATCTTAAGGAACTCCATGAGTTGCATGAAAATTGGCTGATCCACGGCG
CTTCCCCGCGGCCCGCTCCTGTCCTCGTTCTGAATGCCGACCTTGATCTCAATACCAT
CGGAGCAGAGTATGAACGGAGTGAGACATCCATATTAACCGATTTTGATCGAGA
ACACGAACCAACACGCTATTCTGACCTCGCCGGCGAAGCGGGCTAAGACGGACTTC
TAA

Protein sequence of *Dm*-dNK (from *Drosophila melanogaster*, Uniprot ID: Q540Z9)

MAEAASCARKGTKYAEGTQPFTVLIEGNISGKTTYLNHFKEYKNDICLLTEPVEKWRN
VNGVNLELMYKDPKKWAMPFQSYVTLMLQSHTAPTNNKKLKIMERSIFSARYCFVEN
MRRNGSLEQGMYNMLEEWYKFIEESIHVQADLIYLRTSPEVAYERIRQRARSEESCVPL
KYLQELHELHEDWLIHQRRPQSKVLVLDADLNLENIGTEYQRSESSIFDAISSNQQPSP
VLVSPSKRQRVAR*

DNA sequence of *Dm*-dNK

ATGGCCGAGGCCGCATCATGTGCTCGAAAAGGAACTAAGTACGCCGAGGGCACCCA
ACCCTTCACGGTACTCATTGAAGGGAATATTGGTTCAGGTAAAACGACGTACTTAAA
CCATTTTGAGAAATACAAGAATGACATTTGTCTTTTGACGGAGCCTGTAGAGAAGTG
GCGTAACGTGAATGGCGTCAATCTGTTAGAATTGATGTATAAAGACCCGAAGAAAT
GGGCCATGCCATTTCAAAGCTACGTTACTTTAACGATGTTGCAGTCGCATACCGCGC
CGACCAACAAGAACTCAAGATAATGGAAAGATCTATCTTCAGTGCGCGCTATTGTT
TCGTTGAGAACATGCGTCGCAACGGAAGTCTGGAACAAGGCATGTATAATACTCTG
GAAGAATGGTATAAGTTCATCGAGGAGTCTATACACGTCCAGGCCGACTTGATAATC
TATTTACGGACCTCTCCAGAAGTGGCCTATGAACGAATACGACAACGTGCACGCAGT
GAGGAGAGTTGTGTCCCCCTTAAGTACCTGCAAGAGTTACACGAACTGCATGAGGA
CTGGCTGATACACCAGCGTCGCCCCCAAAGTTGCAAGGTTTTAGTGCTGGACGCAGA
CCTTAACCTTGAGAATATTGGCACAGAGTATCAGCGAAGTGAGTCTTCTATATTCTGA
CGCTATTTCTTCGAACCAACAACCGTCACCTGTTCTTGTGTCACCTTCAAAGAGACA
ACGCGTCGCGCGCTAA

Protein sequence of *Hs*-dCK (from *Homo sapiens*, Uniprot ID: P27707)

MATPPKRSCPSFSASSEGTRIKKISIEGNIAAGKSTFVNILKQLCEDWEVVPEPVARWCNV
QSTQDEFEEELTMSQKNGGNVLQMMYEKPERWSFTFQTYACLSRIRAQLASLNGKLKDA
EKPVLFFERSVYSDRYIFASNLYESECNMNETEWTIYQDWHDDWMNNQFGQSLELDGIIYL
QATPETCLHRIYLRGRNEEQGIPLEYLEKLHYKHESWLLHRTLKTNFDYDLQEVPIITLDV
NEDFKDKYESLVEKVKEFLSTL*

DNA sequence of *Hs*-dCK

ATGGCGACTCCACCGAAACGAAGTTGTCCATCTTTCTCGGCTAGCAGTGAGGGGACA
CGCATAAAGAAGATCTCAATTGAGGGCAACATCGCCGCTGGGAAGTCCACGTTTCGT
TAATATACTGAAGCAGCTCTGCGAGGACTGGGAAGTTGTGCCGGAGCCTGTGGCCC
GATGGTGCAATGTGCAGAGTACCCAAGACGAATTTGAAGAGCTCACGATGAGCCAA
AAGAATGGCGGAAACGTGCTTCAGATGATGTACGAGAAGCCAGAACGGTGGTCGTT
CACGTTTCAAACATACGCCTGTCTGAGCCGCATCCGCGCTCAATTAGCATCGCTCAA
TGGCAAGCTCAAGGATGCTGAGAAACCTGTCTTATTCTTCGAGCGCTCTGTCTACTC
TGACCGTTACATCTTCGCGAGTAACTTGTACGAGAGCGAGTGTATGAATGAGACTGA
GTGGACGATATATCAAGACTGGCACGATTGGATGAACAATCAATTCGGCCAATCTCT

GGAGTTAGACGGAATCATATACCTTCAGGCGACGCCTGAAACGTGTTTGCACCGGAT
CTATCTTCGTGGCCGGAACGAGGAGCAAGGCATCCCCCTAGAATATCTCGAAAAGCT
GCATTACAAACACGAATCCTGGTTGTTGCACCGTACTTTGAAGACGAATTTTCGATTA
TCTCCAGGAAGTCCCAATCTTGACCTTGACGTCAACGAGGACTTTAAGGACAAGTA
CGAATCCCTCGTCGAAAAGGTCAAGGAGTTCCTGAGTACCTTGTGA

Protein sequence of *Tm*-TK (from *Thermotoga maritima*, Uniprot ID: Q9WYN2)

MSGKLTVITGPMYSGKTTELLSFVEIYKLGKKKVAVFKPKIDSRVHSTMIVSHSGNGVE
AHVIERPEEMRKYIEEDTRGVFIDEVQFFNPSLFEVVKDLLDRGIDVFCAGLDLTHKQNP
FETTALLSLADTVIKKKAVCHRCGEYNATLTLKVAGGEEEIDVGGQEKYIAVCRDCYN
TLKKRV*

DNA sequence of *Tm*-TK

ATGAGCGGGAAACTGACAGTCATAAACCGGTCCGATGTATAGTGGTAAAACAACTGA
GCTGCTCAGTTTCGTTGAAATCTACAAGCTCGGAAAGAAGAAAGTCGCGGTATTCAA
ACCTAAGATCGACTCGCGTTACCACAGCACTATGATAGTTAGCCATAGCGGGAACG
GTGTGGAAGCGCACGTGATCGAACGTCCTGAAGAAATGCGTAAGTATATCGAGGAA
GACACGCGTGCGGTATTCATCGACGAAGTCCAATTCTTCAACCCCTCTTTATTTCGAG
GTCGTAAAAGATCTCTTAGACCGTGGCATAGACGTGTTCTGTGCGGGTTTGGATCTG
ACACACAAGCAGAACCCCTTCGAGACCACCGCCTTACTGTTGTCTTTGGCCGATACT
GTAATTAAGAAGAAAGCGGTCTGCCACCGCTGCGGGGAGTATAATGCGACCTTGAC
TCTTAAGGTCGCTGGTGGCGAAGAGGAGATCGATGTTGGCGGTCAAGAGAAGTATA
TAGCCGTCTGCCGCGACTGTTATAACACTTTAAAGAAGAGAGTGTGA

Protein sequence of *Sc*-AK (from *Saccharomyces cerevisiae*, Uniprot ID: B3LG61)

MSSSESIRMVLIGPPGAGKGTQAPNLQERFHAAHLATGDMMLRSQIAKGTQLGLEAKKIM
DQGGVLVSDDIMVNMKDELNNPACKNGFILDGFPRPTIPQAEKLDQMLKEQGTPLEKAI
ELKVDDELLVARITGRLIHPASGRSYHKIFNPPKEDMKDDVTGEALVQRSDDNADALKK
RLAAYHAQTEPIVDFYKKTGIWAGVDASQPPATVWADILNKLKGD*

DNA sequence of *Sc*-AK

ATGAGCAGTTCGAAAGCATTCGTATGGTGCTGATAGGTCCACCTGGAGCGGGCAA
GGGGACACAAGCACCTAATCTGCAAGAGCGCTTCCACGCTGCCCACCTTGCGACCG
GCGACATGCTGCGCAGTCAGATTGCAAAGGGCACTCAATTAGGGCTGGAAGCCAAG
AAGATTATGGATCAGGGTGGACTCGTGTCCGACGACATCATGGTAAACATGATAAA
GGACGAACCTACCAACAACCCAGCTTGTAAGAACGGATTCATTCTGGATGGTTTCCC
GCGAACAATACCGCAGGCAGAAAAGTTAGATCAAATGCTCAAGGAACAGGGCACA
CCATTAGAGAAGGCCATTGAGCTTAAAGTAGACGACGAACCTCCTCGTAGCACGCAT
CACCGGTAGATTAATACACCCAGCATCGGGTCGGTCCTACCATAAGATTTTCAATCC
CCCAAAGAAGACATGAAGGATGACGTTACTGGGGAAGCACTTGTTCAAAGATCAG
ATGACAACGCCGATGCACTTAAGAAACGGCTTGCCGCTTATCATGCGCAAACCTGAG
CCCATCGTCGACTTCTATAAGAAGACGGGAATCTGGGCTGGTGTGATGCAAGTCAG
CCCCCGGCTACGGTATGGGCCGACATCTTGAACAAGTTGGGAAAGGATTAA

Protein sequence of *Ec*-AK (from *Escherichia coli* O139:H28, Uniprot ID: A7ZIN4)

MRIILLGAPGAGKGTQAQFIMEKYGIPQISTGDMLRAAVKSGSELGKQAKDIMDAGKLV
TDELVIALVKERIAQEDCRNGFLLDGFPRPTIPQADAMKEAGINVDYVLEFDVPDELIVDR

IVGRRVHAPSGRVYHVKNPPKVEGKDDVTGEELTTRKDDQEETVRKRLVEYHQMTAP
LIGYYSKEAEAGNTKYAKVDGTPVAEVRADLEKILG*

DNA sequence of *Ec*-AK

ATGCGTATAATATTGTTGGGTGCACCGGGTGCCGGCAAAGGAACACAAGCCCAATT
CATCATGGAGAAGTATGGTATTCCACAAATCAGTACAGGCGACATGTTGAGAGCAG
CAGTTAAAAGCGGCTCAGAGCTGGGTAAGCAGGCTAAAGACATAATGGATGCAGGC
AAGCTGGTTACTGATGAACTTGTAATAGCACTTGTGAAGGAGCGCATAGCTCAAGA
AGATTGTCGGAACGGCTTTCTGCTCGATGGCTTCCCTCGCACTATTCCGCAGGCGGA
CGCAATGAAAGAGGCTGGTATCAACGTAGATTATGTACTTGAATTTGACGTCCTCCGA
CGAGTTGATTGTCGATCGTATCGTGGGCCGTAGAGTACATGCACCCTCAGGGCGTGT
ATACCATGTGAAGTTTAACCCACCTAAGGTAGAGGGTAAGGACGACGTGACAGGTG
AGGAGCTGACTACCCGTAAAGACGATCAAGAGGAAACGGTCCGAAAGCGCCTTGTA
GAGTACCACCAGATGACAGCGCCACTGATTGGATACTACAGTAAGGAAGCAGAGGC
GGGGAACACTAAGTATGCGAAAGTGGACGGAACGAAACCAGTTGCCGAGGTGCGTG
CAGACTTAGAGAAGATATTAGGTAA

Protein sequence of *Bf*-GMPK (from *Branchiostoma floridae*, Uniprot ID: C3YEM4)

MALPRPVVICGPSGSGKSTLYNKLLKEFPGVFQLSVSHTTRQPRPGELNGREYHFINRDQ
FQENIKQGDFFLEWAEFSGNIYGTSSKALEEVQSNNVIPILDIDTQGVNRVKKASLEAVYI
FIKPPSIDVLEKRLRSRKTETEEALQKRLSAARNELEYGLKPGNFQHIITNDDLDVAYEKL
KGILIKSQMPLAMATGSSSSSVNSFLDKPAASATTVNSSSQD*

DNA sequence of *Bf*-GMPK

ATGGCTTTGCCTAGACCTGTCTGATCTGTGGGCCTTCTGGAAGTGGCAAATCCACA
TTATACAATAAATTGCTGAAAGAGTTCCAGGTGTATTCCAAGTGTCCGTAAAGTCAC
ACTACTCGGCAGCCCAGACCGGGAGAGCTGAACGGCCGTGAATATCACTTCATCAA
CCGAGACCAATTCCAAGAAAACATTAAGCAAGGGGATTTCTTAGAGTGGGCAGAAT
TCAGTGGGAACATTTACGGTACCAGTAAGAAGGCTCTCGAAGAGGTACAATCGAAC
AATGTCATTCCGATCTTAGATATAGACACCCAGGGAGTTCGTAACGTAAAGAAGGC
GTCGCTTGAGGCAGTTTACATCTTCATTAAGCCGCCGAGCATTGACGTCTTAGAGAA
GAGACTTAGAAGTCGCAAGACGGAACGGAAGAAGCATTACAAAAGCGGCTTAGC
GCAGCTCGTAATGAGCTTGAGTATGGACTTAAGCCGGGGAATTTCCAACACATTATC
ACCAATGACGATCTGGACGTTGCGTACGAGAAGCTCAAAGGTATACTCATCAAATCT
CAAATGCCTCTGGCAATGGCCACAGGCAGCTCATCTAGCGTCGTAAACAGTTTCCTG
GACAAACCGGCAGCGAGTGCCACCACGGTCAATTTCGAGCTCCCAAGATTAA

Protein sequence of *Hs*-GMPK (from *Homo sapiens*, Uniprot ID: Q16774)

MSGPRPVVLSGPSGAGKSTLLKRLLEHSGIFGFSVSHTRNPRPGEENGKDYYFVTREV
MQRDIAAGDFIEHAEFSGNLYGTSKVAVQAVQAMNRCVLDVDLQGVNRNIKATDLRPIY
ISVQPPSLHVLEQRLRQRNTETEEESLVKRLAAAQADMESKEPGLFDVVIINDSLDQAYA
ELKEALSEEIKKAQRTGA*

DNA sequence of *Hs*-GMPK

ATGTCGGGGCCCCGTCCGGTTGTTCTTAGTGGTCCTAGTGGTGCAGGTAAGAGCACA
TTGCTCAAACGGCTGTTGCAAGAGCATTCCGGTATCTTCGGGTTCTCGGTATCACAT
ACCACTCGTAACCCGCGTCCAGGGGAAGAGAATGGCAAGGACTATTATTCGTAAC
GCGCGAAGTTATGCAACGTGACATAGCCGCTGGTGATTTTCATCGAGCATGCTGAATT
TTCTGGCAACCTGTACGGCACCTCGAAGGTTGCAGTGCAAGCAGTGCAGGCAATGA

ACCGCATTGTGTACTCGACGTAGACCTGCAAGGGGTGAGAAACATTAAGGCCACT
GACTTACGGCCCATATATATTTCTGTTCAACCGCCAAGTCTTCACGTCTTAGAGCAA
CGGTTGAGACAACGTAACACCGAGACCGAAGAGTCATTAGTTAAGCGTCTGGCCGC
AGCGCAAGCTGACATGGAGAGTTCAAAGGAGCCAGGCTTGTTTGACGTCGTAATCA
TCAACGACAGTCTTGATCAAGCGTATGCTGAATTGAAAGAGGCCCTTTCAGAGGAG
ATTAAGAAGGCACAGAGAACAGGAGCCTAA

Protein sequence of *Ec*-GMPK (from *Escherichia coli* O121, Uniprot ID: A0AAP9SJ55)

MAQGTLYIVSAPSGAGKSSLIQALLKTQPLYDTQVSVSHTTRQPRPGEVHGEHYFFVNH
DEFKEMISRDAFLEHAEVFGNYYGTSREAIEQVLATGVDVFLDIDWQGAQQIRQKMPH
ARSIFILPPSKIELDRRLRGRGQDSEEVIAKRMAQAVAEMSHYAEYDYLIVNDDFDALT
DLKTIIRAERLRMSRQKQRHDALISKLLAD*

DNA sequence of *Ec*-GMPK

ATGGCACAAAGGCACCTTATACATCGTTAGTGCTCCTTCCGGCGCAGGAAAGTCGTCT
CTGATTCAGGCTTTACTGAAGACGCAGCCACTGTACGACACCCAAGTGAGTGTCTCC
CACACCACGCGTCAACCCAGACCTGGGGAAGTTCACGGGGAGCACTACTTCTTTGTC
AACCACGACGAGTTCAAGGAGATGATATCTCGCGATGCCTTCCTGGAGCATGCTGA
AGTGTTTCGGGAATTATTATGGAACCTCTAGAGAGGCAATTGAGCAGGTGCTCGCCAC
CGGCGTTGACGTCTTCCTTGATATTGACTGGCAGGGAGCCCAACAGATTCGCCAGAA
GATGCCGCACGCCCCGAAGCATTTTCATACTGCCCCCTTCCAAGATAGAGCTGGATCG
TCGCTTAAGAGGACGGGGTCAAGATAGCGAGGAAGTGATCGCTAAGCGAATGGCAC
AAGCGGTTGCTGAGATGAGTCATTACGCCGAGTACGACTACTTGATCGTAAACGAC
GATTTTGATACAGCCTTAACGGACTTGAAGACTATCATTTCGCGCGGAGAGACTGCGC
ATGTCACGTCAGAAGCAGCGTCACGACGCCTTGATCTCAAAGTTACTTGCAGACTGA

Protein sequence of *Hs*-UMP-CMPK (from *Homo sapiens*, Uniprot ID: P30085)

MKPLVVFVLGGPGAGKGTQCARIVEKYGYTHLSAGELLRDERKNPDSQYGELIEKYIKE
GKIVPVEITISLLKREMDQTMAANAQKNKFLIDGFPRNQDNLQGWNKTMDGKADVSFV
LFFDCNNEICIERCLERKGSSGRSDDNRESLEKRIQTYLQSTKPIIDL YEEMGKVKKIDAS
KSVDEVFDEVVQIFDKEG*

DNA sequence of *Hs*- UMP-CMPK

ATGAAACCCTTGGTGGTATTTCGTATTGGGTGGTCCGGGCGCAGGCAAGGGCACCCA
GTGCGCCCGTATAGTGAGAAATACGGATACACGCATTTATCCGCGGGCGAACTGC
TTCGCGACGAGCGGAAGAACCCTGACTCTCAATACGGGGAACTTATTGAGAAATAC
ATCAAGGAAGGGAAGATAGTTCCCGTAGAGATTACTATCTCGTTACTTAAGCGGGA
GATGGATCAAACGATGGCTGCCAACGCCCAAAGAATAAGTTCTTAATTGATGGTTT
CCCTCGTAACCAAGACAATCTGCAGGGCTGGAATAAAACAATGGATGGTAAGGCAG
ACGTGAGTTTCGTCCTTTTCTTCGACTGCAATAATGAGATCTGTATCGAACGCTGTTT
AGAACGAGGAAAGTCTAGCGGGCGATCTGATGATAACCGCGAAAGCCTTGAAAAGC
GTATTCAAACGTATCTGCAATCTACTAAGCCGATCATAGACCTCTACGAAGAGATGG
GCAAGGTCAAGAAGATAGATGCGAGTAAGAGTGTTGACGAAGTATTTGATGAAGTG
GTACAGATCTTCGACAAGGAAGGCTGA

Protein sequence of *Bs*-CMPK (from *Bacillus subtilis*, Uniprot ID: A3F452)

MEKKLSIAIDGPAAAGKSTVAKIVAEKKSYYIDTGAMYRAITYAALQENVDLTDEEKL
AELLKRTDIELITTKDGQKV FVNGTDVTEAIRTEISNQVSIAAKHRSVREEMVKRQQQL

GEKGGVMDGRDIGTHVLPNAEVKIFLLASVEERAKRRYEENVKKGFDVNYETLIEEIA
RRDKLDSEREVSPLRKAEDALEIDTTSLSIQEVADKILEAVEQKSR*

DNA sequence of *Bs*-CMPK

ATGGAAAAGAAGCTCAGTATAGCAATAGACGGACCCGCTGCGGCTGGAAAATCAAC
AGTGGCTAAAATTGTCGCAGAGAAGAAGTCGTACATTTACATTGACACAGGCGCTA
TGTATAGAGCGATAACATACGCAGCCCTTCAAGAAAACGTGGACTTGACAGACGAA
GAGAAGCTGGCGGAGTTGCTCAAGCGCACCGACATCGAACTTATCACAACAAAGGA
CGGGCAGAAAGTGTTTGTAAACGGCACAGACGTAAGTGAAGGCCATCCGCACCGACG
AGATATCAAATCAGGTCTCGATCGCAGCTAAGCACCGCTCTGTACGGGAAGAAATG
GTAAAGAGACAACAGCAATTGGGCGAAAAGGGTGGCGTCGTAATGGATGGGCGTG
ATATAGGTACCCATGTTCTTCCCAACGCGGAAGTGAAAATTTTCTTATTGGCAAGCG
TGGAAGAACGGGCCAAGCGTCGTTATGAAGAGAACGTCAAGAAAGGGTTCGACGTA
AATTATGAAACTTTGATTGAGGAGATAGCTAGACGGGACAAGTTAGACTCAGAAAG
AGAGGTCTCCCCTCTGCGGAAGGCCGAAGACGCGCTGGAAATCGATACGACATCCC
TTAGCATCCAAGAAGTAGCAGATAAGATTTTAGAGGCCGTCGAACAAAAGTCACGA
TAG

Protein sequence of *At*-CMPK (from *Arabidopsis thaliana*, Uniprot ID: O04905)

MGSVDAANGSGKKPTVIFVLGGPGSGKGTQCAIYIVEHYGYTHLSAGDLLRAEIKSGSEN
GTMIQNMIEGKIVPSEVTIKLLQKAIQENGNDKFLIDGFPRNEENRAAFEKVTEIEPKFV
LFFDCPEEEMEKRLLLGRNQGREDDNIETIRKRFKVFLESSLPVIHYEAKGKVRKINAAK
PIEAVFEEVKAIFSPEAEKVEA*

DNA sequence of *At*-CMPK

ATGGGTTCTGTGGATGCTGCAAATGGCTCTGGAAAGAAGCCCACTGTTATATTCGTC
CTCGGTGGACCTGGTAGTGGAAAAGGCACGCAATGCGCCTACATCGTTGAACATTA
CGGTTACACTCATTTGAGTGCTGGGGATCTCTTGCGTGCGGAGATCAAGTCGGGGCTC
AGAAAACGGAACGATGATTCAAAATATGATCAAGGAAGGAAAGATCGTGCCTAGTG
AGGTGACAATCAAGCTTTTGCAGAAGGCGATCCAAGAGAATGGAAACGATAAGTTC
CTGATTGATGGATTCCACGGAACGAGGAGAACCAGGGCCGCATTCGAGAAGGTAAC
AGAGATTGAGCCGAAGTTCGTTCTGTTCTTTGACTGCCCAGAAGAGGAAATGGAGA
AGCGGTTGCTGGGTGCGCAATCAAGGACGAGAGGACGATAATATTGAAACCATTCGG
AAACGCTTCAAGGTATTTCTTGAATCCTCTCTGCCGTTATACATTATTATGAAGCTA
AGGGCAAGGTACGGAAGATTAATGCGGCCAAGCCGATTGAGGCAGTGTTGAGAGAA
GTAAAGGCGATATTTAGCCCCGAAGCAGAGAAAGTAGAAGCCTAG

Protein sequence of *Mt*-CMPK (from *Mycobacterium orygis*, Uniprot ID: A0A829C8P9)

MSRLSAAVVAIDGPAGTGKSSVSRRLARELGARFLDTGAMYRIVTLAVLRAGADPSDIA
AVETIASTVQMSLGYDPDGDSCYLAGEDVSVEIRGDAVTRAVSAVSSVPAVRTRLVELQ
RTMAEGPGSIVVEGRDIGTVVFPDAPVKIFLTASAETRARRRNAQNVAAGLADDYDGV
LADVRRRDHLDSTRAVSPLQAAGDAVIVDTSMDTEAEVVAHLLELVTRRSEAVR*

DNA sequence of *Mt*-CMPK

ATGTCTCGTCTGAGTGCAGCTGTTGTCGCTATTGATGGACCCGCCGGAAGTGGTAAG
AGTTCCGTCTCTCGTCGCTTAGCACGGGAAGTGGGTGCACGCTTCCTGGATACGGGC
GCTATGTACCGTATAGTCACTTTGGCGGTGCTTCGCGCCGGGGCCGACCCTTCGGAT
ATCGCCCGCGTTGAGACGATTGCCTCAACTGTACAGATGAGCCTGGGTACGACCCT
GACGGCGACAGTTGCTACTTAGCAGGAGAAGATGTCTCAGTGGAGATCCGCGGTGA

TGCAGTGACGCGAGCCGCTCTCCGCTGTATCCAGTGTCCCTGCGGTTAGAACCCGGCT
CGTTGAATTGCAACGGACAATGGCCGAAGGTCCGGGTTCTATCGTTGTAGAGGGTA
GAGACATCGGAACCGTTGTGTTCCCTGATGCACCAGTCAAGATCTTTCTCACGGCGT
CGGCAGAGACGCGTGCAAGAAGACGCAACGCTCAAAATGTTGCAGCGGGACTTGCG
GACGACTATGATGGCGTTCTGGCCGACGTAAGACGCAGAGATCACCTGGACTCTAC
GAGAGCAGTTTCTCCGTTACAAGCTGCTGGTGTATGCGGTGATCGTGGATACGTCTGA
CATGACTGAGGCTGAGGTAGTCGCCCACCTGCTGGAACCTGTAACACGGAGATCCG
AAGCCGTTTCGTTGA

Protein sequence of *Tm*-UMPK (from *Thermotoga maritima*, Uniprot ID: Q9X1U0)

MRVLVKLSGEALSGEGGRGFDPERVNYIVNEIKSAIEEGFKIGIVVGAGNLFRGVELKNL
TMTRADQIGLLGTVMNSVYLKDIFERSGLKARIYSQIVNLPDERVNYDSIESALRENSIL
IFAGGTSNPFFTTDTAAVLRAQEMRAKL VVKATKVDGVYDKDPKKFPDAKKIPHLTFSE
AMKMGLKVMDAEAFALCKKLGITVKVINFFEPGTLKALKGEDVGSTVVPD*

DNA sequence of *Tm*-UMPK

ATGCGAGTTCTGGTTAAGTTGTCAGGAGAAGCCTTATCAGGGGAAGGTGGCCGCGG
CTTTGACCCGGAAGAGTCAACTACATCGTTAACGAGATTAAGTCTGCGATAGAAG
AGGGCTTCAAGATTGGGATCGTTGTAGGGGCGGGGAATTTGTTCCGCGGAGTTGAA
CTTAAGAACCTGACCATGACACGTGCGGACCAAATTGGTCTCTTGGGGACCGTGATG
AACTCTGTATACCTGAAAGACATCTTCGAACGATCCGGACTTAAGGCACGGATATAC
TCGCAAATTGTAAACCTGCCGGACGTAGAAAGAGTTAACTATGACAGCATTGAGAG
TGCACTGCGCGAGAACTCTATTCTTATTTTCGCAGGCGGCACCTCAAACCCCTTCTTC
ACGACGGACACAGCTGCGGTGCTGAGAGCGCAAGAGATGCGTGCAAAATTAGTAGT
CAAGGCCACGAAGGTAGATGGAGTATACGACAAGGACCCTAAGAAAGTTCCCCGATG
CCAAGAAGATCCCTCATTTGACCTTCAGTGAAGCTATGAAGATGGGTCTTAAGGTTA
TGGACGCAGAGGCGTTCGCCTTATGTAAGAAGCTTGGCATAACGGTTAAAGTCATA
AATTTCTTTGAGCCGGGCACGTTGCTTAAGGCACTGAAGGGCGAGGACGTGGGTCT
ACGGTGGTTCCCGACTAA

Protein sequence of *Ec*-TmK (from *Escherichia coli*, Uniprot ID: B1XA08)

MRSKYIVIEGLEGAGKTTARNVVVETLEQLGIRDMVFTREPGGTQLAEKLRLSLVLDIKSV
GDEVITDKAEVLMFYAARVQLVETVIKPALANGTWVIGDRHDLSTQAYQGGGRGIDQH
MLATLRDAVLGDFRPDLTLYLDVTPEVGLKRARARGELDRIEQESFDFNRTRARYLEL
AAQDKSIHTIDATQPLEAVMDAIRTTVTHWVKELDA*

DNA sequence of *Ec*-TmK

ATGCGCTCTAAATATATCGTCATCGAAGGTCTCGAAGGCGCAGGCAAAACCACCGC
TCGAAACGTCGTGGTTGAAACATTAGAGCAGCTGGGTATTCGCGACATGGTGTTTAC
ACGTGAGCCTGGCGGGACCCAGCTGGCTGAAAAGCTGCGGTCAATTGGTTTTGGACAT
TAAGTCTGTAGGTGACGAGGTGATTACCGACAAAGCTGAAGTGCTTATGTTTTATGC
GGCACGTGTCCAATTGGTGGAGACCGTTATTAAGCCGGCCCTGGCAAACGGAACGT
GGGTATATCGGTGACCGGCACGATTTATCGACTCAAGCGTACCAAGGTGGCGGGCGT
GGGATAGACCAGCACATGCTGGCGACGCTCCGGGACGCCGTTTTAGGTGACTTCCG
ACCAGACCTGACACTGTACCTTGACGTCACACCAGAGGTGGGACTCAAGCGCGCGC
GTGCTCGGGGAGAACTTGACCGTATCGAGCAAGAGAGCTTCGACTTCTTCAACCGCA
CTCGGGCACGGTATCTGGAACCTGGCGGCCCAAGACAAGTCCATTCATACGATTGATG

CGACTCAACCACTCGAAGCAGTTATGGATGCAATCAGAACAACGGTAACCCACTGG
GTGAAAGAGCTGGACGCTTGA

Protein sequence of *Tm*-TmK (from *Thermotoga maritima*, Uniprot ID: Q9X0I3)

MFITFEGIDGSGKSTQIQLLAQYLEKRGKKVILKREPGGTETGEKIRKILLEEEVTPKAELF
LFLASRNLLVTEIKQYLSEGYAVLLDRYTDSSVAYQGFGRNLGKEIVEELNDFATDGLIP
DLTFYIDVDVETALKRKGLNRFEKREFLERVREGYLVLAREHPERIVVLDGKRSIEEIH
RDVVREVKRRWKLDV*

DNA sequence of *Tm*-TmK

ATGTTTCATTACCTTTGAGGGCATAGACGGCAGCGGGAAGAGCACCCAAATACAACCT
CTTGGCGCAATACTTAGAGAAGAGAGGGAAGAAGGTAATCCTGAAACGTGAGCCAG
GCGGCACGGAGACCGGGGAGAAAATCCGTAAGATTTTACTTGAAGAGGAAGTAACG
CCGAAGGCGGAGCTTTTCTTATTCTTAGCAAGCCGCAACCTGCTGGTCACCGAGATC
AAGCAATACCTGAGCGAAGGATACGCCGTTCTCTTGGACCGCTATACTGATAGCTCC
GTGGCCTATCAAGGGTTCGGACGAAACCTGGGGAAGGAGATAGTGGAAGAGCTGAA
CGACTTCGCCACCGATGGCCTGATTCCCGACCTTACCTTTTACATTGATGTAGACGTC
GAGACCGCGCTTAAGCGTAAAGGCGAGTTAAACCGCTTTGAGAAGCGTGAGTTCTT
AGAACGCGTTCGTGAGGGGTACCTTGTCTTAGCGCGAGAGCACCTGAGCGTATTGT
TGTGCTGGACGGTAAGCGGAGCATCGAAGAGATACATCGTGACGTTGTTTCGCGAGG
TCAAGCGGCGATGGAAGTTAGACGTCTGA

Protein sequence of *Tm*-AcK (from *Thermotoga sp. 2812B*, Uniprot ID: A0AAN0SKJ1)

MRVLVINSGSSSIKYQLIEMEKEKVLCKGIAERIGIEGSRLVHRVGDEKHVIERELPDHEE
ALKLILNTLVDEKLGVIKDLKEIDAVGHRVVHGGERFKESVLVDEEVLKAIEEVSP LAPL
HN PANLMGIKAAMKLLPGVPNVAVFD TAFHQ TIPQKAYLYAIPY EY EY EY KY KIRRYGFH
GTSHRYVSKRAAEILGKKLEELKIITCHIGNGASVAAVKYGKCVDTSMGFTPLEGLVMG
TRSGDLDP AIPFFIMEKEGISPQEMYDILNKKSGVYGLSKGFSSDMRDIEEAALKGDEWC
KLVL E IYDYRIAKYIGAYAAAMNGVDAIVFTAGVGENSPITREDVCSYLEFLGVKLDKQ
KNEETIRGKEGIISTPDSRVKVLVVP TNEELMIARDTKEIVEKIGR*

DNA sequence of *Tm*-AcK

ATGCGTGTCTGCTGTTATAAATTCAGGCTCTTCGTCAATTAAGTATCAGTTGATCGAG
ATGGAAGGCGAGAAGGTGCTCTGTAAAGGTATCGCAGAACGGATAGGAATCGAGG
GATCGCGGTTAGTACACCGAGTAGGGGACGAGAAGCACGTTATTGAGCGGGAGTTG
CCAGATCACGAGGAAGCACTGAAGCTTATTCTCAACACTCTTGTTGATGAGAAGCTG
GGAGTAATTAAGGATTTAAAAGAGATAGATGCCGTCGGGCACCGTGTTGGTGATGG
CGGTGAGCGCTTCAAGGAGTCGGTACTCGTTGACGAAGAGGTTTTAAAGGCTATTGA
AGAGGTTTCCCCACTCGCCCCGCTGCATAATCCCGCTAACCTCATGGGAATAAAGGC
TGCCATGAAATTACTTCCAGGAGTACCAAATGTAGCAGTATTTCGATACTGCGTTCCA
CCAAACAATCCCACAAAAGGCATATCTGTATGCGATTCTTACGAGTACTATGAAAA
GTACAAGATACGACGTTACGGTTTCCACGGTACTTCACACAGATACGTATCTAAGCG
TGCTGCTGAAATACTGGGCAAGAACTCGAAGAGTTAAAGATCATAACGTGCCACA
TAGGGAATGGAGCTAGCGTTGCTGCCGTCAAGTACGGCAAGTGTGTGGATACGTCG
ATGGGATTACACCACTGGAAGGACTCGTCATGGGCACACGCTCGGGTGACCTCGA
TCCGGCGATCCCATTCTTCATAATGGAGAAGGAAGGAATTAGTCCTCAGGAGATGTA
CGACATCCTCAACAAGAAGTCTGGGGTATATGGCCTGAGCAAAGGTTTCTCAAGCG
ATATGAGAGATATTGAGGAAGCGGCTCTGAAGGGTGACGAGTGGTGTAAGTCTCGTC
CTGGAGATTTACGATTATCGGATTGCCAAATACATTGGTGCGTACGCCGCCGCAATG

AACGGAGTCGACGCAATTGTTTTCACTGCGGGAGTCGGGGAAAACAGCCCCATAAC
ACGCGAAGACGTTTGCTCTTACTTAGAATTCTTGGGAGTAAAGTTGGATAAGCAAAA
GAACGAGGAAACAATACGGGGAAAAGAGGGTATAATCTCGACTCCTGATTCGCGTG
TAAAGGTGCTGGTGGTACCTACGAATGAAGAGCTGATGATCGCTCGTGATACGAAG
GAAATCGTCGAAAAGATTGGTTCGCTGA

Protein sequence of *Ec*-AcK (from *Escherichia coli*, Uniprot ID: W1XDL9)

MSSKLVLVLNCGSSSLKFAIIDA VNGEEYLSGLAECFHLPEARIKWKMDGNKQEAALGA
GAHSEALNFIVNTILAQKPELSAQLTAIGHRIVHGGEKYTSSVVIDESVIQGIKDAASFA
PLHNPAHLIGIEEALKSFPQLKDKNVA VFD TAFHQTMPEESYLYALPYNLYKEHGIRRY
GAHGTSHFYVTQEAAKMLNKPVEELNIITCHLGNNGGSVSAIRNGKCVDTSMGLTPLEGL
VMGTRSGDIDPAIIFHLHDTLGMSVD AINKLLTKESGLLGLTEVTSDCRYVEDNYATKE
DAKRAMDVYCHRLAKYIGAYTALMDGRLDA VVFTGGIGENAAMVRELSLGLGLVLF
EVDHERNLAARFGKSGFINKEGTRPA VVIPTNEELVIAQDASRLTA*

DNA sequence of *Ec*-AcK

ATGAGCTCTAAGCTGGTTCTTGTCTTAATTGTGGGTCTTCTAGCCTTAAATTCGCAA
TTATAGACGCGGTGAACGGAGAGGAGTACCTCAGTGGTTTGGCGGAATGTTTTACC
TGCCTGAGGCGCGTATTAAGTGGAAGATGGATGGTAACAAGCAGGAAGCGGCATTG
GGTGCTGGTGCGGCACACTCAGAAGCGTTAAATTTTATAGTCAACACTATCTTGGCA
CAGAAGCCCGAACTTTCGGCACAGCTTACTGCTATAGGTCACCGTATCGTCCACGGC
GGGGAAAAGTACACGTCGAGTGTCGTGATCGACGAGAGCGTGATACAGGGTATTAA
GGACGCCGCTAGCTTTGCACCGTTACACAATCCAGCACACTTAATCGGGATAGAAG
AAGCTTTAAAGAGTTTTCCCCAACTGAAGGACAAGAATGTCGCTGTGTTTGACACCG
CATTCCACCAGACAATGCCCGAAGAGAGCTACCTGTACGCACTGCCTTACAACCTCT
ACAAGGAGCACGGAATCCGACGGTACGGAGCTCACGGGACCTCACACTTCTACGTG
ACACAAGAGGCTGCCAAGATGCTGAATAAGCCTGTTGAGGAGCTGAACATAATAAC
TTGTCATCTCGGAAACGGTGGATCAGTTAGCGCGATACGGAATGGCAAATGCGTCG
ACACCTCAATGGGTCTGACCCCGCTTGAGGGCTTGGTCATGGGGACACGGAGTGGC
GATATTGATCCAGCAATAATCTTTACCTGCACGATACGTTAGGCATGTCCGTAGAC
GCCATAAATAAGTTATTGACTAAAGAGAGCGGGCTGTTAGGTTTAACCGAAGTGAC
GAGCGACTGTCGATACGTGGAAGACAATTACGCTACGAAGGAAGATGCTAAGCGAG
CTATGGACGTGTACTGTCACCGCTTGGCAAATAACATTGGTGCCTATACGGCTCTGA
TGGATGGGCGTCTGGACGCCGTGGTGTCTTACTGGTGGGATAGGCGAGAATGCTGCA
ATGGTGCAGAGCTTAGTCTTGGCAAGTTGGGAGTCCTTGGCTTTGAAGTAGACCAC
GAACGTAATCTGGCTGCCAGATTTGGAAAATCAGGGTTTATTAACAAAGAGGGGAC
CCGTCCGGCAGTAGTAATCCCCACAAATGAGGAGTTGGTAATTGCACAAGACGCTTC
ACGACTTACCGCTTAA

Protein sequence of *Gs*-PK (from *Geobacillus stearothermophilus*, Uniprot ID: Q02499)

MKRKTKIVCTIGPASESVDKLVQLMEAGMNVARLNFSGHDHEEHGRRIANIREAAKRT
GRTVAILLDTKGPEIRTHNMENGAIELKEGSKLVISMSEVLGTPEKISVTYPSLIDDVSVG
AKILLDDGLISLEVNAVDKQAGEIVTTVLNGGVLKNKKGVNVPGVKNLPGITEKDRA
DILFGIRQGIDFIAASFVRRASDVLEIRELLEAHDALHIQIIAKIENEEGVANIDEILEAADG
LMVARGDLGVEIPAEVPLIQKLLIKKCNMLGKPVITATQMLDSMQRNPRPTRAESDV
ANAI FDGTD AVMLSGETAAGQYPVEAVKTMHQIALRTEQALEHRDILSQRTKESQTTIT
DAIGQSV AHTALNLDVAAIVTPTVSGKTPQMVAKYRPKAPIIAVTSNEAVSRRLALVWG

VYTKEAPHVNTTDEMLDVAVDAAVRSLVKHGD LVVITAGVPVGETGSTNLMKVHVI
SDLLAKGQGIGRKS AFGKAVVAKTAEERQKMVDGGIL VTVSTDADMMMPAIEKAAAI
TEEGGLTSHA AVVGLSLGIPVIVGVENATTLFKD GQEITVDGGFGAVYRGHASVL*

DNA sequence of *Gs*-PK

ATGAAGCGCAAAACCAAGATAGTTTGCACGATAGGTCCAGCCTCGGAAAGTG TAGA
TAAGCTGGTGCAGTTGATGGAAGCTGGGATGAATGTGGCACGCTTGAAC TTTAGTCA
CGGTGATCACGAGGAACATGGTCGTCGCATAGCTAACATCCGTGAGGCTGCTAAGC
GTACGGGCCGTACAGTAGCGATACTGCTGGATACGAAAGGCC CAGAGATCCGGACT
CACAACATGGAGAACGGCGCTATCGAGTTGAAAGAGGGCTCCAAGCTGGTGATCTC
AATGTCAGAGGTCCTCGGAACGCCCCGAAAAGATTTCTGTTACTTACCC CAGCTTGAT
CGACGATGTGTGCGTCGGTCGGCGCCAAGATACTCTTAGATGACGGGCTCATCTCTCTCGA
AGTGAACGCTGTGGACAAGCAAGCGGGTGAAATCGTAACCACCGTCCTGAACGGCG
GTGTTCTGAAGAACAAGAAGGGTGTTAACGTTCTTGCGTGAAAGTCAATCTCCCTG
GCATAACAGAAAAGGACCGCGCTGATATACTGTTCCGTATACGTCAAGGGATCGAT
TTCATTGCTGCTTCGTTTCGTTTCGTCGAGCCTCTGACGTCCTGGAGATCCGAGA ACTGC
TGGAAGCGCACGATGCGTTACACATTCAGATAATCGCTAAGATCGAAAATGAAGAG
GGCGTTGCCAACATCGATGAAATATTAGAGGCGGCGGACGGGCTGATGGTTGCCCG
TGGCGACCTGGGCGTTGAAATCCCAGCTGAAGAAGTGCCTTTAATTCAA AAGCTGTT
GATTAAGAAGTGCAACATGTTGGGAAAGCCTGTTATTACAGCAACACAGATGCTTG
ACAGTATGCAGCGTAACCCCCGTCCAACCCGCGCTGAGGCAAGTGACGTCGCGAAC
GCCATATTTGACGGCACCGATGCCGTTATGTTATCTGGCGAGACGGCGGCAGGTCAA
TACCCTGTTGAGGCGGTTAAGACCATGCATCAGATCGCGCTGCGCACAGAGCAAGC
TCTTGAGCACCGTGACATACTCAGTCAACGAACTAAGGAATCTCAGACAACAATAA
CCGACGCTATCGGTCAATCGGTTGCACACACGGCTTTAACTTAGATGTAGCCGCGA
TAGTACCCCCACCGTAAGCGGAAAGACACCCGAGATGGTGGCCAAGTATAGACCG
AAAGCTCCTATAATTGCTGTTACCTCGAATGAAGCGGTGAGTCGGCGTTTAGCTCTC
GTTTGGGGCGTGTA CTAATAAGAGGCACCCCATGTCAATACCACAGATGAGATGCT
GGACGTGGCAGTTGACGCAGCGGTCCGCTCAGGACTTGTTAAGCACGGGGACTTGG
TTGTCATCACGGCAGGCGTCCCTGTTGGAGAAACAGGGTCTACTAATTTAATGAAGG
TGCATGTAATTTCA GATCTGCTTGCGAAGGGCCAAGGTATCGGTCGAAAGAGCGCCT
TCGGGAAGGCGGTCTGTTGGCGAAGACCGCCGAGGAAGCTCGGCAA AAGATGGTGGA
CGGCGGCATCTTAGTCACCGTTTCCACGGATGCGGATATGATGCCTGCGATAGAGAA
AGCTGCTGCCATAATTACGGAAGAGGGTGGCTTAACCTCTCACGCGGCGGTAGTGG
GTTTATCTCTTGGGATTCCAGTGATTGTGGGTGTAGAAAATGCTACGACTCTCTTTAA
GGACGGGCAAGAGATCACAGTTGACGGCGGCTTCGGTGCGGTGTACCGAGGGCACG
CTTCAGTTCTGTGA

Protein sequence of *Ef*-NDK (from *Escherichia fergusonii*, Uniprot ID: B7LKC0)

MAIERTFSIIKPNVA VAKNVIGNIFARFEAAGFKIVG TKMLHLTVEQARGFYAEHDGKPF
DGLVEFMTSGPIVSVLEGENAVQRHRDLLGATNPANALAGTLRADYADSLTEN GTHG
SDSVESAAREIAYFFGEGEVCP RTR*

DNA sequence of *Ef*-NDK

ATGGCCATAGAACGAACATTCTCGATTATCAAACCTAATGCAGTAGCTAAGAACGT
AATTGGTAATATCTTCGCTCGCTTCGAAGCTGCTGGTTTTAAGATCGTTGGC ACAA
GATGCTGCACCTGACCGTTGAACAGGCAAGAGGGTTCTACGCAGAGCACGACGGTA
AGCCCTTCTTCGACGGACTCGTGGAGTTTATGACTTCGGGACCCATCGTGGTCTCAG

TTCTTGAAGGGGAGAACGCAGTGCAACGGCACCGTGACCTGCTCGGTGCTACTAATC
CAGCTAACGCGTTGGCGGGCACGCTCCGCGCTGATTATGCAGACTCACTTACAGAA
AACGGCACACATGGTTCAGATTCGGTGGAGAGCGCAGCACGCGAGATTGCCTATTT
CTTTGGGGAAGGCGAGGTGTGTCCACGTACACGTTGA

Protein sequence of *Ec*-GalK Y371H (from *Escherichia coli*, Uniprot ID: P0A6T3)

MSLKEKTQSLFANAFGYPATHTIQAPGRVNLIGEHTDYNDGFVLPICAIDYQTVISCAPRD
DRKVRVMAADYENQLDEFSLDAPIVAHENYQWANYVRGVVKHLQLRNNNSFGGVDMV
ISGNVPQGAGLSSSASLEVAVGTVLQQLYHLPLDGAQIALNGQEAENQFVGCNCGIMDQ
LISALGKKDHALLIDCRSLGTKAVSMPKGVAVVIINSNFKRTLVGSEYNTRREQCETGAR
FFQQPALRDVTIEEFNAVAHELDPIVAKRVRHILTENARTVEAASALEQGDLKRMGELM
AESHASMRDDFEITVPQIDTLVEIVKAVIGDKGGVRMTGGGFGGCIVALIPEELVPAVQQ
AVAEQYEAKTGIKETFHVCKPSQGAGQC*

DNA sequence of *Ec*-GalK Y371H

ATGTCACTGAAAGAGAAGACTCAGTCGTTGTTCGCCAACGCATTTGGCTACCCCGCG
ACCCACACAATACAAGCCCCTGGTCGAGTGAATCTTATCGGTGAGCACACAGACTA
TAATGACGGTTTTGTATTGCCGTGTGCCATTGATTACCAAACCGTGATCAGTTGCGCT
CCAAGAGACGATCGAAAAGTTCGTGTGATGGCGGCAGACTACGAGAATCAACTCGA
CGAGTTCTCATTGGACGCGCCCATTGTGGCCCATGAAACTACCAAGTGGGCCAACTA
CGTAAGAGGCGTTGTGAAGCACTTGCAGCTGCGCAATAATAGCTTTGGCGGTGTGG
ACATGGTGATAAGTGGCAATGTCCCCCAAGGCGCCGGATTGAGCTCCTCAGCTTCTT
TGGAAGTAGCTGTTGGCACCGTACTGCAGCAGCTCTATCATCTGCCCCTGGACGGTG
CCCAAATCGCGCTCAATGGCCAAGAGGCCGAAAATCAGTTCGTTGGCTGCAATTGTG
GCATCATGGATCAATTGATTTCCGGCTTTAGGTAAGAAGGACCACGCCCTCTTGATAG
ACTGCCGCTCCCTCGGAACCAAAGCGGTCTCCATGCCAAAGGGTGTGCGCTTGTC
TTATAAATTCAAACCTTCAAGCGTACGTTAGTAGGGAGCGAATACAACACCCGGAGA
GAACAATGTGAGACTGGTGCACGTTTCTTCCAACAGCCGGCTCTTCGTGACGTAACG
ATCGAGGAATTCAATGCAGTAGCTCACGAACTGGACCCAATTGTGGCGAAGCGTGT
ACGGCACATATTAACGGAGAACGCACGAACTGTTGAAGCAGCTTCTGCATTGGAAC
AGGGTGACCTGAAGAGAATGGGAGAGCTGATGGCAGAGAGTCATGCTTCAATGAGA
GACGATTTTGAGATAACGGTACCGCAGATAGACACCTTGTTGAGATTGTGAAGGC
AGTTATCGGCGATAAGGGTGGCGTGCGTATGACCGGTGGTGGTTTCGGCGGATGCAT
TGTTGCGCTCATCCCGGAAGAGTTAGTTCGGCGGTCCAGCAGGCCGTCGCGGAGC
AGTACGAGGCAAAGACTGGCATCAAGGAGACGTTTCACGTGTGCAAACCTTCACAA
GGAGCCGGCCAATGTAA

Protein sequence of *Ec*-AroL (from *Escherichia coli* HVH 36, Uniprot ID: A0A7U9P159)

MTQPLFLIGPRGCKTTVGMALADSLNRRFVDTDQWLQSQLNMTVAEIVEREEWAGFR
ARETAALEAVTAPSTVIATGGGIILTEFNRFMQNNGIVVYLCAPVSVLVNRLQAAPPEED
LRPTLTGKPLSEEVQEVLEERDALYREVAHIIDATNEPSQVISEIRSALAQTINC*

DNA sequence of *Ec*-AroL

ATGACGCAGCCTTTATTTCTGATTGGCCCTCGCGGGTGTGGTAAGACAACGGTGGGA
ATGGCCCTCGCCGACTCTCTCAACCGAAGATTCGTAGACACAGATCAATGGCTGCAG
AGCCAATTAAATATGACGGTCGCCGAAATAGTTGAGAGAGAGGAGTGGGCAGGCTT
CCGTGCAAGAGAGACTGCGGCGTTAGAGGCCGTCACCGCTCCTTCTACTGTTATAGC
GACAGGTGGTGGTATCATTCTTACAGAGTTTAATCGTCACTTTATGCAAAACAACGG

AATTGTCGTATACCTGTGTGCACCCGTGAGCGTACTGGTGAATCGTTTGCAAGCAGC
ACCCGAGGAAGACCTTCGACCGACGCTCACGGGTAAGCCCTTATCAGAGGAAGTTC
AGGAAGTACTTGAGGAGCGCGACGCGTTGTACCGAGAGGTAGCGCACATCATTATC
GACGCCACAAACGAGCCAAGCCAAGTTATCTCAGAAATTCGTTCCGCACTGGCTCA
GACGATAAACTGCTAA

Protein sequence of *Tk-I3P* (from *Thermococcus kodakarensis*, Uniprot ID: Q5JDA3)

MKCLVVGHVVRDIVKKGNKVLRLGGGAYYSALALSRFCDVEILTSFSNLPEEWIKELE
SMAKLQVVPSETTTTTYELTYLDGNRRRLKLLERASPIEELPDGEYDVLLMNPVAREVPP
ALVTSALKKFPFVAVDIQGFIRSSSPGEIQYQPIDGSFLKGVKILHADLGEYQYLQGFSP
FVDVLLLSNGPEPGKAFLHGREYTFEPVHVGVDESTGAGDVFLGAFTGFYSQCPFVQAL
KRAAAFTALFLKNRSVDFSMDDVNELAMKVEVKRV*

DNA sequence of *Tk-I3P*

ATGAAATGTTTGGTAGTGGGACACGTGGTAAGAGACATCGTAAAGAAGGGTAACAA
GGTGTGGAGCGGTTAGGGGGGGGTGCCTACTACTCGGCTCTGGCGCTGTCGAGATT
CTGTGACGTCGAGATTCTGACTTCTTTCTCCAATTTACCGGAAGAGTGGATCAAGGA
GTTGGAGTCGATGGCGAAATTACAAGTGGTCCCGTCCGAGACTACTACAATTATGA
GTAAACCTACTTAGACGGTAATCGTCGCCGGCTGAAGTTATTAGAACGAGCTTCCCC
GATTGAGGAGCTTCTGACGGAGAATACGATGTGCTTTTAATGAACCCTGTTGCTCG
TGAGGTACCACCCGCTCTGGTTACTTCTGCATTAAAGAAGTTTCCATTCGTAGCGGTT
GACATTCAGGGATTTCATCAGAAGCTCTTCCCCTGGCGAGATTCAATACCAGCCGATC
GACGGCAGCTTCTTAAAAGGTGTCAAGATCTTACACGCGGACCTTGGCGAGTACCA
ATACCTGCAAGGGTTTTCCCCTGAGTTCGTCGACGTATTACTTCTGTCTAATGGGCCT
GAGCCAGGTAAGGCGTTCCTTCACGGGCGCGAGTATACTTTCGAACCTGTCCATGTA
GGAGTGGATGAGAGTACTGGTGCAGGTGACGTTTTCTGGGCGCCTTCACAGGTTTC
TATTCCCAATGCCCGTTCGTACAGGCATTGAAGCGGGCAGCTGCGTTTACGGCTTTA
TTCCTCAAGAATCGTTCGGTCGACTTCAGCATGGACGACGTCAACGAGCTTGCAATG
AAGGTTGAAGTGAAGCGTGTATAA

Protein sequence of *Ec-NadD_A86W_Y118N* (from *Escherichia coli*, Uniprot ID: P0A752)

MKSLQALFGGTFDPVHYGHLKPVETLANLIGLTRVTIIPNNVPPHRPQPEANSVQRKHM
LELAIAADKPLFTLDERELKRNAPSYT**W**QTLKEWRQEQQGPDVPLAFIIGQDSLTLTFPT**W**NE
YETILDNAHLIVCRRPGYPLEMAQPQYQQWLEDHLTHNPEDLHLQPAGKIYLAETPWFN
ISATHIRERLQNGESCEDLLPEPVLTYINQQGLYR*

DNA sequence of *Ec-NadD_A86W_Y118N*

ATGAAGAGCTTACAAGCCTTGTTTGGTGGCACATTCGATCCGGTTCATTATGGACAC
CTTAAGCCTGTTGAGACGCTGGCCAACTGATCGGCCTCACTCGTGTGACAATTATT
CCAAACAATGTGCCGCCTCATCGCCACAAACCGGAAGCCAATTCCGTGCAAAGAAA
GCACATGTTAGAATTAGCAATTGCCGATAAACCCTTATTCCTCTGGATGAACGAGA
GCTCAAGAGAAACGCCCCAAGCTATACTTGGCAGACTCTCAAGGAGTGGAGACAAG
AGCAAGGGCCAGACGTTCTTTAGCATTTCATCATCGGTCAAGACAGTTTGTAACT
TTCCTACTTGGAACGAATACGAGACGATATTGGACAACGCACATTTAATCGTTTGCC
GTCGTCCTGGATACCCTTTAGAGATGGCACAGCCGACGTACCAACAGTGGTTGGAA
GACCATTTGACACACAACCCCGAAGATCTGCACTTGCAACCTGCGGGTAAAATTTAC
CTCGCAGAAACGCCTTGGTTCAACATAAGCGCAACTATCATTTCGTGAGCGTTTACAA

AATGGTGAATCCTGTGAAGATCTGCTCCCGGAGCCCGTTCTGACGTATATTAATCAA
CAAGGTTTGTATCGTTGA

Protein sequence of *Sc*-IPYR (from *Saccharomyces cerevisiae*, Uniprot ID: A6ZKV8)

MTYTTTRQIGAKNTLEYKVYIEKDGPVSAFHDIPLYADKENNIFNMVVEIPRWTNAKLEI
TKEETLNPIIQDTKKGKLRFVRNCFPHHGYIHNYGAFPQTWEDPNVSHPETKAVGDNDPI
DVLEIGETIAYTGQVKQVKALGIMALLDEGETDWKVIAIDINDPLAPKLN DIEDVEKYFP
GLLRATNEWFRYKIPDGKPENQFAFSGEAKNKKYALDIKETHDSWKQLIAGKSSDSKG
IDLNTVTLPTPTYSKAASDAIPPASPKADAPIDKSIDKWFFISGSV*

DNA sequence of *Sc*-IPYR

ATGACATACTACCCGCCAGATCGGGGCCAAGAACACATTAGAATATAAAGTCTA
CATCGAAAAGGACGGGAAGCCAGTAAGTGCTTTCCACGACATCCCCCTTTACGCTG
ACAAGGAAAATAACATATTTAACATGGTTGTAGAGATCCCGCGGTGGACTAACGCT
AAGCTGGAGATCACCAAAGAGGAGACCCTGAACCCTATAATCCAAGATACGAAGAA
AGGGAAGCTTAGATTTGTGAGAACTGTTTTCTCATCATGGATATATACATAATTA
CGGCGCGTTTCCCCAGACCTGGGAAGACCCTAACGTCAGCCATCCCGAGACTAAAG
CAGTTGGTGACAATGATCCGATAGATGTCCTGGAGATTGGAGAAACCATTGCCTATA
CCGGACAGGTAAAACAGGTTAAAGCCCTTGGGATCATGGCTCTGCTGGACGAAGGC
GAAACCGACTGGAAGGTTATCGCGATCGACATCAACGACCCACTGGCGCCGAAGTT
GAACGATATTGAAGACGTAGAGAAGTATTTCCCGGGATTACTCCGTGCAACCAACG
AGTGGTTTTCGCATATATAAGATACCGGATGGGAAGCCAGAAAACCAATTCGCGTTC
AGCGGTGAGGCTAAGAATAAGAAGTACGCGTTGGACATCATAAAGGAGACCCACGA
CTCGTGGAAGCAGCTTATCGCGGGCAAGAGTAGCGACAGTAAGGGCATCGACTTAA
CGAACGTGACACTCCCAGACACACCTACGTATAGCAAGGCTGCATCAGATGCTATC
CCGCCGGCGAGTCCCAAAGCAGATGCTCCGATAGATAAGTCGATAGACAAGTGGTT
CTTCATTTCCGGATCTGTTTGA

Protein sequence of *Ec*- IPYR (from *Shigella dysenteriae*, Uniprot ID: A0A2S8D5I0)

MSLLNVPAGKDLPEDIYVVIEIPANADPIKYEIDKESGALFVDRFMSTAMFYPCNYGYIN
HTLSLDGDPVDVLVPTPYPLQPGSVIRCRPVGVLKMTDEAGEDAKLVAVPHSKLSKEYD
HIKDVNDLPELLKAQIAHFFEHYKDLEKKGKWKVEGWENAEAAKAEIVASFERAKNK*

DNA sequence of *Ec*- IPYR

ATGAGCCTTTTAAACGTCCCTGCGGGCAAAGACTTGCCAGAAGATATATATGTAGTC
ATTGAGATTCCTGCAAACGCTGACCCGATAAAGTATGAGATTGACAAGGAGTCTGG
TGCTCTGTTTGTGATCGCTTTATGAGCACCGCAATGTTCTACCCGTGCAATTATGGA
TACATCAATCACACCCTCTCACTGGACGGCGACCCTGTTGATGTTCTTGTCCCGACC
CCGTACCCCTTCAACCCGGTAGCGTCATTCGCTGCAGACCTGTGGGTGTCTTGAAG
ATGACTGATGAGGCGGGCGAAGATGCCAAGCTTGTAGCTGTGCCGCACAGTAAGTT
GTCTAAGGAATACGACCATATTAAGGACGTAAATGATCTGCCAGAGCTCCTGAAGG
CCCAAATAGCTCATTTCTTGAACATTACAAGGACCTGGAGAAGGGTAAATGGGTG
AAGGTGGAAGGATGGGAAAACGCAGAGGCCGCTAAAGCGGAGATTGTAGCGTCATT
TGAGCGCGCAAAGAATAAGTGA

Protein sequence of *Km*-NRK (from *Kluyveromyces marxianus*, Uniprot ID: W0TD38)

MTTTKVKLIAISGCSSSGKTTLAKFLANAIPGCILIHEDDFYKPDSEIPINEKYGVADWDC
PEALDLDAFKRELDLIKTGSIKTKLIHNENVDDIGKFNIKQEDWDALRAKLSSVIESDLK

VVLVDGFMIFNDEELMRKFDIRIFVRAPYEVL SRRRHARAGYKTLESFWVDPPYYFDEF
VYRAYREEHKHLFVNEDVEGSLRSDAGLFELINDDETEITTALNTIADYIVSHLDAN*

DNA sequence of *Km*-NRK

ATGACGACTACCAAGGTGAAGTTAATCGCCATCAGCGGCTGTTCTAGCTCGGGGAA
GACCACCTTAGCCAAGTTCCTGGCCAACGCAATTCCAGGCTGCATCCTGATCCATGA
GGACGACTTCTACAAACCGGACAGCGAGATTCCAATAAACGAAAAGTACGGTGTCTG
CTGATTGGGATTGTCCTGAAGCCCTGGATCTGGATGCCTTCAAGAGAGAACTGGATC
TGATTAAAACCACTGGGAGCATCAAAACGAAGTTGATTACAAACGAGAATGTAGAT
GATATCGGTAAATTCAATATAAAGCAGGAAGACTGGGACGCCTTACGGGCCAAGCT
GTCCTCAGTCATCGAGAGCGACCTTAAGGTGGTACTGGTGGACGGGTTTATGATCTT
CAACGACGAGGAAGTATGCGCAAGTTCGACATACGTATATTCGTACGCGCTCCCTA
TGAAGTGCTTTCTCGAAGACGGCACGCACGAGCTGGCTATAAGACCTTGGAGAGCTT
TTGGGTCGACCCACCCTACTACTTTGATGAGTTTGTTCACAGAGCGTACCGCGAGGA
GCACAAGCATTATTCGTCAATGAGGACGTTGAAGGGAGTTTACGGTCAGACGCCG
GACTTTTTCGAATTAATTAACGATGACGAGACCGAGATTACGACCGCATTAACACA
ATCGCAGATTACATTGTAAGCCATCTCGACGCAAATTAA

Protein sequence of *Hs*-NMNAT (from *Homo sapiens*, Uniprot ID: Q9HAN9)

MENSEKTEVLLACGSFNPITNMHLRLFELAKDYMNGTGRYTVVKGIISPVGDAYKKK
GLIPAYHRVIMAE LATKNSKWVEVD TWESLQKEWKETLKVLRHHQE KLEASDCDHQQ
NSPTLERPGRKRKWTETQDSSQKKSLEPKTKAVPKVKLLCGADLLESFAVPNLWKSEDI
TQIVANYGLICVTRAGNDAQKFIYESDVLWKHRSNIHVVNEWIANDISSTKIRRALRRGQ
SIRYLV PDLVQEYIEKHNLYSSESEDRNAGVILAPLQRNTAEAKT*

DNA sequence of *Hs*-NMNAT

ATGGAAAACAGCGAGAAGACAGAAGTTGTATTATTGGCGTGTGGGTCCTTCAACCC
TATAACAAATATGCACTTACGTCTTTTCGAACCTTGCTAAGGATTACATGAACGGGAC
CGGAAGATACACCGTGGTCAAGGGCATTATAAGCCCAGTTGGGGATGCGTACAAGA
AGAAGGGCCTGATACCTGCTTATCACCGGGTCATTATGGCCGAATTAGCGACTAAGA
ACTCCAAGTGGGTGGAAGTCGACACTTGGGAGTCTCTGCAAAAGGAGTGGAAAGGAA
ACATTAAAGGTGTTACGTCACCACCAAGAGAACTCGAAGCATCCGATTGCGACCA
CCAACAAAACAGCCCAACGCTGGAGCGTCCGGGTAGAAAGCGCAAGTGGACTGAG
ACGCAGGACTCCTCTCAAAAGAAATCGTTAGAGCCTAAGACCAAGGCAGTGCCGAA
AGTGAAATTACTGTGTGGAGCGGACTTATTGGAATCTTTCGCTGTTCCGAATCTGTG
GAAGAGCGAGGATATCACACAGATTGTCGCTAACTACGGCCTGATATGCGTAACTC
GCGCAGGAAATGACGCCCAAAAGTTTATCTATGAGTCTGACGTTTTGTGGAAGCACC
GTAGTAATATCCACGTTGTAAACGAATGGATAGCTAACGATATATCGTCAACAAAG
ATACGTAGAGCTTTGCGTCGGGGACAATCTATTCGTTACTTAGTTCCGGACTTAGTA
CAAGAGTATATCGAGAAGCACAACTTTACAGCAGCGAGTCCGAGGACCGGAACGC
GGGTGTAATACTTGCTCCCTTGCGAGAGAAACACTGCGGAAGCGAAGACTTAA

Protein sequence of *Ec*-NADK (from *Escherichia coli*, Uniprot ID: B1XBT5)

MNNHFKCIGIVGHPRHPTALTTHEMLYRWLCTKGYEVIVEQQIAHELQLKNVKTGTLAE
IGQLADLAVVVGGDGNMLGAARTLARYDIKVIGINRGNLGLTDLDPDNAQQQLADVL
EGHYISEKRFLLEAQVCQQDCQKRISTAIN EVVLHPGKVAHMI EF EYIDEIFA FSQRS DG
LIISTPTGSTAYSLSAGGPILTPSLDAITLVPMFPHTLSARPLVINSSSTIRLRFSHRRNDLEI
SCDSQIALPIQEGEDVLIRRCDYHLNLIHPKDYSYFNTLSTKL GWSKKLF*

DNA sequence of *Ec*-NADK

ATGAATAACCATTTTAAATGCATTGGTATTGTAGGACACCCACGGCACCCCTACCGCC
CTTACTACCCACGAGATGTTGTACCGGTGGTTATGCACCAAAGGTTACGAGGTTATA
GTCGAGCAACAAATCGCACATGAGTTACAATTAAGAATGTTAAACTGGTACGCT
GGCAGAAATAGGACAGCTGGCTGACCTTGCCGTTGTCGTGGGCGGGGACGGGAACA
TGTTAGGTGCCGCCCGTACGCTCGCGCGATATGATATTAAAGTCATCGGGATTAACA
GAGGCAATCTGGGATTCTTAACAGATTTAGATCCTGACAATGCTCAACAGCAATTAG
CAGACGTCCTTGAGGGGCATTATATTTCTGAGAAAAGATTTCTGCTGGAAGCCCAAG
TATGTCAGCAGGACTGTCAAAAGCGGATTAGTACAGCGATCAATGAGGTGGTACTG
CACCCCGGGAAAGTGGCGCACATGATTGAATTTGAGGTTTACATAGACGAAATATTT
GCCTTTTCTCAGCGGTCCGATGGCCTGATCATATCCACACCGACGGGCAGTACGGCC
TATTCATCTCAGCCGGTGGTCCTATTCTTACTCCGTCTTTGGACGCTATCACTCTCG
TACCAATGTTCCACACACGTTAAGCGCTCGTCCGCTCGTAATAAACTCATCATCGA
CCATCCGCCTGCGCTTTAGTCACCGTCGTAACGACCTGGAGATATCATGCGATTCTC
AAATAGCTCTGCCCATAACAAGAGGGTGAAGACGTATTGATTCGCCGTTGTGATTACC
ATCTGAACCTGATCCACCCGAAGGACTACAGCTACTTCAACACATTAAGTACCAAGT
TAGGATGGTCGAAGAAGTTGTTTTAA

Protein sequence of *Bs*-NADK (from *Bacillus sp. TSA-4*, Uniprot ID: A0AA96ZNA9)

MKFAVSSKGDQVSDTLKSKIQAYLLDFDMELDENEPEIVISVGGDGTLLEYAFHRYSDRL
DKTAFVGVHTGHLGFYADWVPHEIEKLVLAIKTPYHTVEYPLLEVIVTYHENEREERY
LALNECTIKSIEGSLVADVEIKGQLFETFRGDGLCLSTPSGSTAYNKALGGAIHPSIRAIQ
LAEMASINNRVFRVTGSPLLLPSHHDCMIKPRNEVDFQVTIDHLTLLHKDVKSIRCQVAS
EKVRFARFRPFPFWKRVQDSFIGKGE*

DNA sequence of *Bs*-NADK

ATGAAGTTTGCGGTATCGTCCAAGGGAGACCAAGTTTTCGGATACACTGAAGAGCAA
GATCCAAGCATACCTGTTGGACTTCGACATGGAGTTGGACGAGAACGAGCCTGAGA
TAGTCATCTCAGTAGGCGGTGACGGGACGCTGTTATACGCTTTCCACCGTTACTCTG
ACCGACTTGATAAGACTGCGTTTGTGGGTGTACATACGGGACATTTAGGATTTTACG
CAGATTGGGTCCCTCATGAGATCGAGAACTGGTTCTGGCCATCGCAAAGACCCCGT
ACCACACCGTCGAATACCCACTCTTAGAGGTTATCGTAACGTATCACGAGAATGAGC
GAGAAGAGCGTTACTTAGCATTGAATGAATGCACAATCAAGTCAATAGAGGGTTCT
TTGGTCGCCGACGTTGAGATTAAAGGGCAATTGTTTCGAAACTTTTCGTGGGGATGGA
CTTTGTTTATCTACACCAAGTGGAAGCACAGCTTATAACAAGGCCCTGGGTGGCGCA
ATAATACATCCATCAATACGCGCCATCCAGCTGGCAGAGATGGCAAGCATCAATAA
TCGTGTCTTTCGCACTGTAGGCTCGCCTCTCCTGTTACCGAGTCACCATGACTGTATG
ATTAAGCCACGAAACGAGGTTGATTTTCAGGTTACCATAGACCACCTTACACTGCTT
CATAAAGATGTGAAGTCTATTCGGTGCCAAGTAGCGTCAGAGAAGGTTTCGATTCGCT
CGATTTTCGTCCGTTTCCGTTCTGGAAACGTGTGCAAGACTCCTTCATAGGGAAGGGT
GAGTAA

Protein sequence of SRC (obtained from Addgene, plasmid #79700, N-term His10-TEV sequence included, organism: *Homo sapiens*, Uniprot ID: P12931)

MKSSHHHHHHHHHHENLYFQSNAQTQGLAKDAWEIPRESLRLEVKLGGQCFGEVWMG
TWNGTTRVAIKTLKPGTMSPEAFLQEAQVMKKLRHEKLVQLYAVVSEPIYIVTEYMSK
GSLDLFLKGETGKYLRPLQLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENLVC

KVADFGLARLIEDNEYTARQGAKFPIKWTAPEAAALYGRFTIKSDVWSFGILLTELTTKGR
VPYPGMVNVREVLDDQVERGYRMPCEPCESLHDLMCQCWRKEPEERPPSSTCRPSWRT
TSRPPSPVPAGETLITLEVDTRDRGAPPVSAVPRVRADRC*

Full plasmid of SRC (SRC gene highlighted in bold)

TTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTAAATGTCATGAT
AATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCC
TATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCC
TGATAAATGCTTCAATAACATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGT
GTCGCCCTTATCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAAC
GCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTACATCG
AACTGGATCTCAACAGCGGTAAAGATCCTTGAGAGTTTTTCGCCCCGAAGAACGTTTTTC
CAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTGTTGACG
CCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGT
ACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGC
AGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATC
GGAGGACCGAAGGAGCTAACCGCTTTTTTGCAACATGGGGGATCATGTAACCTCG
CCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACA
CCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACCTATTAACCTGGCGAACTA
CTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCA
GGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGA
GCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCC
CTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAA
ATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACCTGTCAGACC
AAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAGGAT
CTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAATCCCTTAACGTGAGTTTTCG
TTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTT
TTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACACCGCTACCAGCGGTGGTT
TGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACCTGGCTTCAGCAGA
GCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAG
AACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCT
GCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGAT
AAGGCGCAGCGGTGCGGCTGAACGGGGGGTTTCGTGCACACAGCCCAGCTTGGAGCG
AACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGC
TTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGG
AGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCG
GGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGA
GCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGC
CTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACC
GCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTC
AGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTG
CGGTATTTACACCCGCATATATGGTGCCTCTCAGTACAATCTGCTCTGATGCCGCA
TAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCC
GACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCG
CTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTACCCGT
CATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTCGTGAAGC

GATTCACAGATGTCTGCCTGTTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGC
GTAAATGTCTGGCTTCTGATAAAGCGGGCCATGTAAAGGGCGGTTTTTTCTCTGTTTGG
TCACTGATGCCTCCGTGTAAGGGGGATTTCTGTTTCATGGGGGTAATGATACCGATGA
AACGAGAGAGGATGCTCACGATACGGGTACTGATGATGAACATGCCCGGTTACTG
GAACGTTGTGAGGGTAAACAACCTGGCGGTATGGATGCGGCGGGACCAGAGAAAAAT
CACTCAGGGTCAATGCCAGCGCTTCGTTAATACAGATGTAGGTGTTCCACAGGGTAG
CCAGCAGCATCCTGCGATGCAGATCCGGAACATAATGGTGCAGGGCGCTGACTTCC
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ACATGAGAA

Protein sequence of STK3 (obtained from Addgene, plasmid #79689, N-term His10-TEV sequence included, organism: *Homo sapiens*, Uniprot ID: Q13188)

MKSSHHHHHHHHHHENLYFQSNAEDSLTKQPEEVFDVLEKLGEFSYGSVFKAIHKESG
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 IRLRNKTLIEDEIATILKSTLKGLEYLHFMRKIHRDIKAGNILLNTEGHAKLADFGVAGQL
 TDTMAKRNTVIGTPFWMAPEVIQEIGYNCVADIWSLGITSIEMAEGKPPYADIHPMRAIF
 MIPTNPPPTFRKPELWSDDFDVFVKCLVKNPEQRATATQLLQHPFIKNAKPVSIILRDLIT
 EAMEIKAKRHEEQQRELEEE*

Full plasmid of STK3 (STK3 gene highlighted in bold)

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Protein sequence of Ar-AC (from *Arthrobacter* sp. CGMCC 3584, Genbank: AEX08664.1)
 MNDEDQQERVDSITPPPATDAHPATGTMSAERLAMKALEARLLGGERKLRRREVAAGA
 GLSLLSARKLWRALGFNFGDEDVAFTERDQAALSTVVDLVRSGKLTEEAAISVTRSIG
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 NAGVQRLAVRAEAGLQASEEGREGDEDDAPLPLARAVGFADLVSYTSLSRMNEKTLA
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 AMVWGRILSRLGDIYGPTVNLAARLTTLADPGTVLVDSMTASALEHDERFVLEPQPPEN
 VRGFGEIRPVRLGRGLGKGLVLD*

DNA sequence of *Ar-AC*

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Protein sequence of PsiK (adapted from Lewis's recent work¹)

MASMTGGQQMGRGSMAFDLKTEDGLITYLTKHLSLDVDTSGVKRLSGGFVNVTWRIK
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DNA sequence of PsiK

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GAGCGTATAAATTTTCGTGAAGAAAGGTGTAGCGGCTTTCCACGACGCGCGTGGGAA
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Protein sequence of T4-PNK (from *Enterobacteria phage T4*, Uniprot ID: P06855)

MKKIILTIGCPGSGKSTWAREFIAKNPGFYNNRDDYRQSIMAHEERDEYKYTKKKEGIV
TGMQFD TAKSILYGGDSVKGVII SDTNLNPERRLA WETFAKEYGWKVEHKVFDVPWTE
LVKRNSKRGTKAVPIDVLRSMYKSMREYLGLPVYNGTPGKPKAVIFDVDGTLAKMNG
RGPYDLEKCDTDVINPMVVELSKMYALMGYQIVVSGRESGTKEDPTKY YRMTRKWV
EDIAGVPLVMQCQREQGDTRKDDVVKEEIFWKHIAPHFDVKLAIDDR TQVVEMWRRIG
VECWQVASGDF*

DNA sequence of T4-PNK

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A

3. Cloning, Expression and Purification of Enzymes

pET28a(+)-based expression vectors for kinases were obtained via DNA synthesis from Twist Bioscience, unless otherwise noted. The codon optimized DNA sequence was inserted between the NdeI and XhoI restriction sites within the commercial pET28a(+) vector. Expression vectors of SRC/ STK3/YopH/Lambda phosphatase were obtained from Addgene. The product number are SRC (#79700), STK3(#79689), YopH (residues 164-468, #79749), Lambda Phosphatase (#79748). The expression vectors were extracted using a Wizard® Plus SV Minipreps DNA Purification System (Promega) and gene sequences were confirmed via Sanger sequencing (Epoch Life Science). The resulting vector was directly transformed into electro-competent *E. coli* strain BL21(DE3) cells via electroporation. *Note: the expression vector for SRC was co-transformed with that for YopH in the same E. coli strain, and the expression vector for STK3 was co-transformed with that for Lambda phosphatase in the same E. coli strain.* All strain stocks were stored as a 25% glycerol solution at -80 °C.

500 mL TB containing corresponding antibiotics (50 µg/mL Kanamycin for pET-28a(+)-based enzymes, 100 µg/mL Ampicillin and 50 µg/mL Spectinomycin for SRC+YopH or STK3+Lambda phosphatase) was inoculated with an overnight culture (2 mL, LB_{Kan} or LB_{Amp+Spec}) of recombinant *E. coli* BL21(DE3) cells. The cultures were shaken at 250 rpm at 37 °C for roughly 2.5 h or until an optical density of 0.6-1.0 was reached. Cultures were cooled on ice for 20 min and then induced by adding IPTG to a final concentration of 25 µM. The cultures were allowed to continue to shake at 250 rpm for another 20 hours at 22 °C. Cell were harvested by centrifugation (4 °C, 15 min, 3,000 × g). The cell pellet was resuspended in KPi buffer (50 mM, pH 8.0, 4 mL/g of cell wet weight) and lysed by sonication (5 min, on 1s, off 4s, 60% amplification). The lysate was centrifuged at 15,000 × g for 30 min at 4 °C to remove cell debris. The collected supernatant was filtered and subjected to a Ni-NTA chromatography step using a Ni Sepharose column (HisTrap-HP, GE healthcare, Piscataway, NJ). The protein was first washed with buffer A (50 mM Tris-HCl, 300 mM NaCl, 25 mM imidazole, pH 8.0) and then eluted from the column with buffer B (50 mM Tris-HCl, 300 mM NaCl, 300 mM imidazole, pH 8.0). Purified protein was buffer exchanged into storage buffer (100 mM Tris-HCl, 300 mM NaCl, 5% glycerol, pH = 7.4) using a centrifugal filter (MW cut-off 10 kDa). Protein concentrations were determined by A280 with calculated extinction coefficients as obtained at <https://web.expasy.org/protparam>. For most enzymes used in this work, the concentration was adjusted to 25 mg/mL using storage buffer. For storage, proteins were aliquoted into 100 µL, flash frozen on liquid N₂, and stored at -80 °C.

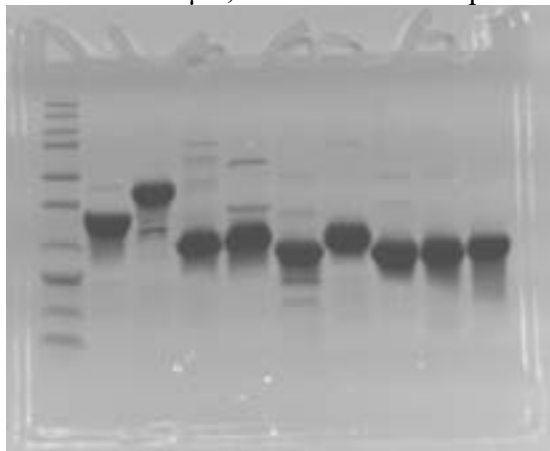


Figure S1. Representative SDS-gel (1). From left to right: Protein ladder, *Ta*-NK, *Hb*-CK, *Ag*-dNK, *Dm*-dNK, *Tm*-TK, *Sc*-AK, *Hs*-GMPK, *Ec*-GMPK, *Hs*-UMP-CMPK.

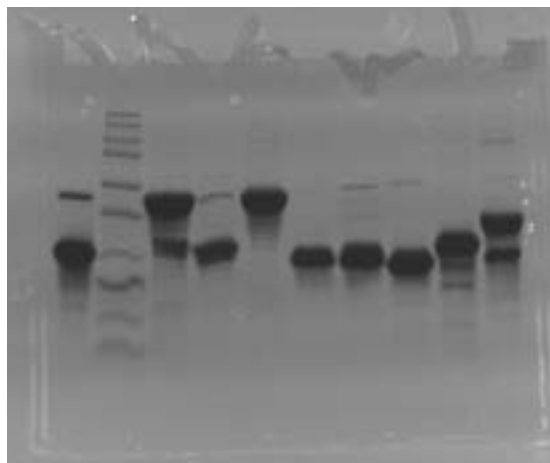


Figure S2. Representative SDS-gel (2). From left to right: *Bs*-CMPK, Protein ladder, *Tm*-AcK, *Bf*-GMPK, *Ec*-AcK, *Ec*-TmK, *Ec*-NadD(mutant), *Ec*-IPYR, *Km*-NRK, *Ec*-NADK.

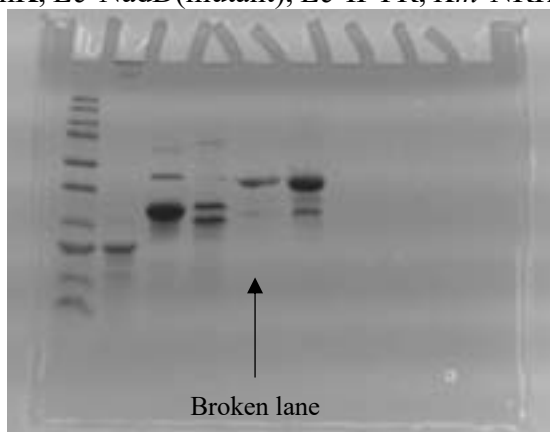


Figure S3. Representative SDS-gel (3). From left to right: Protein ladder, *Ec*-AroL, *Tk*-I3P, *Hs*-NMNAT, *Ec*-GalK (Y371H).

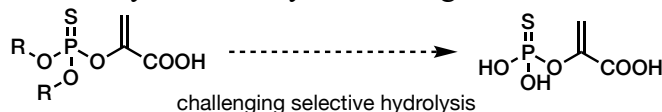
4. Analysis, Synthesis and Characterization of Thiophosphate Donor

4.1 Consideration of thiophosphate donors

We considered the thiophosphate counterparts of several known phosphate donors in biological systems. The choice was made based on thermodynamic properties, availability of corresponding kinases, complexity of regeneration system and prices of donors.

Here are the considerations:

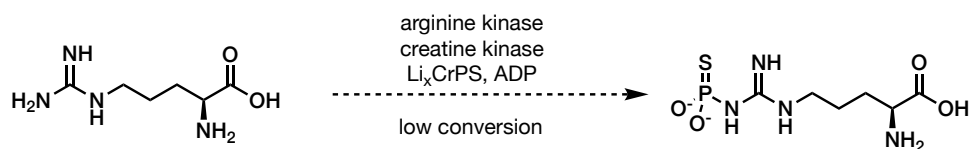
1. Thiophosphoenol pyruvate (thio-PEP): the preparation of thio-PEP is synthetically challenging as the selective hydrolysis of protecting group while keeping the pyruvate motif is not a trivial issue. We were also concerned by the stability of this reagent due to its enol ether motif.



2. Thio-1,3-diphosphoglycerate ((thio)-1,3-BPG): the enzymatic synthesis of (thio)-1,3-BPG was considered at the beginning. However, based on Whitesides' system², several expensive enzymes are required, and immobilization of these enzymes are beyond our expertise.

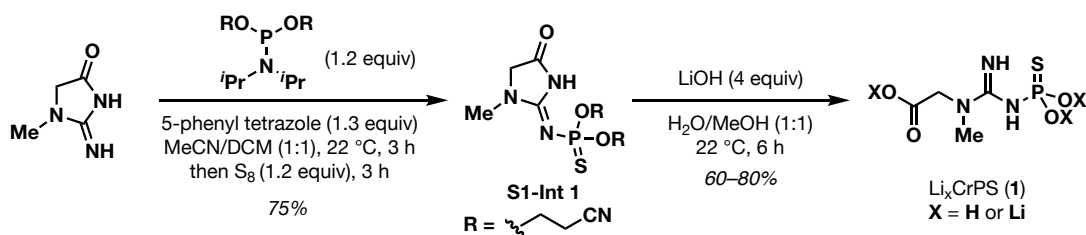
3. Carbamoyl thiophosphate: The synthesis could be challenging because of the good leaving group ability of the carbamoyl motif.

4. Arginine thiophosphate: We think selective functionalization of arginine's guanidine to install only one thiophosphate group would require multi-step synthesis and careful reverse phase chromatography. This would affect the practicality of the overall transformation. The free energy of arginine phosphate hydrolysis is slightly better than ATP, as such, the overall equilibrium of the thiophosphate transfer might be favored. We later found the arginine thiophosphate cannot be generated when arginine was reacted with creatine thiophosphate and ADP in the presence of creatine kinase and arginine kinase, suggesting arginine thiophosphate might be a thiophosphoryl donor with more favorable thermodynamic properties but also more challenging to use.



Considering the above, we think acetyl thiophosphate and creatine thiophosphate are the most practical donors and we focused on their synthesis.

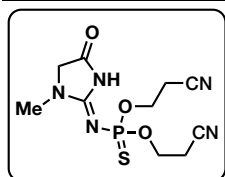
4.2 Creatine thiophosphate and derivatives



A flame-dried 250 mL round bottom flask equipped with a magnetic stir bar was charged with creatinine (10 mmol, 1.13 g) and 5-phenyl tetrazole (13 mmol, 1.9g, 1.3 equiv.). Under nitrogen, the solid was suspended in MeCN/DCM (1:1, 150 mL), followed by addition of bis(2-cyanoethyl)-*N,N*-diisopropyl phosphoramidite (12 mmol, 1.2 equiv., 3.0 mL). After the addition, the solid was observed to mostly dissolve within 10 minutes and white precipitate reformed after 20 minutes. After stirring at room temperature for 3 hours, sulfur (12 mmol, 384 mg, 1.2 equiv.) was added and the mixture was allowed to stir at room temperature for 3 more hours. The mixture was filtered through a pad of celite and washed with MeCN and was concentrated under reduced pressure. The crude product was purified with silica column chromatography to afford **S1-Int 1** (2.36 g, 75%). *Note: To obtain high purity, column chromatography needed to be performed twice, first using EtOAc/hexanes (50:50 to 80:20 to 100:0) and second using DCM/MeOH (99:1 to 97:3).* Single crystal of **S1-Int 1** was obtained by slow diffusion of DCM into its MeCN solution.

In a 100 mL round bottom flask equipped with a magnetic stir bar, creatinine thiophosphate ester **S1-Int 1** (4 mmol, 1.26 g) was suspended in H₂O/MeOH (1:1, 25 mL) and the suspension was cooled down in an ice-water bath. LiOH (16 mmol, 383.2 mg, 4 equiv.) was added in small portions over 3 minutes. After addition, the reaction was warmed up to room temperature and stirred for 6 hours. LC-MS was used to monitor the consumption of the **S1-Int 1**. The reaction was concentrated under reduced pressure to afford a viscous pale-yellow liquid. The crude product was dissolved in small amount of water (roughly 3 mL), then the solution was added to 30 mL NaClO₄ solution (1 M in acetone), during which a sticky precipitate formed. The mixture was centrifuged for 10 min (4000 rpm) and supernatant liquid was carefully decanted, another 30 mL NaClO₄ solution was added and centrifuged again. The sticky solid was re-dissolved in small amount of water and lyophilized to afford pale yellow solid, Li_xCrPS (**1**). Generally, the yield of creatine thiophosphate ranged from 60% to 80%. The solid form is highly hygroscopic. For this reason, the solid was dissolved in water and the creatine thiophosphate concentration was determined by ³¹P NMR with methyl phosphonic acid as the internal standard. The concentration was adjusted to 0.6 M and the solution was stored as 100 μL aliquots at -20 °C.

Characterization of **S1-Int 1**



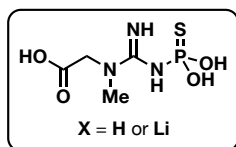
¹H NMR (600 MHz, CD₃CN): δ 9.84 (brs, 1H), 4.18 (dt, *J* = 9.1, 6.0 Hz, 4H), 3.91 (s, 2H), 2.95 (s, 3H), 2.78 (td, *J* = 6.0, 0.7 Hz, 4H).

¹³C NMR (151 MHz, CD₃CN): δ 170.8, 156.4 (d, *J* = 11.3 Hz), 62.3 (d, *J* = 5.1 Hz), 52.5 (d, *J* = 2.1 Hz), 30.5, 20.1 (d, *J* = 8.3 Hz).

³¹P NMR (243 MHz, CD₃CN): δ 64.0 (p, *J* = 9.0 Hz).

HRMS [ESI] C₁₀H₁₅N₅O₃PS ([M+H]⁺) calculated 316.0628 found 316.0628.

Characterization of Li_xCrPS (**1**)

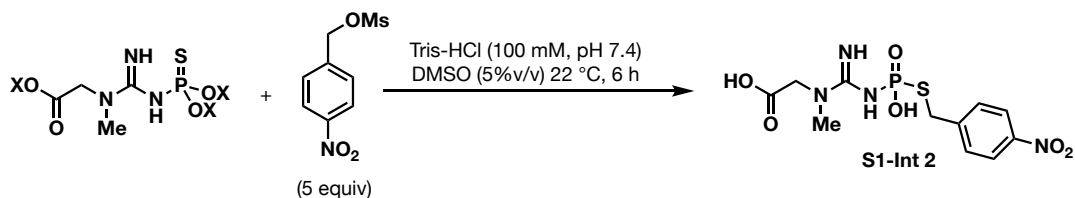


¹H NMR (600 MHz, D₂O): δ 3.89 (s, 2H), 2.92 (s, 3H).

¹³C NMR (151 MHz, D₂O): δ 176.4, 156.3 (d, *J* = 2.3 Hz), 53.5, 36.2.

³¹P NMR (243 MHz, D₂O): δ 35.0.

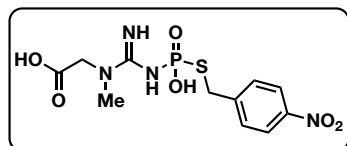
HRMS [ESI] C₄H₁₁N₃O₄PS ([M+H]⁺) calculated 228.0202 found 228.0200.



To further elucidate the structure of creatine thiophosphate, alkylation of thiophosphate was chosen as derivatization. In a one-dram vial equipped with a magnetic stir bar, Li_xCrPS (0.05 mmol) was dissolved in 1 mL Tris-HCl solution (100 mM, pH 7.4) and 50 μL was added as co-solvent. To this solution, 4-nitrobenzyl methanesulfonate (0.25 mmol, 5 equiv.) was added and the mixture was allowed to stir at room temperature for 6 hours. The solution was quenched by the addition of methanol (3 mL) and filtered through a pad of celite. After concentration under reduced pressure, the crude mixture was dissolved in small amount of water and purified by ion exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, gradient 0:100 to 25:75), fractions containing product (monitored by LC-MS) was collected, concentrated under reduced pressure and lyophilized to afford pale-yellow solid. The solid was further purified by C18 reverse phase column using (MeOH/water, gradient from 0:100 to 100:0). Desired product was collected and concentrated under reduced pressure (temp. ≤ 40 °C). Lyophilization was applied to remove residual water.

Single crystal of **S1-Int 2** was obtained by slow evaporation of its methanol/acetonitrile (1:3, v/v) solution.

Characterization of **S1-Int 2**



¹H NMR (600 MHz, D₂O): δ 8.26 – 8.23 (m, 2H), 7.71 – 7.65 (m, 2H), 4.14 (d, *J* = 17.0 Hz, 2H), 3.63 (brs, 2H), 2.82 (brs, 3H).

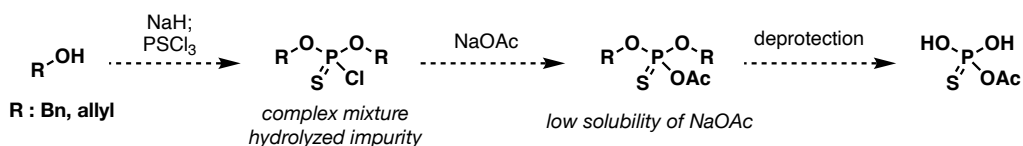
¹³C NMR (151 MHz, D₂O): δ 173.5, 154.7 (d, *J* = 3.1 Hz), 146.7, 146.6 (d, *J* = 3.0 Hz), 129.7, 123.8, 53.9, 37.1, 33.9 (d, *J* = 3.9 Hz).

³¹P NMR (243 MHz, D₂O): δ 12.7 (t, *J* = 17.0 Hz).

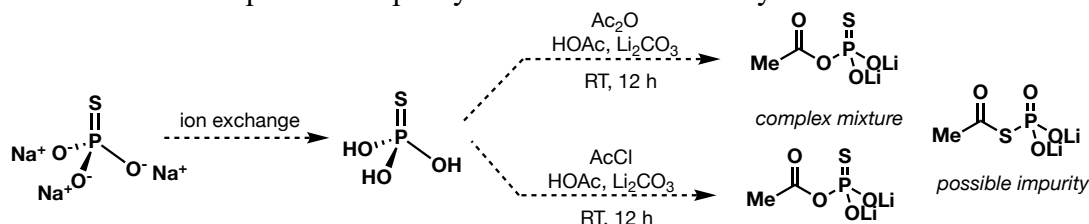
HRMS [ESI] C₁₁H₁₆N₄O₆PS ([M+H]⁺) calculated 363.0523 found 363.0523.

4.3 Unsuccessful attempts to prepare acetyl thiophosphate and creatine thiophosphate

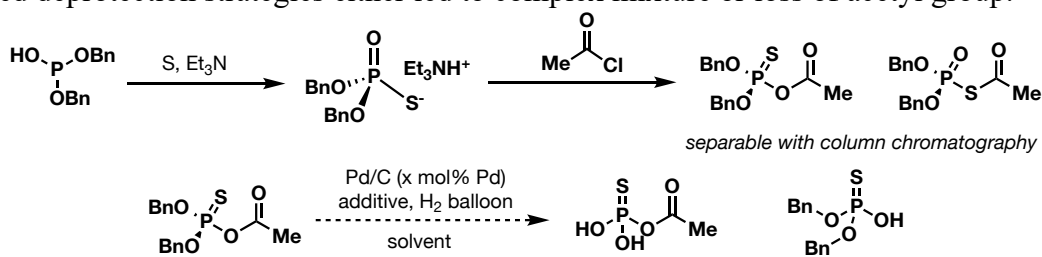
We first tried to synthesize protected thiophosphate ester from alcohol and thiophosphoryl chloride, but complex mixture was obtained and hydrolyzed impurity prevented purification.



We then attempted to directly carry out acetylation with thiophosphate salt, but complex mixture was often obtained. One possible impurity could result from acetylation at sulfur.



We later developed a two-step synthesis of the acetyl thiophosphate benzyl ester. However, all attempted deprotection strategies either led to complex mixture or loss of acetyl group.

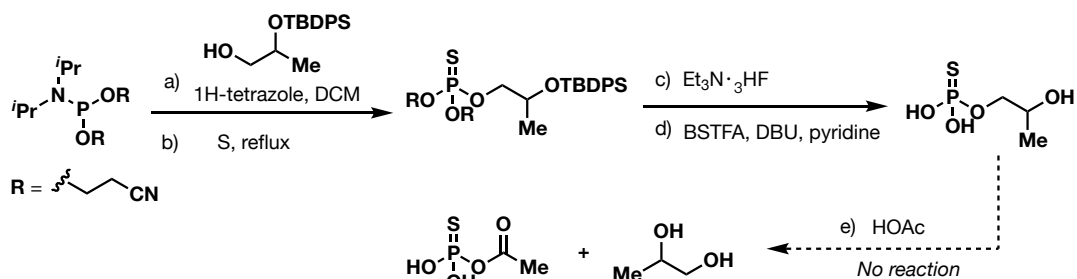


entry	x	additive	solvent	results
1	25	-	MeOH	SM consumed, loss of acetyl group
2	12.5 or 20	-	THF	SM recovered
3	12.5	-	EtOAc	SM recovered
4	5	ZnCl ₂ (1 equiv)	THF	SM consumed, loss of acetyl group
5	5	BF ₃ ·Et ₂ O (1 equiv)	THF	SM consumed, loss of acetyl group

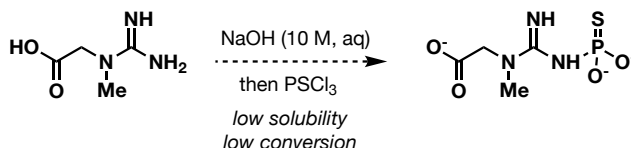


entry	condition	results
1	LiCl (3 eq), RT, THF	SM consumed, loss of acetyl group
2	LiCl (3 eq), 60 °C, THF	SM consumed, loss of acetyl group
3	Ir photocatalyst, DDQ (0.2 eq) O ₂ , MeCN, blue LED, RT	SM partially consumed, complicated mixture
4	Ir photocatalyst, DDQ (2.0 eq) O ₂ , MeCN, blue LED, RT	SM fully consumed, complicated mixture
5	DDQ (0.5 equiv), ^t BuO-NO (4 eq) O ₂ , DCM/H ₂ O, green LED, RT	SM partially consumed, complicated mixture
6	TMSBr (4.5 eq), pyridine (11.5 eq) DCM, RT then H ₂ O	SM consumed, loss of acetyl group

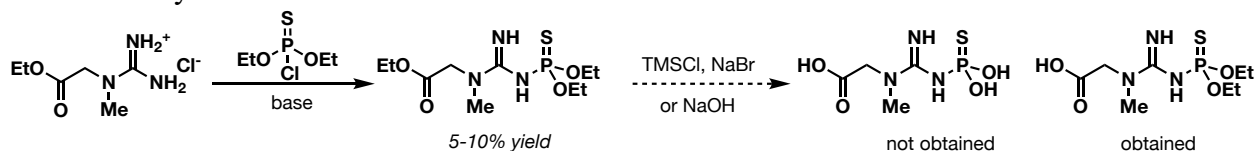
Another attempt to synthesize acetyl thiophosphate donor was not successful due to low reactivity of acetic acid to displace propanediol from the phosphorus center.



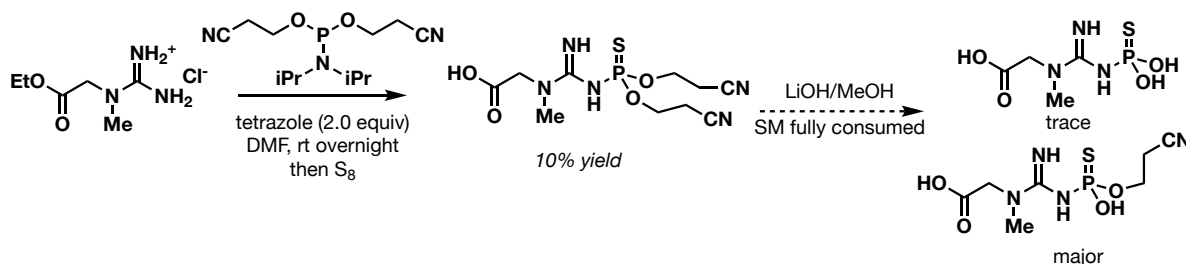
Attempts to synthesize creatine thiophosphate from creatine was not successful due to its low solubility in most of solvents.



Using ester protected creatine, we were able to prepare creatine thiophosphate ethyl ester with poor yields. But the deprotection either led to loss of thiophosphate motif or only ester hydrolysis to the carboxylic acid.



Our last unsuccessful attempt commenced with phosphination of protected creatine and sulfurization, affording protected thiophosphate ester with simultaneous loss of ethyl ester in a poor yield. Base induced deprotection afford trace amount of desired product, while the major product was half deprotected intermediate.



A 500-mL round bottom flask equipped with a magnetic stir bar was charged with guanosine (7.1 mmol, 2.0 g) and acetone (200 mL). To the stirring suspension, 2,2-dimethoxypropane (10.4 mL, 85 mmol) and *p*-toluenesulfonic acid monohydrate (1.34 g, 7.0 mmol) were added and the reaction mixture was stirred at room temperature for 12 hours. The reaction mixture was concentrated by evaporation and then dissolved in water. Solid NaHCO₃ was added carefully in three portions, and the solution was stirred for 2 hours. Saturated NaHCO₃ solution (10 mL) was added and stirred for an additional 2 hours. The suspension was filtered, and the product was washed with water (2 x 100 mL) and dried under vacuum to afford **S51-Int 1** as a white solid, which was used directly for the next step without further purification.

In a 100 mL round bottom flask, **S51-Int 1** (3.1 mmol, 1.00 g) and imidazole (9.3 mmol, 633 mg, 3 equiv.) was dissolved in DMF (12 mL). To this solution was added TBDPSCl (6.2 mmol, 1.704 g, 2 equiv.) and the reaction was allowed to stir at room temperature for 4 hours. The reaction was diluted concentrated under reduced pressure and purified with silica column chromatography (MeOH/DCM, 5% to 10%) to afford **S51-Int 2** as a white solid (1.57 g, 90%).

¹H NMR (600 MHz, MeOD) δ 7.81 (s, 1H), 7.63 – 7.59 (m, 2H), 7.56 – 7.51 (m, 2H), 7.44 – 7.39 (m, 1H), 7.39 – 7.32 (m, 3H), 7.29 – 7.24 (m, 2H), 6.03 (s, 1H), 5.33 – 5.28 (m, 1H), 5.01 (dd, J = 6.3, 3.3 Hz, 1H), 4.33 (td, J = 5.5, 3.3 Hz, 1H), 3.82 (d, J = 5.5 Hz, 2H), 1.57 (s, 3H), 1.36 (s, 3H), 0.99 (s, 9H).

In a 50 mL round bottom flask, acetic acid (12 mL) was diluted with water (3 mL), followed by the addition of the **S51-Int 2** (1 mmol, 561 mg). The reaction mixture was heated to 80 °C and stirred for 12 hours. The reaction mixture was concentrated under reduced pressure and diluted with a small amount of methanol. The crude product was purified with silica column chromatography (MeOH/DCM, 10% to 20%) to afford **S51-Int 3** as a white solid (468 mg, 90%).

¹H NMR (600 MHz, MeOD) δ 7.84 (s, 1H), 7.69 – 7.64 (m, 4H), 7.45 – 7.32 (m, 6H), 5.88 (d, J = 4.8 Hz, 1H), 4.45 (t, J = 5.0 Hz, 1H), 4.11 (q, J = 4.0 Hz, 1H), 3.90 (ddd, J = 64.5, 11.6, 3.5 Hz, 2H), 1.04 (s, 9H). There is one proton buried under water peak.

Isolated **S51-Int 3** (0.85 mmol, 445 mg) was suspended in anhydrous MeCN (10 mL), followed by addition of benzoyl anhydride (771 mg, 3.41 mmol, 4.0 equiv.) and DMAP (0.74 g, 0.17 mmol, 0.2 equiv.). The reaction vessel was equipped with an air condenser and the reaction mixture was refluxed for 3 h. The reaction mixture was concentrated under reduced pressure and diluted with small amount of acetonitrile. The crude product was purified with silica column chromatography (MeOH/DCM, 5% to 10%) to afford **S51-Int 4** as a white solid (465 mg, 75%).

¹H NMR (600 MHz, MeOD) δ 8.02 (dd, J = 8.3, 1.4 Hz, 2H), 7.97 (dd, J = 8.3, 1.4 Hz, 2H), 7.93 (s, 1H), 7.92 – 7.89 (m, 2H), 7.72 – 7.69 (m, 1H), 7.69 – 7.67 (m, 2H), 7.48 – 7.40 (m, 4H), 7.40 – 7.36 (m, 5H), 7.33 – 7.28 (m, 2H), 6.36 (d, J = 5.0 Hz, 1H), 6.27 (t, J = 4.5 Hz, 1H), 6.21 – 6.14 (m, 1H), 4.56 (dt, J = 5.1, 3.4 Hz, 1H), 4.07 (ddd, J = 45.2, 11.7, 3.4 Hz, 2H), 1.07 (s, 9H).

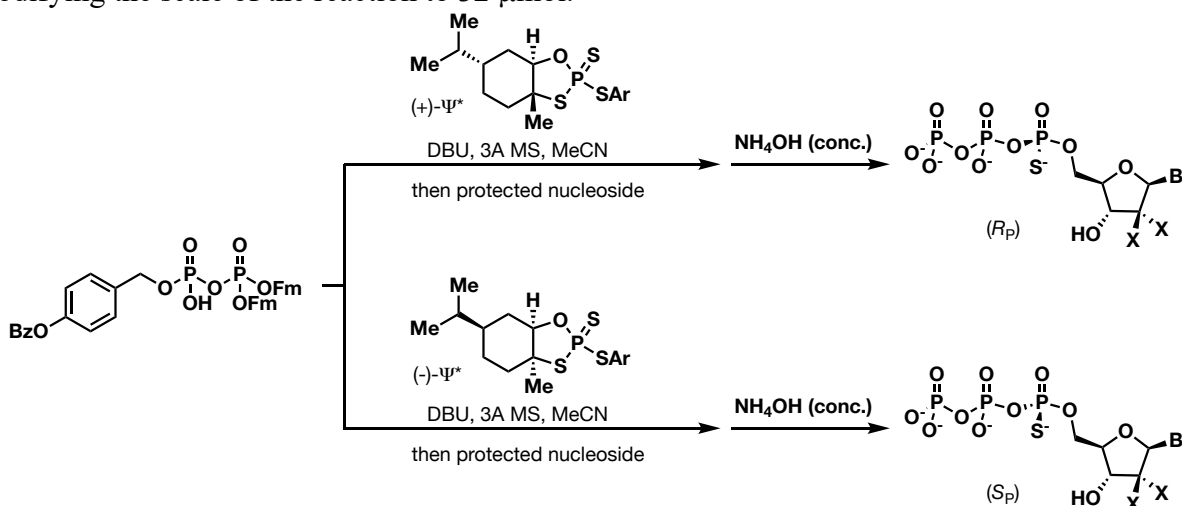
S51-Int 4 (0.53 mmol, 386 mg) was added to a solution of MeOH containing 3% HCl (made by adding 1.6 mL of acetyl chloride to 40 mL of MeOH). The reaction was stirred at room temperature for 6 hours until consumption of all starting material. The mixture was then concentrated and

purified with silica column chromatography (MeOH/DCM, 8% to 15%) to afford **S51** as a white solid (149.2 mg, 57%) which is consistent with literature report ⁶.

¹H NMR (600 MHz, DMSO) δ 10.70 (s, 1H), 8.07 (s, 1H), 7.96 – 7.92 (m, 2H), 7.81 – 7.76 (m, 2H), 7.68 (tt, J = 7.4, 1.3 Hz, 1H), 7.61 (tt, J = 7.4, 1.3 Hz, 1H), 7.55 – 7.47 (m, 2H), 7.43 – 7.38 (m, 2H), 6.52 (s, 2H), 6.26 (d, J = 6.7 Hz, 1H), 6.11 (dd, J = 6.7, 5.6 Hz, 1H), 5.84 (dd, J = 5.6, 2.9 Hz, 1H), 5.49 (t, J = 5.5 Hz, 1H), 4.49 (q, J = 3.4 Hz, 1H), 3.80 (ddd, J = 12.1, 5.6, 3.7 Hz, 2H).

5.2 Preparation of standard samples

To determine the absolute configuration of the P atom (S_P or R_P configuration) of nucleosides α -thio triphosphates, the following standards were synthesized following reported procedure⁶, while modifying the scale of the reaction to 32 μ mol.



A flame dried 1-dram vial with a stir bar was charged with pyrophosphate (24 mg, 32 μ mol, 1.0 equiv.). The vial was closed with a Teflon septum screw cap, evacuated and backfilled with argon. Anhydrous MeCN (0.3 mL) and DBU (19 μ L, 4.0 equiv.) were added and the mixture was stirred for 10 min. Subsequently, freshly flame-dried 3 Å molecular sieves (25 mg) and Ψ^* reagent 11 (15.9 mg, 36 μ mol, 1.2 equiv.) were added and the reaction was stirred at room temperature for 15 min. After that, protected nucleoside (64 μ mol, 2.0 equiv.) was added, followed by another portion of DBU (38 μ L, 8.0 equiv.) and the mixture was stirred for 3 to 6 h. Upon completion of the reaction, the resulting mixture was filtered, the solid residue was washed with $\sim$ 1 mL of MeCN and the filtrate was concentrated under reduced pressure to $\sim$ 0.25 mL. Concentrated aq. NH_3 solution (1.0 mL) was added to the residue and the reaction was stirred at room temperature (or at 40 °C) for 16 h. Following quenching and purification were identical to literature procedures.

5.3 Assignment of stereochemistry

Relative chromatographic mobility of the obtained pairs of epimers (fast-eluting epimer: S_P ; slow-eluting epimer: R_P) and products obtained from enzymatic reactions are compared. The stereochemistry of phosphorus atom was assigned based on retention time.

Analytical method

HPLC analyses were conducted on a C18 column (Kinetex 5 μ m EVO C18 100 Å, LC Column 100 $\times$ 2.1 mm)

Solvent A: 0.1 M triethylammonium acetate (TEAA) in H_2O

Solvent B: MeCN

Flow rate: 0.3 mL/min

Temperature: 40°C

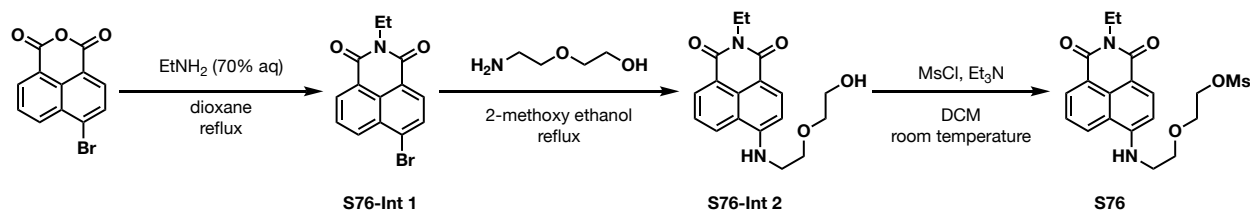
HPLC method 1

Time (min)	Solvent A (%)	Solvent B (%)
0	98	2
14	92	8
17	5	90

HPLC method 2

Time (min)	Solvent A (%)	Solvent B (%)
0	99	1
8	95	5
11	5	90

5.4 Preparation of fluorophore S76 for oligonucleotide derivatization



4-Bromo-1,8-naphthalic anhydride (1.38 g, 5.0 mmol) and EtNH₂ (70 % solution in H₂O, 0.47 mL, 6.0 mmol) were heated under reflux in dioxane (50 mL) for 9 h. The solution was cooled to rt and poured into iced water (500 ml), and the precipitate collected by filtration. The residue was washed with H₂O (200 ml, twice) and the solvent removed in vacuo to afford **S76-Int 1** (1.44 g, 95%) as cream solid.

¹H NMR (600 MHz, CDCl₃) δ 8.65 (d, *J* = 7.3 Hz, 1H), 8.56 (d, *J* = 8.5 Hz, 1H), 8.41 (d, *J* = 7.8 Hz, 1H), 8.03 (d, *J* = 7.8 Hz, 1H), 7.84 (t, *J* = 7.9 Hz, 1H), 4.24 (q, *J* = 7.2 Hz, 2H), 1.33 (t, *J* = 7.1 Hz, 3H).

To a round-bottom flask with stir bar was charged **S76-Int 1** (304 mg, 1.0 mmol), 2-(2-aminoethoxy) ethanol (0.40 mL, 4.0 mmol, 4.0 equiv.), and 2-methoxy-ethanol (10 mL). The resulting mixture was heated under reflux for 8 h. The reaction mixture was cooled to room temperature then concentrated under reduced pressure. The residue thus obtained was purified with silica column chromatography (EtOAc/MeOH, 9:1) to afford **S72-Int 2** as a yellow solid (274.5 mg, 84%).

¹H NMR (600 MHz, CDCl₃) δ 8.61 (dd, *J* = 7.3, 1.1 Hz, 1H), 8.49 (dd, *J* = 8.4, 1.2 Hz, 1H), 8.17 (dd, *J* = 8.4, 1.2 Hz, 1H), 7.65 (dd, *J* = 8.4, 7.3 Hz, 1H), 6.76 (d, *J* = 8.4 Hz, 1H), 4.25 (q, *J* = 7.1 Hz, 2H), 3.94 (t, *J* = 5.1 Hz, 2H), 3.90 – 3.85 (m, 2H), 3.76 – 3.71 (m, 2H), 3.63 (t, *J* = 5.1 Hz, 2H), 1.34 (t, *J* = 7.1 Hz, 3H).

To a solution of **S76-Int 2** (164 mg, 0.5 mmol) and methanesulfonyl chloride (0.043 mL, 0.55 mmol, 1.1 equiv.) in DCM (10 mL) was added Et₃N (0.14 mL, 1.0 mmol, 2.0 equiv.). After stirring at 0 °C for 2 h, the mixture was warmed to room temperature and allowed to be stirred for 2 h. After the reaction was completed, the mixture was diluted with water (50 mL), the organic phase separated was dried over sodium sulfate and concentrated to afford homemade **S76** (171.6 mg, 84%).

¹H NMR (600 MHz, CDCl₃) δ 8.59 (d, *J* = 6.3 Hz, 1H), 8.47 (d, *J* = 8.4 Hz, 1H), 8.24 (d, *J* = 7.4 Hz, 1H), 7.67 – 7.62 (m, 1H), 6.71 (d, *J* = 8.4 Hz, 1H), 4.48 – 4.43 (m, 2H), 4.23 (q, *J* = 7.1 Hz, 2H), 3.92 (t, *J* = 5.1 Hz, 2H), 3.88 – 3.79 (m, 2H), 3.61 (t, *J* = 5.0 Hz, 2H), 3.06 (s, 3H), 1.32 (t, *J* = 7.1 Hz, 3H).

¹³C NMR (151 MHz, CDCl₃) δ 164.7, 164.1, 149.3, 134.3, 131.3, 129.9, 126.7, 125.1, 123.3, 120.8, 111.3, 104.9, 69.1, 68.9, 68.7, 43.5, 38.1, 35.3, 13.6.

6. Optimization of Reaction Conditions

Table S1. Initial reactivity tests of nucleoside kinases

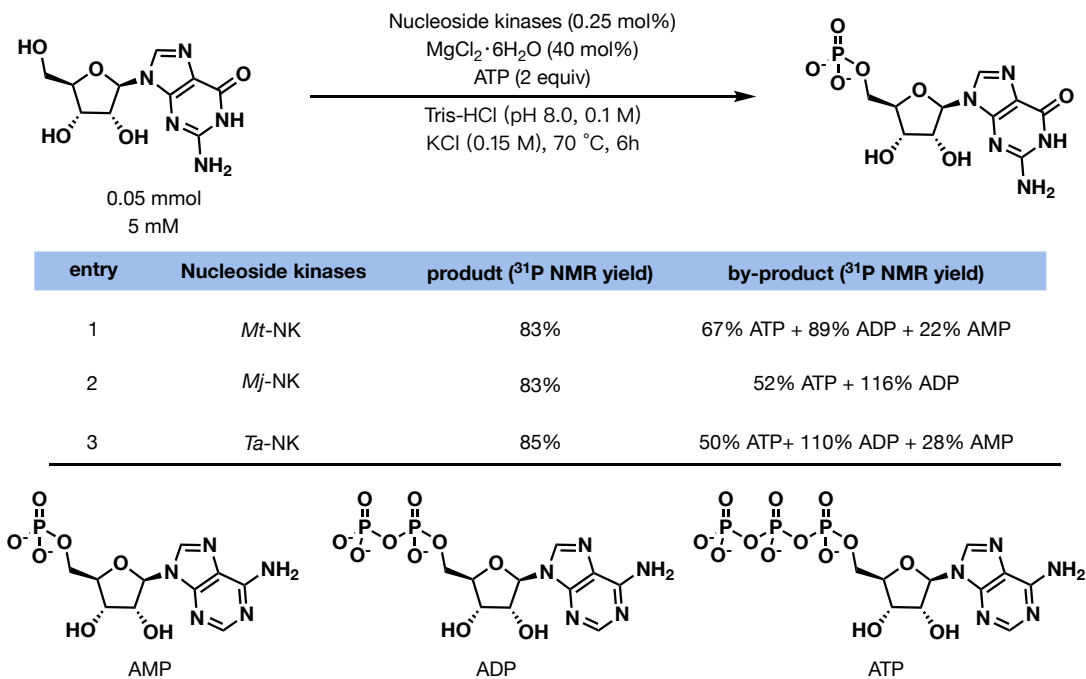
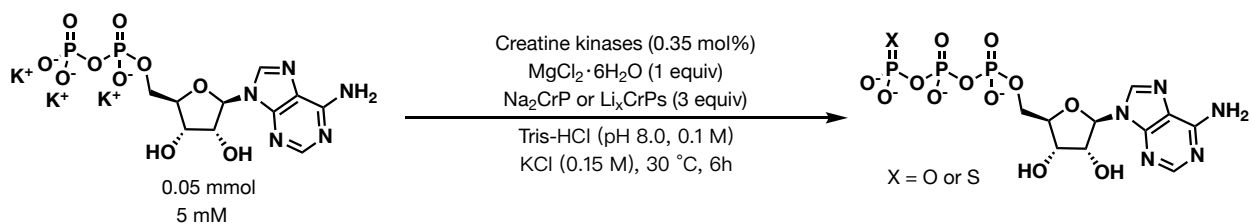


Table S2. Attempts to capture ATP or ATP γ S using creatine kinases



entry	Creatine kinases	phosphate donors	Results
1	<i>Hb</i> -CK	Na ₂ CrP	LC-MS detected, low NMR yield
2	<i>Hm</i> -CK	Na ₂ CrP	LC-MS detected, low NMR yield
3	<i>Rm</i> -CK	Na ₂ CrP	LC-MS detected, low NMR yield
4	<i>Hb</i> -CK	Li _x CrPs	LC-MS detected, low NMR yield
5	<i>Hm</i> -CK	Li _x CrPs	LC-MS detected, low NMR yield
6	<i>Rm</i> -CK	Li _x CrPs	LC-MS detected, low NMR yield

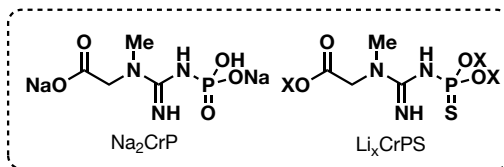
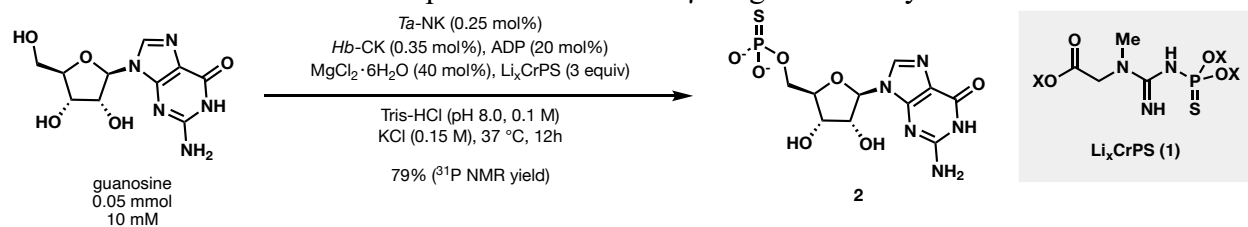


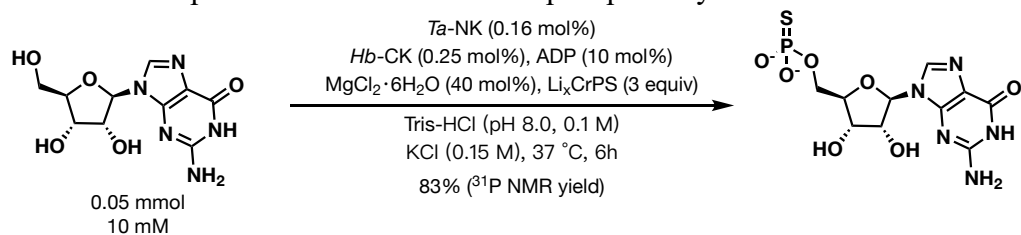
Table S3. Reaction conditions optimization for ATP γ S regeneration system



entry	variation from standard conditions	³¹ P NMR yield	conversion
1	10 mol% ADP	83%	>95%
2	2 mol% ADP + 1.2 eq. Li _x CrPS	80%	>95%
3	0.16 mol% <i>Ta</i> -NK + 0.25 mol% <i>Hb</i> -CK	82%	>95%
4	entry 3 + 5 mol% ADP + 1.2 eq Li _x CrPS	81%	>95%
5	6 h instead of 12 h	80%	>95%
6	0.04 mol% <i>Ta</i> -NK + 0.063 mol% <i>Hb</i> -CK	74%	>95%
7	<i>Hm</i> -CK instead of <i>Hb</i> -CK	77%	>95%
8	<i>Rm</i> -CK instead of <i>Hb</i> -CK	74%	>95%
9	<i>Mt</i> -NK instead of <i>Ta</i> -NK	72%	>95%
10	<i>Mj</i> -NK instead of <i>Ta</i> -NK	57%	>95%
11	no enzymes	<2%	<2%

NMR yield was determined by quantitative ³¹P NMR using methyl phosphonic acid as internal standard. Conversion was determined by ¹H NMR using methyl phosphonic acid as internal standard.

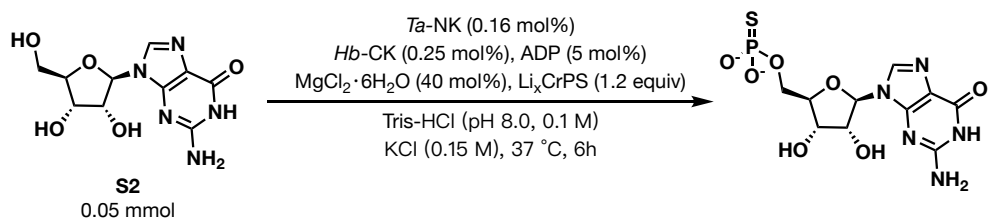
Table S4. Additional optimization for 5'-monothiophosphate synthesis



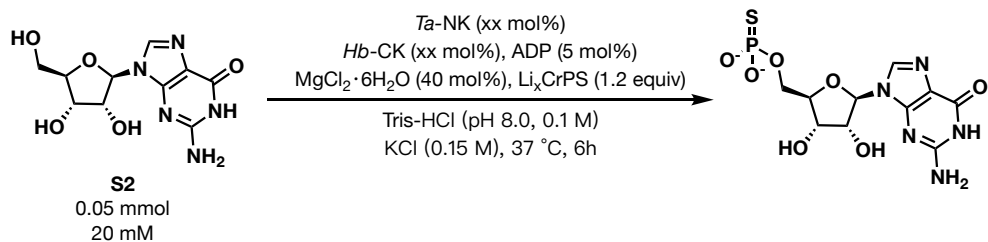
entry	variation from standard condition	^{31}P NMR yield
1	0.05 M Tris-HCl pH 7.0	80%
2	0.05 M Tris-HCl pH 8.0	76%
3	0.05 M Tris-HCl pH 9.0	<1%
4	0.1 M Tris-HCl pH 7.0	70%
5	0.1 M Tris-HCl pH 8.0	78%
6	Na_xCrPS instead of Li_xCrPS	82%

NMR yield was determined by quantitative ^{31}P NMR using methyl phosphonic acid as internal standard

Table S5. Effect of substrate concentration and enzyme loading.



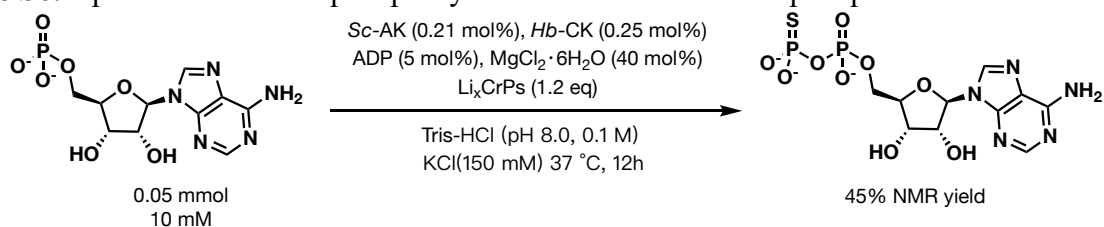
entry	Concentration of S2	³¹ P NMR yield
1	10 mM	79%
2	20 mM	78%
3	5 mM	79%



entry	enzymes	³¹ P NMR yield
1	<i>Ta</i> -NK (0.08 mol%)+ <i>Hb</i> -CK (0.125 mol%)	79%
2	<i>Ta</i> -NK (0.04 mol%)+ <i>Hb</i> -CK (0.0625 mol%)	39%

NMR yield was determined by quantitative ³¹P NMR using methyl phosphonic acid as internal standard

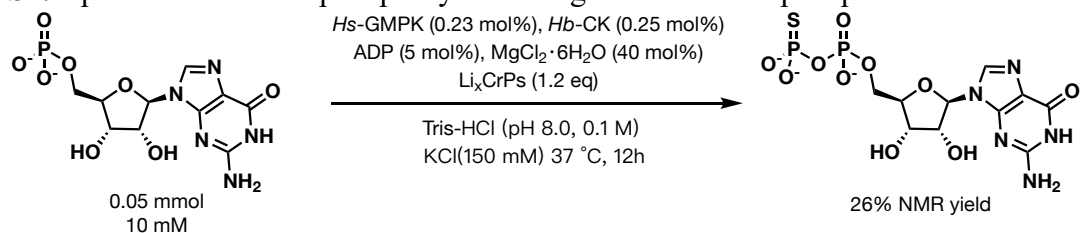
Table S6. Optimization of thiophosphorylation of adenosine monophosphate



entry	variation from previous condition	^{31}P NMR yield	conversion
1	using Ec-AK	40%	79%
2	Fe^{2+} or Zn^{2+} instead of Mg^{2+}	<2%	<5%
3	Mn^{2+} or Co^{2+} instead of Mg^{2+}	trace	<5%
4	DTT (5 mol%) as additive	46%	78%
5	addition of enzyme in 3 batches	46%	59%
6	6 h instead of 12h	58%	86%
7	48 h instead of 12h	35%	80%
8	5 mM instead of 10 mM	33%	42%
9	20 mM instead of 10 mM	18%	65%

NMR yield was determined by quantitative ^{31}P NMR using methyl phosphonic acid as internal standard

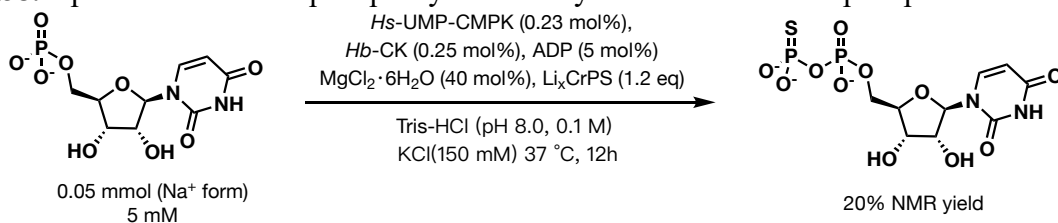
Table S7. Optimization of thiophosphorylation of guanosine monophosphate



entry	variation from previous condition	³¹ P NMR yield	conversion
1	<i>Bf</i> -GMPK instead	<2%	<2%
2	<i>Ec</i> -GMPK instead	<2%	<2%
3	run in a 0.01 mmol scale	36%	77%
4	entry 3 + pH 7.0 Tris-HCl, 30 °C	43%	82%
5	entry 3 + pH 7.4 Tris-HCl, 30 °C	46%	77%
6	entry 3 + pH 8.0 Tris-HCl, 30 °C	43%	74%
7	entry 6 + 6 hours of reaction time	55%	88%

NMR yield was determined by quantitative ³¹P NMR using methyl phosphonic acid as internal standard

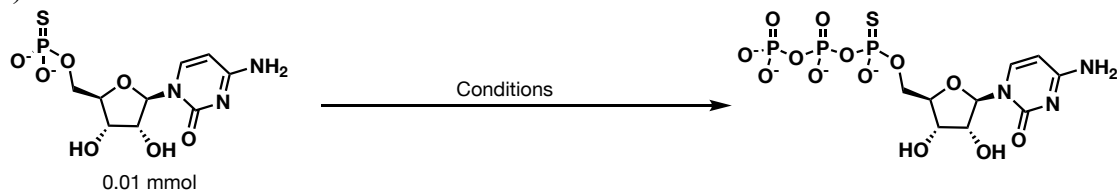
Table S8. Optimization of thiophosphorylation of cytidine/uridine monophosphate



entry	variation from previous condition	³¹ P NMR yield
1	cytidine monophosphate instead of uridine monophosphate	<10%
2	substrate concentration changed from 10 mM to 2.5 mM	46%
3	10 mM concentration	<5%
4	substrate concentration 1.5 mM	<2%
5	<i>Tm</i> -UMP-K instead of <i>Hs</i> -UMP-CMPK (substrate conc.= 10 mM)	29%
6	Tris-HCl pH 7.0 buffer (substrate conc.= 2.5 mM)	40%
7	2× loading of enzyme	45%
8	DTT (10 mM) additive + entry 2	55%

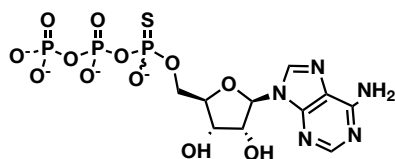
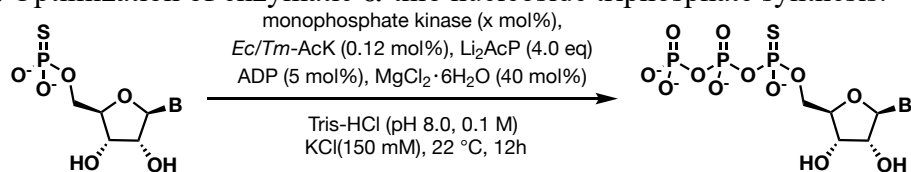
NMR yield was determined by quantitative ³¹P NMR using methyl phosphonic acid as internal standard

Table S9. Initial discovery of enzymatic α -thio nucleoside triphosphate synthesis (pyrimidine-based)

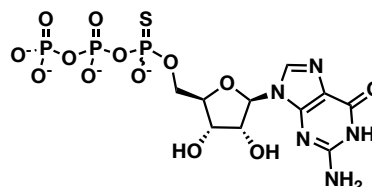


entry	conditions	results
1	enzymes: <i>Hs</i> -UMP-CMPK, <i>Ec</i> -AcK Mg ²⁺ , ATP or ADP (cat), Li ₂ AcP (4 eq), 37 °C pH 8.0 Tris-HCl, 150 mM KCl	CDP α S detected, CTP α S detected
2	enzymes: <i>Hs</i> -UMP-CMPK, <i>Ec</i> -NDK, <i>Hb</i> -CK Mg ²⁺ , ATP or ADP (cat), Na ₂ CrP (4 eq), 37 °C pH 8.0 Tris-HCl, 150 mM KCl	CDP α S detected, CTP α S trace
3	enzymes: <i>Hs</i> -UMP-CMPK, <i>Gs</i> -PK Mg ²⁺ , ATP (cat/1.0 equiv), KPEP (4 eq), 37 °C pH 8.0 Tris-HCl, 150 mM KCl	CDP α S detected, CTP α S not detected
4	enzymes: <i>Hs</i> -UMP-CMPK, <i>Ec</i> -AcK Mg ²⁺ , ATP or ADP (cat), Li ₂ AcP (4 eq), 37 °C pH 7.4 or 7.0 Tris-HCl, 150 mM KCl	CDP α S trace, CTP α S trace
5	enzymes: <i>Hs</i> -UMP-CMPK, <i>Ec</i> -AcK Mg ²⁺ , ATP or ADP (cat), Li ₂ AcP (4 eq), 22 °C pH 8.0 Tris-HCl, 150 mM KCl	CDP α S 11%, CTP α S 30%

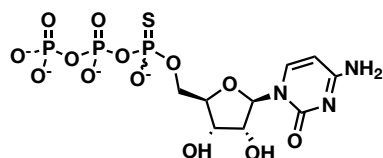
Table S10. Optimization of enzymatic α -thio nucleoside triphosphate synthesis.



enzymes	<i>Ec</i> -AcK	<i>Tm</i> -AcK
<i>Ec</i> -AK	trace pdt	trace pdt
<i>Sc</i> -AK	60% (NMR) dr 2:1	58% (isolated) dr 9:1

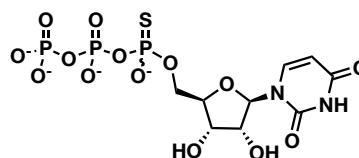


enzymes	<i>Ec</i> -AcK	<i>Tm</i> -AcK
<i>Ec</i> -GMPK	trace pdt	trace pdt
<i>Hs</i> -GMPK	45% (NMR) dr 3:1	58% (NMR) dr 3:1
<i>Bf</i> -GMPK	54% (NMR) dr 8:1	55% (NMR) dr 12:1



enzymes	<i>Ec</i> -AcK	<i>Tm</i> -AcK
<i>Mt</i> - or <i>At</i> -CMPK	<20% (NMR) dr 1:1	<20% (NMR) dr 1:1
<i>Hs</i> -UMP-CMPK	60% (NMR) dr 1.2:1 ^a	60% (NMR) dr 1.6:1 ^{a,b}
<i>Bs</i> -CMPK	60% (NMR) dr 2:1 ^a	<20% (NMR) dr 1:1

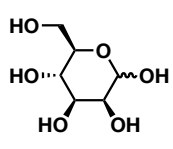
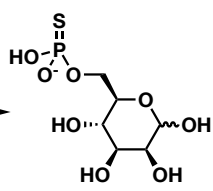
^a 8-10% ATP is not separable from desired product
^b significant tetraphosphate by-product observed



enzymes	<i>Ec</i> -AcK	<i>Tm</i> -AcK
<i>Mt</i> - or <i>At</i> -CMPK	<20% (NMR) dr 1:1	<20% (NMR) dr 1:1
<i>Hs</i> -UMP-CMPK	65% (NMR) dr 1.2:1 ^a	60% (NMR) dr 1.8:1 ^{a,b}
<i>Bs</i> -CMPK	55% (NMR) dr 2:1 ^a	<20% (NMR) dr 1:1

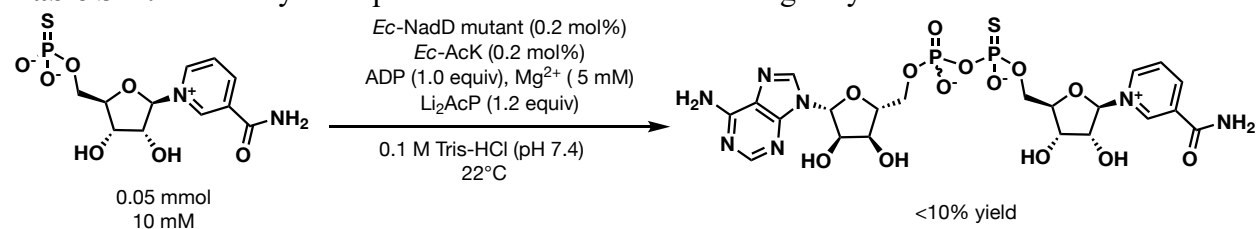
^a 8-10% ATP is not separable from desired product
^b significant tetraphosphate by-product observed

Table S11. Optimization of thiophosphorylation of hexose

 0.05 mmol 10 mM $\xrightarrow[\text{Tris-HCl (pH 8.0, 0.1M)}]{\begin{array}{l} \text{hexokinase (25 U)} \\ \text{Hb-CK (0.25 mol\%), ADP (5 mol\%)} \\ \text{MgCl}_2 \cdot 6\text{H}_2\text{O (40 mol\%), Li}_x\text{CrPS (2.0 equiv)} \end{array}}$ 30 °C, 12h 			
entry	variation from standard condition	³¹ P NMR yield	dr
1	none	46%	~2:1
2	50 U hexokinase 10 mol% ADP, 3.0 eq of Li _x CrPS	49%	~2:1
3	37 °C instead of 30 °C	19%	~2:1
4	22 °C instead of 30 °C	31%	~2:1
5	Hm-CK instead of Hb-CK	40%	~2:1
6	Rm-CK instead of Hb-CK	38%	~2:1
7	pH 7.0 Tris-HCl buffer	62%	~2:1
8	pH 9.0 Tris-HCl	no reaction	-
9	pH 7.0, 50 U hexokinase 2.5 eq of Li _x CrPS	73%	~2:1
10	pH 7.0, 75 U hexokinase 3.0 eq of Li _x CrPS	86%	~2:1
11	pH 7.0, 75 U hexokinase 1.2 eq of Li _x CrPS	86%	~2:1

NMR yield was determined by quantitative ³¹P NMR using methyl phosphonic acid as internal standard

Table S12. Discovery and optimization of NAD⁺ thio-analogue synthesis

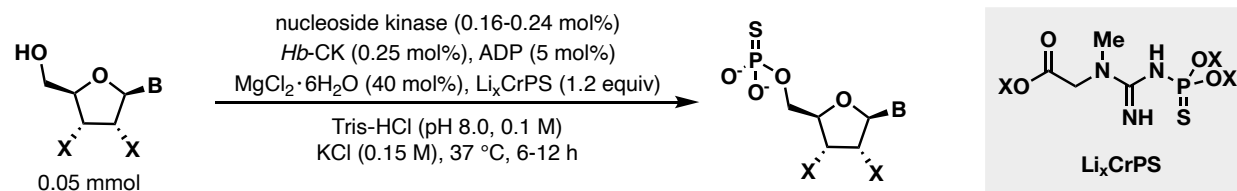


entry	conditions	results
1	ATP (1 equiv) instead of ADP	< 5% yield
2	Mn ²⁺ , Co ²⁺ , Ni ²⁺ instead of Mg ²⁺	5-10% yield
3	2x loading of enzyme	<10% yield
4	slow addition of ADP	< 5% yield
5	pH 7.0 or 8.0 buffer	<10% yield
6	37°C instead of 22°C	< 5% yield
7	entry 1+ <i>Ec</i> -IPYR instead of <i>Ec</i> -AcK	46% yield
8	2x loading of enzyme as entry 7	30%
9	entry 1+ <i>Hs</i> -NMNAT + <i>Ec</i> -IPYR	47% yield, dr = 1:1

NMR yield was determined by quantitative ³¹P NMR using methyl phosphonic acid as internal standard

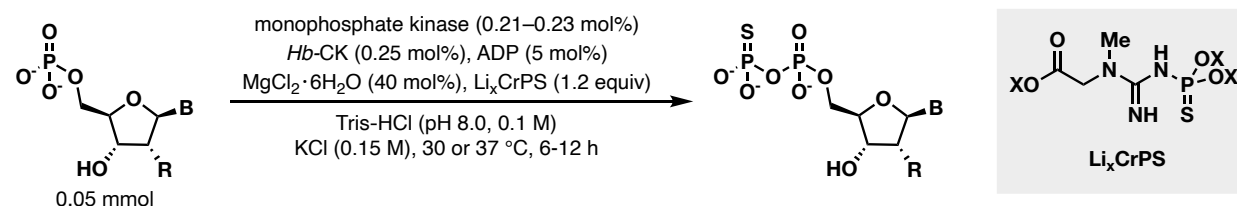
7. General Procedures for Enzymatic Thiophosphorylation

Method A. (Preparation of monothiophosphate)



A 20-mL scintillation vial equipped with a magnetic stir bar was charged with ADP (K^+ salt, hydrate, 1.2 mg, 2.5 μmol , 5 mol%) and the appropriate nucleoside substrate (0.05 mmol). The solids were dissolved in 4.3 mL of reaction buffer (0.1 M Tris-HCl, pH 8.0 and 0.15 M KCl), followed by the addition of MgCl_2 stock solution (50 mM, 400 μL , 0.4 equiv.) and Li_xCrPS (1.2 equiv., ~100 μL). Then to the stirring reaction mixture was added *Ta*-NK or other nucleoside kinases (2.5 mg) and *Hb*-CK (5 mg, 0.25 mol%) sequentially, and a small amount of reaction buffer was added to a final volume of 5.0 mL. The reaction mixture was stirred at 1000 rpm in a heating block with the temperature set at 37 °C for 12 hours. Reaction progress was monitored by LC-MS. After most of the nucleoside was consumed, the vial was removed from the heating block and cooled in an ice-water bath for 5 minutes. To the vigorously stirring reaction mixture was added 15 mL of methanol (white foam precipitate was often observed), and the mixture was stirred for another 2 minutes. The reaction mixture was filtered through a pad of celite to remove all precipitate, the celite pad was washed with water (5 mL) twice, water-methanol mixture (1:1, 5 mL) once and methanol (5 mL) once. The filtrate was combined, and solvent was evaporated overnight under a flow of nitrogen. The crude product was re-dissolved in small amount of water (~ 2 to 3 mL) and purified by ion exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, gradient was specified for each substrate). Fractions containing product (monitored by LC-MS) were combined and the solvent was evaporated under reduced pressure (temp. ≤ 40 °C). The solid residue was dissolved in minimal amount of water and lyophilized to provide pure products.

Method B. (Preparation of β -thio nucleoside diphosphates)

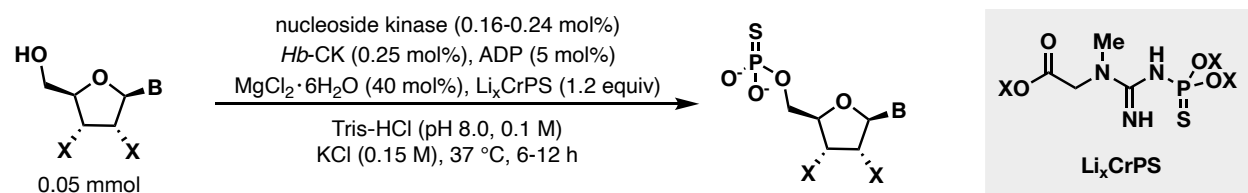


A 20-mL scintillation vial equipped with a magnetic stir bar was charged with ADP (K^+ salt, hydrate, 1.2 mg, 2.5 μmol , 5 mol%) and the appropriate nucleoside monophosphate substrate (0.05 mmol). *Note: Some substrates require DTT as additive and the final concentration of DTT is 10 mM.* The solids were dissolved in 4.3 mL of reaction buffer (0.1 M Tris-HCl, pH 8.0 and 0.15 M KCl), followed by the addition of MgCl_2 stock solution (50 mM, 400 μL , 0.4 equiv.) and Li_xCrPS (1.2 equiv., ~100 μL). Then to the stirring reaction mixture was added the appropriate nucleoside

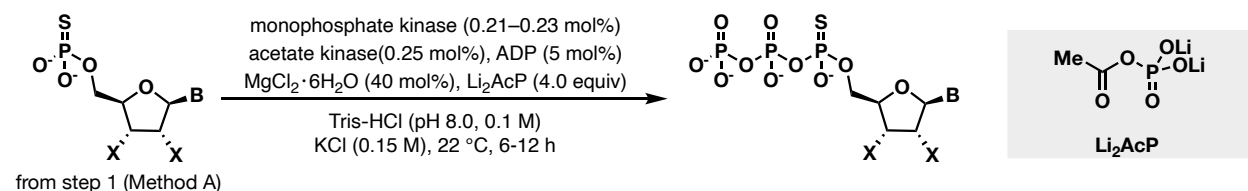
monophosphate kinase (2.5 mg) and *Hb*-CK (5 mg, 0.25 mol%) sequentially, and a small amount of reaction buffer was added to reach a final volume of 5.0 mL. The reaction mixture was stirred at 1000 rpm in a heating block with the temperature set at 30 or 37 °C for 12 hours. Reaction progress was monitored by LC-MS. After the ratio of starting material and product reached a steady state, the vial was removed from the heating block and cooled in an ice-water bath for 5 minutes. To the vigorously stirring reaction mixture was added 15 mL of methanol (white foam precipitate was often observed), and the mixture was stirred for another 2 minutes. The reaction mixture was filtered through a pad of celite to remove all precipitate, the celite pad was washed with water (5 mL) twice, water-methanol mixture (1:1, 5 mL) once and methanol (5 mL) once. The filtrate was combined, and solvent was evaporated overnight under a flow of nitrogen. The crude product was re-dissolved in small amount of water (~ 2 to 3 mL) and purified by ion exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, gradient was specified for each substrate). Fractions containing product (monitored by LC-MS) were combined and the solvent was evaporated under reduced pressure (temp. ≤ 40 °C). The solid residue was dissolved in minimal amount of water and lyophilized to provide pure products.

Method C. (Preparation of α -thio nucleoside triphosphates, two-step sequence)

Most purine-type α -thio triphosphates were prepared with this two-step sequence without further note.



In the first step, each monothiophosphate was prepared from the nucleoside starting material using **Method A**. Ion exchange chromatography purification is recommended to remove unreacted nucleosides and creatine thiophosphate.

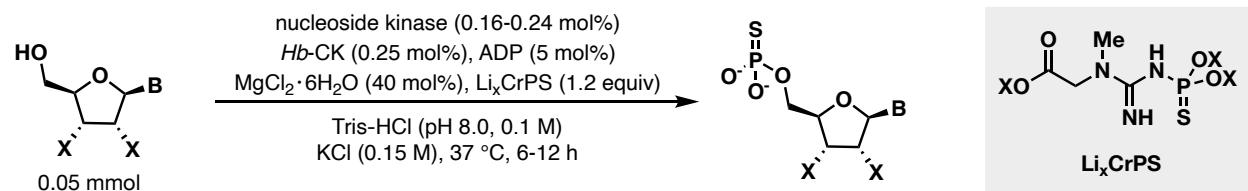


In the second step, a 20-mL scintillation vial equipped with a magnetic stir bar was charged with ADP (K⁺ salt, hydrate, 1.2 mg, 2.5 μ mol, 5 mol%), acetyl phosphate lithium salt (30.4 mg, 0.2 mmol, 4.0 equiv.) and the corresponding nucleoside monothiophosphate product synthesized from the first step (0.05 mmol scale). The solids were dissolved in 4.4 mL of reaction buffer (0.1 M Tris-HCl, pH 8.0 and 0.15 M KCl), followed by addition of MgCl₂ stock solution (50 mM, 400 μ L, 0.4 equiv.). Then to the stirring reaction mixture was added the corresponding nucleoside monophosphate kinase (2.5 mg, 0.15-0.20 mol%) and acetate kinase (2.5 mg, 0.15-0.20 mol%) sequentially. The reaction mixture was stirred at room temperature for 12 hours. Reaction progress was monitored by LC-MS. After most of the nucleoside was consumed, the vial was cooled in an ice-water bath for 5 minutes. To the vigorously stirring reaction mixture was added 15 mL of

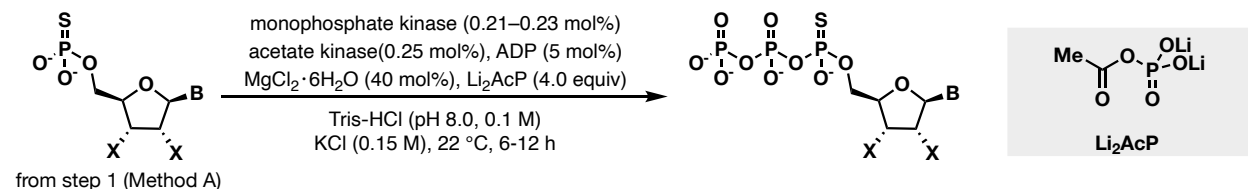
methanol (white foam precipitate was often observed), and the mixture was stirred for 2 additional minutes. The reaction mixture was filtered through a pad of celite to remove all precipitate, the celite pad was washed with water (5 mL) twice, water-methanol mixture (1:1, 5 mL) once and methanol (5 mL) once. The filtrate was combined, and solvent was evaporated overnight under a flow of nitrogen. The crude product was re-dissolved in small amount of water (~ 2 to 3 mL) and purified by ion exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, gradient was specified for each substrate). Fractions containing product (monitored by LC-MS) were combined and the solvent was evaporated under reduced pressure (temp. $\leq 40^\circ\text{C}$). The solid residue was dissolved in minimal amount of water and lyophilized to provide pure products.

Method D. (Preparation of α -thio nucleoside triphosphates, three-step sequence)

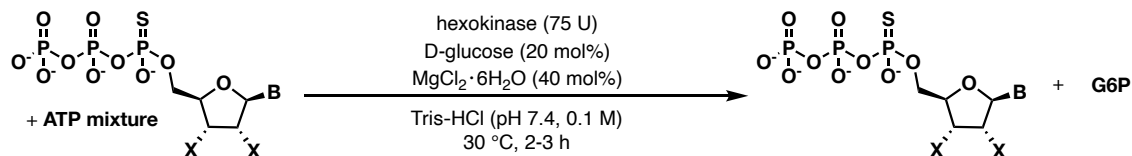
Most pyrimidine-type α -thio triphosphates were prepared from this three-step sequence without further note.



In the first step, each mono thiophosphate was prepared from the nucleoside starting material using **Method A**. Ion exchange chromatography purification is recommended to remove unreacted nucleosides and creatine thiophosphate.



In the second step, each pyrimidine-type α -thio triphosphates was prepared from the mono thiophosphate product from the first step using **Method C**. Ion exchange chromatography purification is recommended to remove unreacted nucleosides and acetyl phosphate.



In the third step, D-glucose and commercially available hexokinase (Sigma-Aldrich item number: H6380) was used to remove ATP impurity. A 20-mL scintillation vial equipped with a magnetic stir bar was charged with D-glucose (1.8 mg, 0.01 mmol, 0.2 equiv.) and the triphosphate product mixture from the second step (0.05 mmol scale nucleoside starting material). The solids were dissolved in 4.5 mL of reaction buffer (0.1 M, pH 7.4, Tris-HCl), followed by the addition of MgCl_2 stock solution (50 mM, 400 μL , 0.4 equiv.). Then to the stirring reaction mixture was added hexokinase (75 U). The reaction mixture was stirred at 1000 rpm in a heating block with the temperature set at 30°C for 2-3 hours. Reaction progress was monitored by LC-MS. After

complete consumption of ATP, the reaction was quenched and purified using the procedure described in the second step. Fractions without ATP contamination was collected and lyophilized to provide products. Improved diastereoselectivity was often observed in this third step, which might be attributed to selective consumption of one isomer by hexokinase in a slower rate.

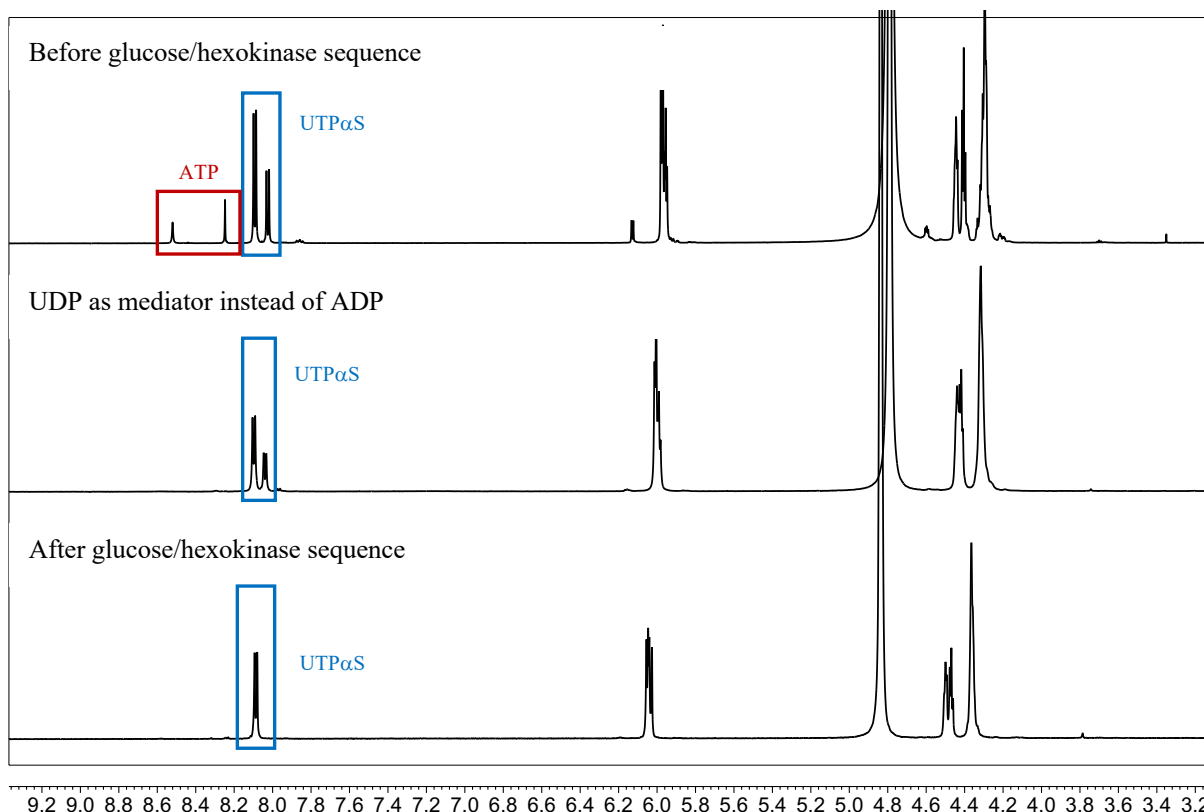
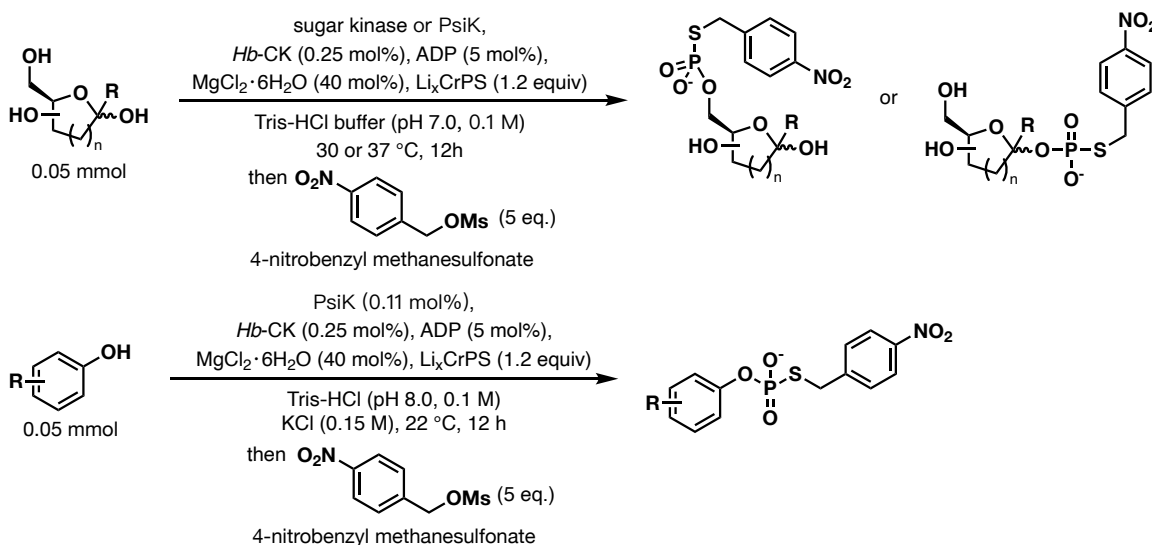


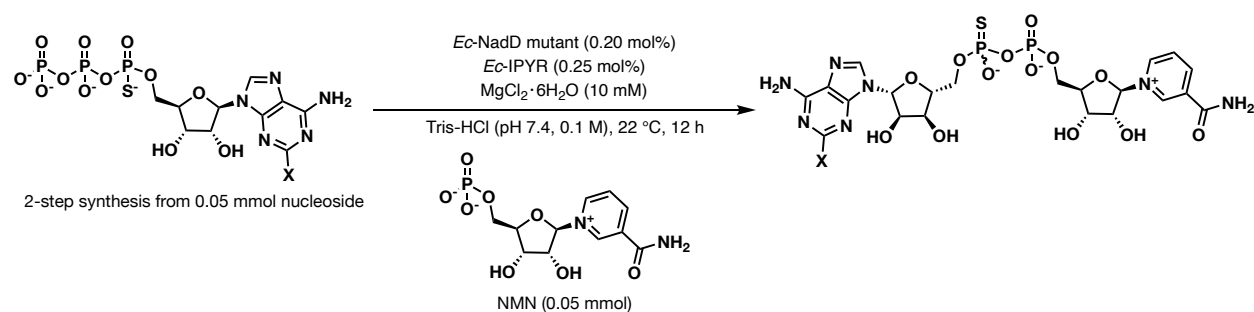
Figure S4. Comparison of products before and after third step (use UTP α S as example).

Method E. (Preparation of sugar or phenol thiophosphate derivatives)



A 20-mL scintillation vial equipped with a magnetic stir bar was charged with ADP (K^+ salt, hydrate, 1.2 mg, 2.5 μ mol, 5 mol%) and the appropriate sugar substrate (0.05 mmol). The solids were dissolved in 4.3 mL of reaction buffer (0.1 M, pH 7.0, Tris-HCl for for sugar molecules; 0.1 M Tris-HCl, pH 8.0 and 0.15 M KCl for phenol molecules), followed by the addition of $MgCl_2$ stock solution (50 mM, 400 μ L, 0.4 equiv for sugar molecules; 1000 μ L, 1.0 equiv for phenol molecules) and Li_xCrPS (1.2 equiv., $\sim$ 100 μ L). Then to the stirring reaction mixture was added the appropriate sugar kinase or PsiK (2.5 mg, 0.11 mol%) and *Hb*-CK (5 mg, 0.25 mol%) sequentially, and a small amount of reaction buffer was added to a final volume of 5.0 mL. The reaction mixture was stirred at 1000 rpm in a heating block with the temperature set at 22 $^{\circ}C$ (PsiK), 30 or 37 $^{\circ}C$ (sugar kinase) for 12 hours. Reaction progress was monitored by ^{31}P NMR. After cooling to room temperature, 1 mL of MeCN was added, followed by the addition of 4-nitrobenzyl methanesulfonate (5 equiv., 0.25 mmol, 57.8 mg). The mixture was stirred at room temperature for 6 hours, and monitored by LC-MS. After most of the free thiophosphate has been alkylated, the vial was cooled in an ice-water bath for 5 minutes. To the vigorously stirring reaction mixture was added 15 mL of methanol (white foam precipitate was often observed), and the mixture was stirred for 2 additional minutes. The reaction mixture was filtered through a pad of celite to remove all precipitate, the celite pad was washed with water (5 mL) twice, water-methanol mixture (1:1, 5 mL) once and methanol (5 mL) once. The filtrate was combined, and the solvent was evaporated overnight under a flow of nitrogen. The crude product was re-dissolved in small amount of water ($\sim$ 2 to 3 mL) and purified by ion exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, gradient was specified for each substrate). Fractions containing product (monitored by LC-MS) were combined and the solvent was evaporated under reduced pressure (temp. $\leq$ 40 $^{\circ}C$). The solid residue was dissolved in minimal amount of water and lyophilized to provide products. For pentose, hexose and inositol, DEAE purified products was redissolved in minimum amount of water and further purified by C18 reverse phase column using (MeCN/water or MeOH/water, gradient from 0:100 to 100:0). The desired product was collected and concentrated under reduced pressure (temp. $\leq$ 40 $^{\circ}C$). Lyophilization was applied to remove residual water.

Method F. (Preparation of α -thio NAD^+ analogues)

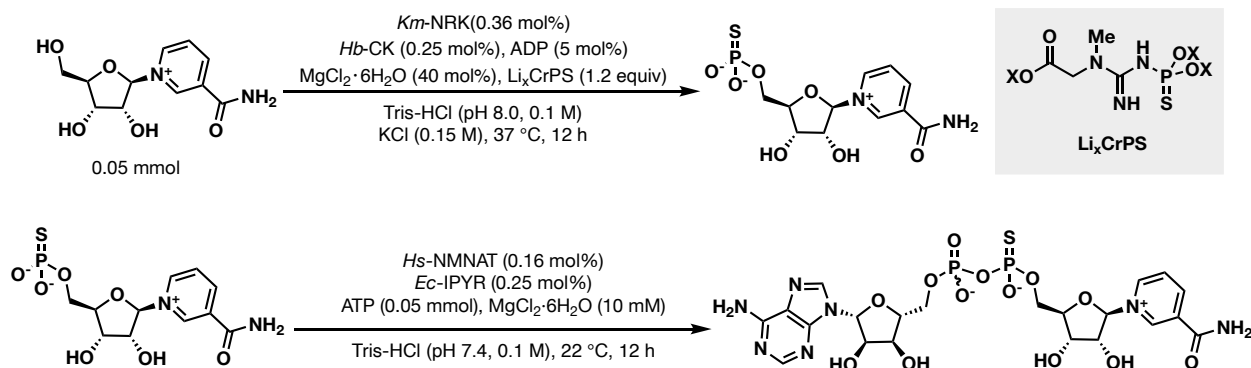


ATP α S and its fluoro derivative were prepared by combining **Method A** and **C** as described previously. Ion exchange chromatography purification is recommended for each step to remove unreacted nucleosides and (thio)phosphate donors. Typically, the yields range from 50% to 60% based on 0.05 mmol adenosine derivatives.

In the third step, a 20-mL scintillation vial equipped with a magnetic stir bar was charged with nicotinamide mononucleotide (0.05 mmol, 16.7 mg) and the ATP α S derivative synthesized above. The solids were dissolved in 3.8 mL of reaction buffer (0.1 M, pH 7.4, Tris-HCl), followed by

addition of MgCl_2 stock solution (50 mM, 1 mL, 1.0 equiv.). Then to the stirring reaction mixture was added *Ec*-IPYR (2.5 mg, 0.25 mol%) and *Ec*-NadD mutant (2.5 mg, 0.2 mol %) sequentially, and a small amount of reaction buffer was added to a final volume of 5.0 mL. The reaction mixture was stirred at 1000 rpm at room temperature for 12 hours. Reaction progress was monitored by LC-MS. After most of the substrates were consumed, the vial was cooled in an ice-water bath for 5 minutes. To the vigorously stirring reaction mixture was added 15 mL of methanol (white foam precipitate was often observed), and the mixture was stirred for 2 additional minutes. The reaction mixture was filtered through a pad of celite to remove all precipitate, the celite pad was washed with water (5 mL) twice, water-methanol mixture (1:1, 5 mL) once and methanol (5 mL) once. The filtrate was combined, and the solvent was evaporated overnight under a flow of nitrogen. Crude product was re-dissolved in small amount of water (~ 2 to 3 mL) and purified by ion exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, gradient was specified for each substrate). Fractions containing product (monitored by LC-MS) were combined and the solvent was evaporated under reduced pressure (temp. $\leq 40^\circ\text{C}$). The solid residue was dissolved in minimum amount of water and further purified by C18 reverse phase column using (MeCN/water or MeOH/water, gradient from 0:100 to 100:0). The desired product was collected and concentrated under reduced pressure (temp. $\leq 40^\circ\text{C}$). Lyophilization was applied to remove residual water.

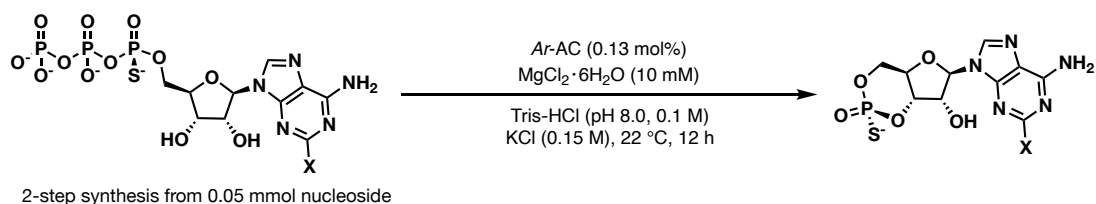
Method G. (Preparation of β -thio NAD^+ analogues)



In the first step, nicotinamide thiophosphate was prepared from nicotinamide riboside (0.05 mmol, 12.8 mg) following **Method A**, in which nucleoside kinase was replaced with *Km*-NRK (5.0 mg, 0.36 mol%). Identical purification was applied and lyophilized nicotinamide thiophosphate was used directly in the second step. The yield of this step ranges from 60% to 70% based on 0.05 mmol nicotinamide riboside.

In the second step, a procedure similar to what was described in **Method F** was employed with the following modifications: *Ec*-NadD mutant was replaced with *Hs*-NMNAT (2.5 mg, 0.16 mol%) and ATP α S was replaced with ATP (0.05 mmol, 27.6 mg). The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 25:75) and then further purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford desired compound after lyophilization.

Method H. (Preparation of cyclic monothiophosphate)



ATP α S and its fluoro derivative were prepared by combining **Method A** and **C** as described previously. Ion exchange chromatography purification is recommended for each step to remove unreacted nucleosides and (thio)phosphate donors. Typically, the yields range from 50% to 60% based on 0.05 mmol adenosine derivatives.

In the third step, a procedure similar to what was described in **Method F** was employed with the following modifications: *Ec*-NadD mutant was replaced with *Ar*-AC (2.5 mg, 0.13 mol%) and ATP α S synthesized was used as the only starting material. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) and then further purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford desired compound after lyophilization.

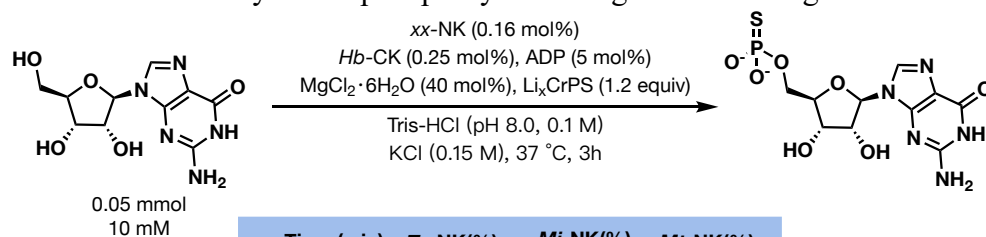
8. Mechanistic studies

8.1 Time course study of different nucleoside kinases

Guanosine was used for time course study with *Ta*-NK, *Mj*-NK and *Mt*-NK following **Method A** (0.05 mmol scale). The loading of 3 nucleoside kinases were all set to 0.16 mol%. At the indicated time point, aliquot of the reaction mixture (500 μ L) was taken out and quenched with methanol (~4 mL) then cooled at 4 °C overnight. The sample was filtered, concentrated and analyzed by NMR with methyl phosphonic acid as internal standard.

Time course study results suggest *Ta*-NK and *Mt*-NK have similar kinetic, while *Mj*-NK has the highest initial reaction rate. They all reach similar conversion and yield after 1 hour, though *Ta*-NK is slightly better than other two at the end of 3h reaction.

Table S13. Time course study of thiophosphorylation of guanosine using different NKs.



Time (min)	<i>Ta</i> -NK(%)	<i>Mj</i> -NK(%)	<i>Mt</i> -NK(%)
0	0	0	0
5	3	43	7
10	11	70	13
15	24	70	21
20	32	70	33
30	44	70	44
60	69	70	70
120	73	71	73
180	80	73	77

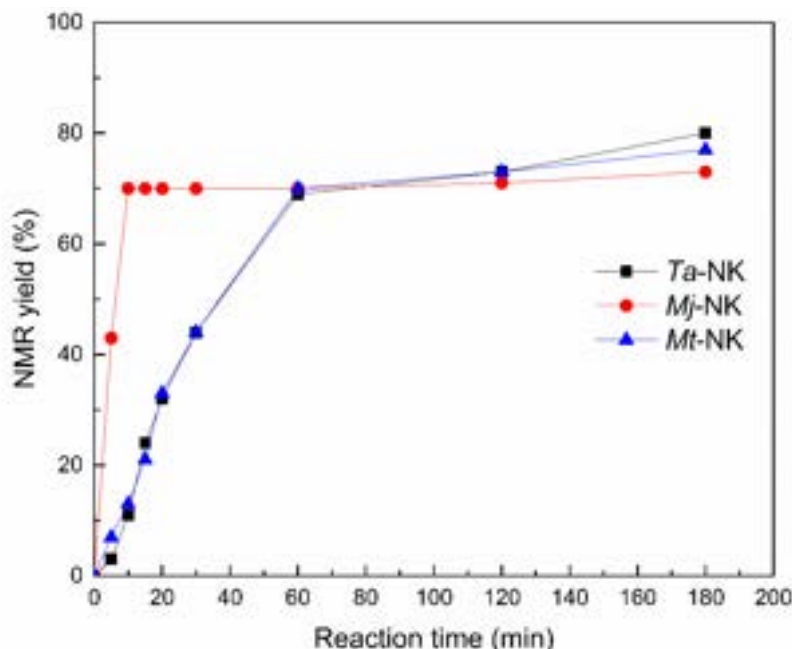


Figure S5. Time course study of thiophosphorylation of guanosine with different nucleoside kinases.

8.2 Reversibility and stability of *Hb*-CK, Li_xCrPS and $\text{ATP}\gamma\text{S}$

We first investigated the stability of ADP, Li_xCrPS and $\text{ATP}\gamma\text{S}$. In a 1.5 mL Eppendorf tube was charged:

1. $\text{ATP}\gamma\text{S}$ (Li^+ salt, 1.0 mg, 1.8 μmol), reaction buffer (200 μL , 0.1 M pH = 8.0 Tris-HCl, 0.15 M KCl, 4 mM MgCl_2).

2. $\text{ATP}\gamma\text{S}$ (Li^+ salt, 1.0 mg, 1.8 μmol), Li_xCrPS (2.6 μmol , 5 μL from 0.52 M stock solution), reaction buffer (195 μL , 0.1 M pH = 8.0 Tris-HCl, 0.15 M KCl, 4 mM MgCl_2).

3. ADP (K^+ salt, 0.8 mg, 1.8 μmol), reaction buffer (200 μL , 0.1 M pH = 8.0 Tris-HCl, 0.15 M KCl, 4 mM MgCl_2).

4. $\text{ATP}\gamma\text{S}$ (Li^+ salt, 1.0 mg, 1.8 μmol), reaction buffer (200 μL , 0.1 M pH = 8.0 Tris-HCl, 0.15 M KCl, 4 mM MgCl_2).

5. $\text{ATP}\gamma\text{S}$ (Li^+ salt, 1.0 mg, 1.8 μmol), D_2O (500 μL)

Tube 1 and 2 were incubated at 37 °C and 500 rpm for 2 hours, tube 3-5 were left at 22 °C for 2 hours. Internal standard (1.8 μmol , methyl phosphonic acid), 300 μL D_2O was added to each tube (except tube 5). The sample was then analyzed by NMR.

Results (Figure S6 and Figure S7):

1. We observed that the signals of β - and γ -phosphorus atom of $\text{ATP}\gamma\text{S}$ become broad when NMR is taken in buffer solution containing Mg^{2+} .

2. In the buffer containing Mg^{2+} , $\text{ATP}\gamma\text{S}$ was rapidly transformed into ADP and lost its terminal thiophosphate (10% $\text{ATP}\gamma\text{S}$ left at 37 °C, 30% $\text{ATP}\gamma\text{S}$ left at 22 °C).

3. $\text{ATP}\gamma\text{S}$ is sensitive to pH change; loss of thiophosphate was observed after addition of methyl phosphonic acid (tube 5). Addition of Li_xCrPS , which is stored in basic solution containing LiOH , also leads to significant change of chemical shifts (tube 2).

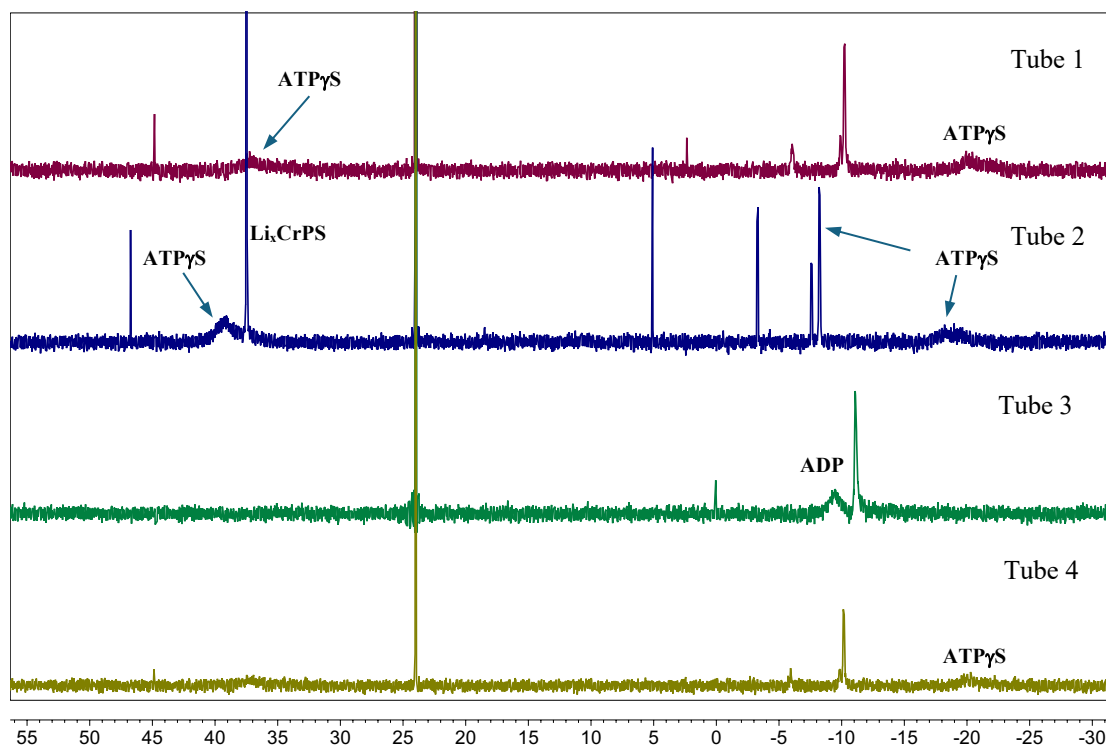


Figure S6. Stability studies of $\text{ATP}\gamma\text{S}$. Methyl phosphonic acid is used as internal standard.

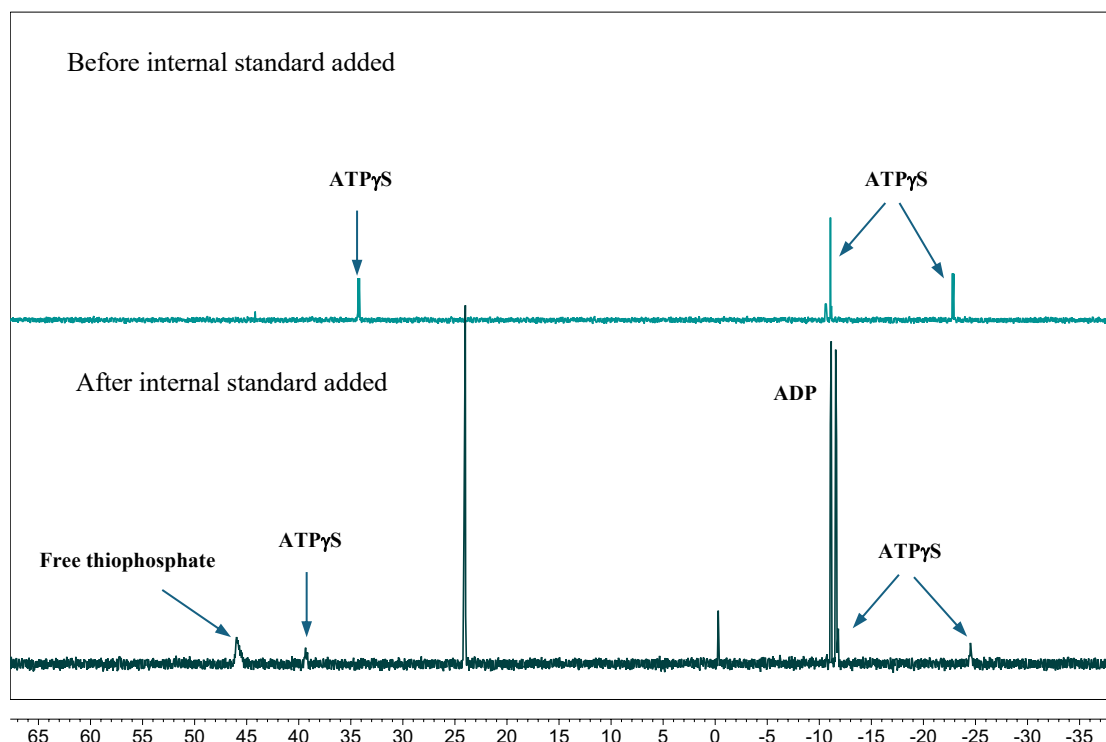


Figure S7. Comparison of ATP γ S before and after addition of methyl phosphonic acid.

We then investigated the reactivity of *Hb*-CK with Li_xCrPS and ATP γ S. In a 1.5 mL Eppendorf tube was charged:

6. ADP (K⁺ salt, 0.8 mg, 1.8 μ mol), Li_xCrPS (2.6 μ mol, 5 μ L from 0.52 M stock solution), *Hb*-CK (0.012 μ mol, 10 μ L from 50 mg/mL stock solution), reaction buffer (185 μ L, 0.1 M pH = 8.0 Tris-HCl, 0.15 M KCl, 4 mM MgCl₂).

7. ATP γ S (Li⁺ salt, 1.0 mg, 1.8 μ mol), creatine (2.6 μ mol, 50 μ L from 52 mM stock solution), *Hb*-CK (0.012 μ mol, 10 μ L from 50 mg/mL stock solution), reaction buffer (140 μ L, 0.1 M pH = 8.0 Tris-HCl, 0.15 M KCl, 4 mM MgCl₂).

The mixture was incubated at 37 °C and 500 rpm for 2 hours, 300 μ L D₂O was added to each tube. The sample was then analyzed by NMR.

Results (Figure S8 and Figure S9):

The interconversion among ADP + Li_xCrPS and ATP γ S + creatine in the presence of creatine kinase is reversible. The enzymatic reaction can reach similar equilibrium regardless of whether it was started from the forward or reverse reaction.

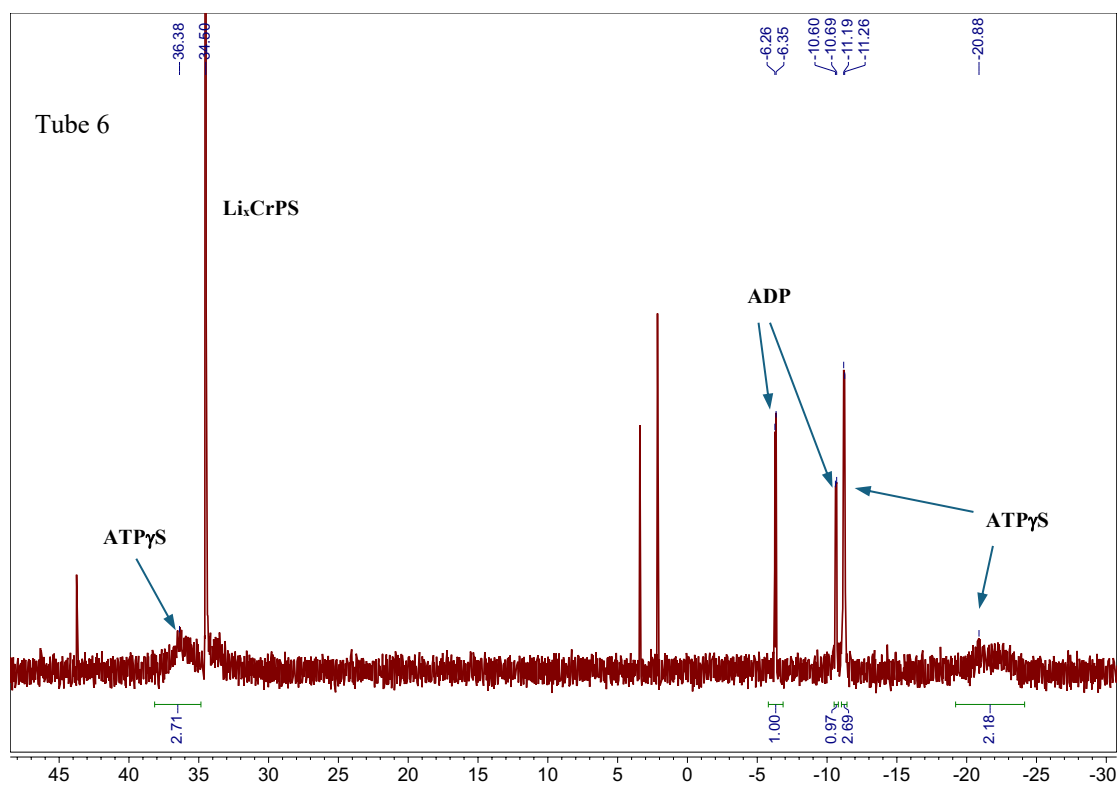


Figure S8. Reversibility studies of ATP_γS regeneration system starting from ADP+ Li_xCrPS.

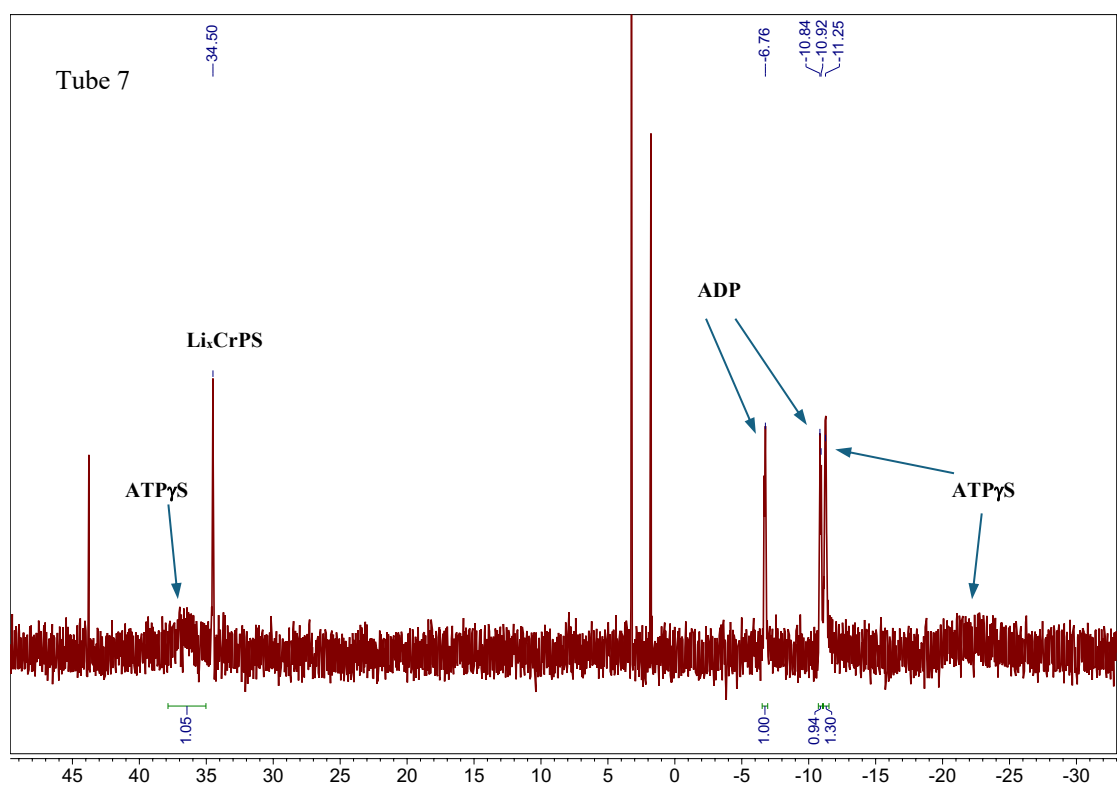
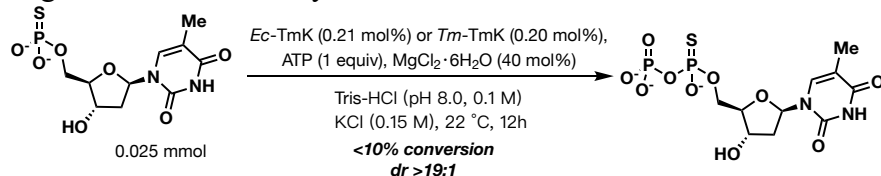


Figure S9. Reversibility studies of ATP_γS regeneration system starting from ATP_γS+creatine.

8.3 Stereochemical origin of NDP

Substrate with high diastereoselectivity



We attempted to investigate the origin of the diastereoselectivity by analyzing the diphosphate intermediate. In a 20-mL scintillation vial equipped with a magnetic stir bar was charged with ATP (Na⁺ salt, hydrate, 13.8 mg, 25 μmol, 1.0 equiv) and thymidine mono thiophosphate synthesized following **Method A** (25 μmol scale). The solids were dissolved in 2.2 mL of reaction buffer (0.1 M Tris-HCl, pH 8.0 and 0.15 M KCl), followed by addition of MgCl₂ stock solution (50 mM, 200 μL, 0.4 equiv.). Then to the stirring reaction mixture was added *Ec*-TmK (2.5 mg, 0.21mol%) or *Tm*-TmK (2.5 mg, 0.20 mol%). The reaction mixture was stirred at room temperature for 12 hours. The reaction was quenched by addition of methanol and crude product was purified using ion exchange chromatography. Fraction containing diphosphate product was collected, concentrated and dried with lyophilization. The sample was then analyzed by NMR.

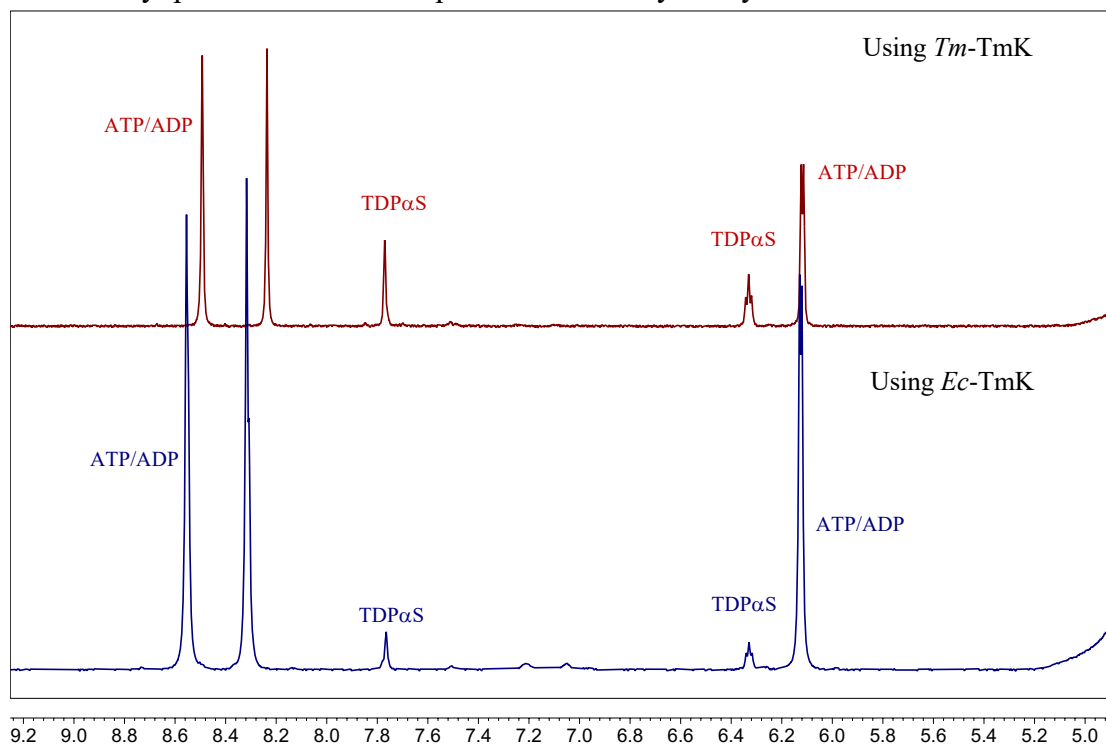
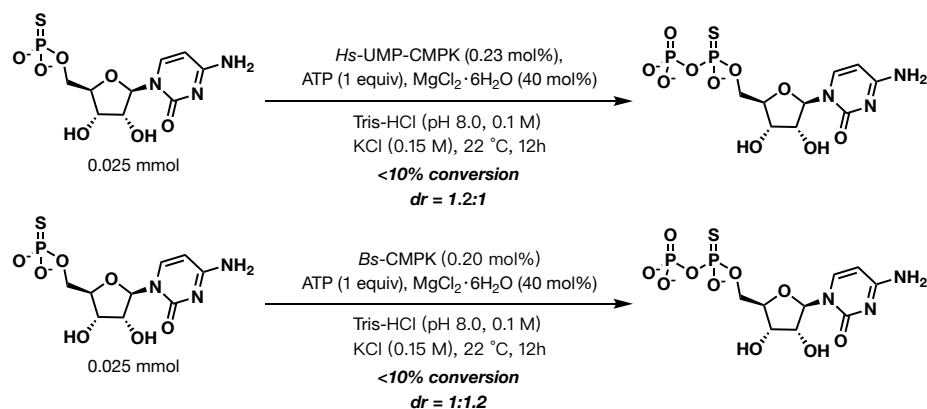


Figure S10. NMR of isolated TDPαS using different monophosphate kinases. High diastereoselectivity was observed for each enzyme.

Substrate with low diastereoselectivity



On the other hand, we also investigated the diastereoselectivity of two cytidine monophosphate kinases using cytidine mono thiophosphate synthesized following **Method A** (25 μmol scale). In contrast, low diastereoselectivity was observed. We think the final diastereoselectivity observed in NTP α S synthesis is mostly governed by the diastereoselectivity of the monophosphate kinase though acetate kinase may have some minor effects on the final diastereoselectivity.

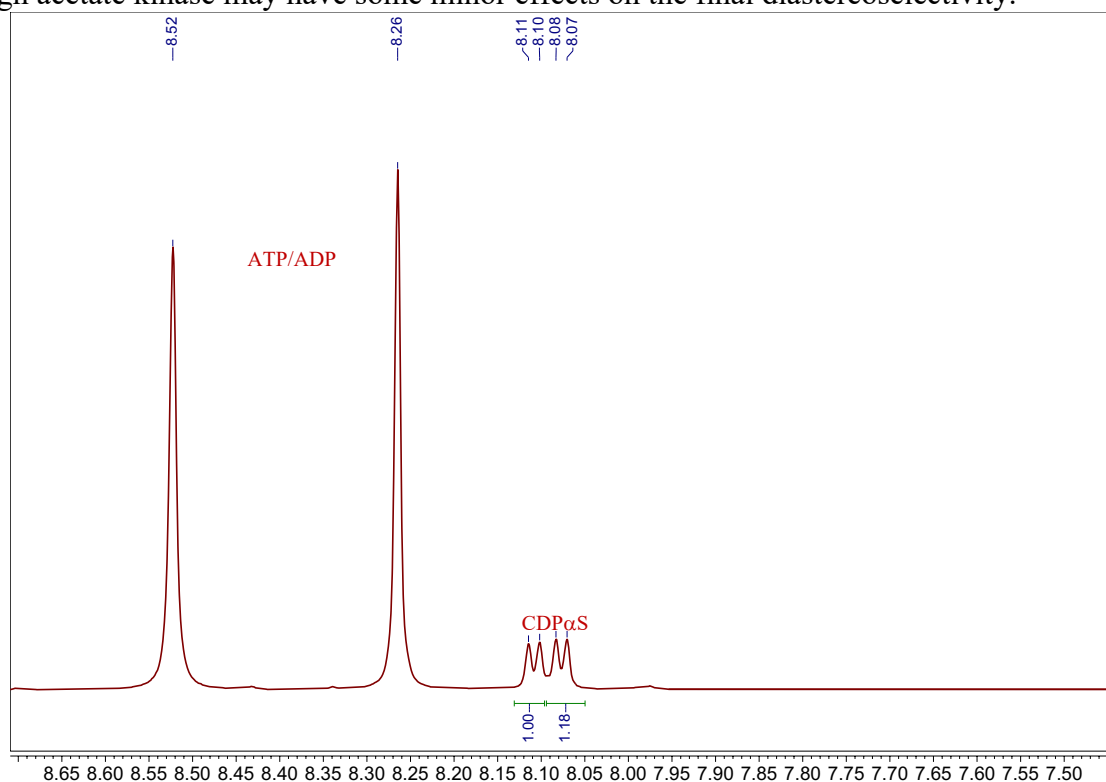


Figure S11. NMR of isolated CDP α S using *Hs*-UMP-CMPK.

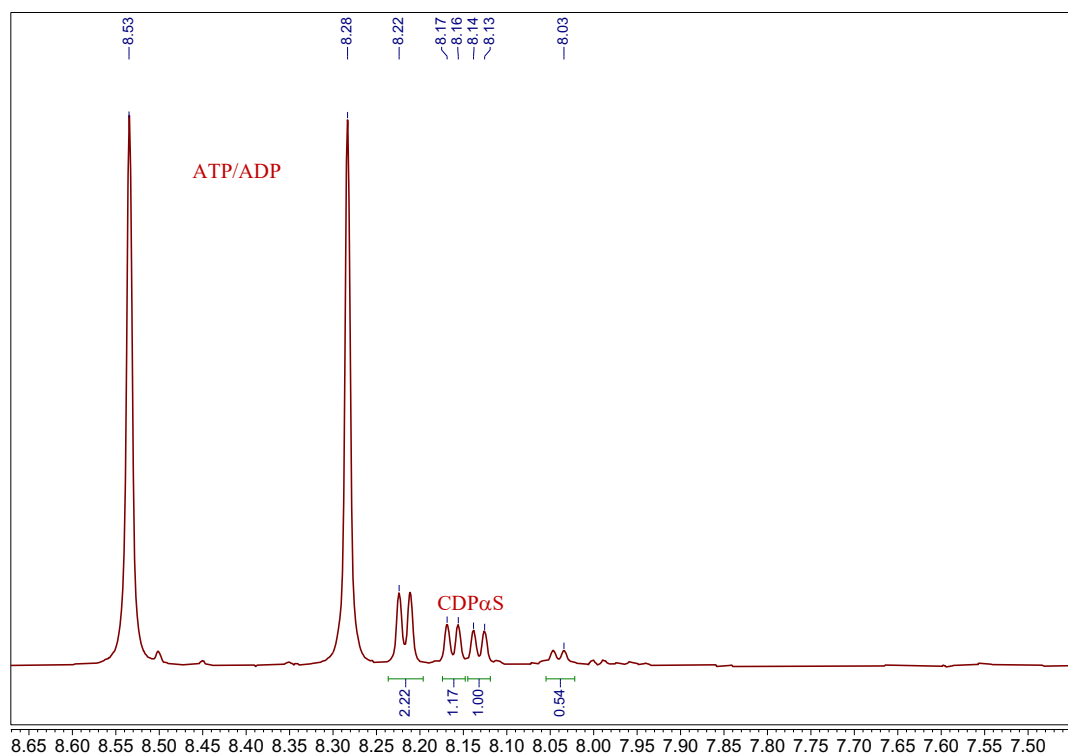


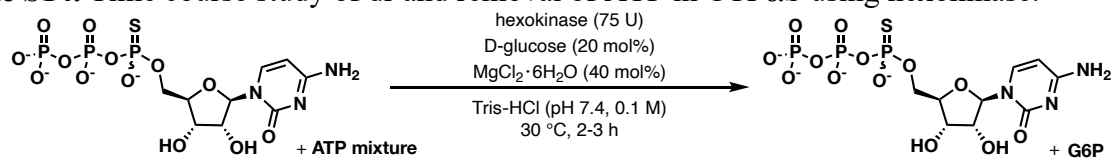
Figure S12. NMR of isolated CDP α S using *Bs*-CMPK. CMPS and CTP α S were also observed.

8.4 Time course study of hexokinase to remove ATP and improve diastereoselectivity

D-glucose (1.8 mg, 0.01 mmol, 0.2 equiv.) and the cytidine triphosphoro- α -thioate product mixture prepared from cytidine following **Method C** (0.05 mmol scale, roughly 60% crude product and 8% ATP impurity) were dissolved in 4.5 mL of reaction buffer (0.1 M, pH 7.4, Tris-HCl), followed by the addition of MgCl₂ stock solution (50 mM, 400 μ L, 0.4 equiv.). Then to the stirring reaction mixture was added hexokinase (75 U). The reaction mixture was stirred at 1000 rpm in a heating block with the temperature set at 30 °C for 3 hours. During the 3 hours, at certain time point, aliquot of the reaction mixture (500 μ L) was taken out and quenched with methanol (~4 mL) then cooled down at 4 °C overnight. The sample was filtered and analyzed by NMR with methyl phosphonic acid as internal standard.

Time course study of hexokinase with CTP α S and ATP mixture suggested that ATP is firstly consumed and the minor isomer CTP α S can be slowly consumed after ATP. The gradual increase of the dr supported our original hypothesis.

Table S14. Time course study of dr and removal of ATP in CTP α S using hexokinase.



Time (min)	CTP α S (major)	CTP α S (minor)	dr	ATP
0	62%	23%	2.5:1	8%
5	58%	14%	4.5:1	<2%
10	58%	12%	4.8:1	<2%
15	61%	12%	5.1:1	<2%
20	60%	11%	5.5:1	<2%
30	56%	10%	5.5:1	<2%
40	59%	9%	6.5:1	<2%
50	60%	9%	6.7:1	<2%
60	58%	8%	7.3:1	<2%
180	60%	6%	10:1	<2%

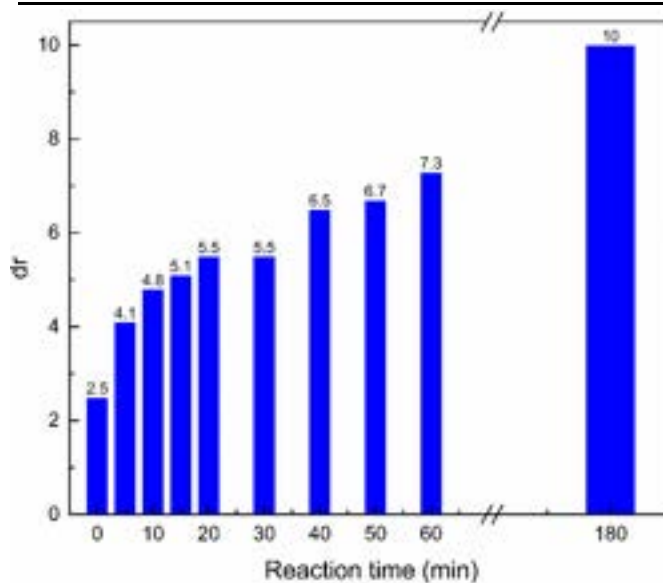


Figure S13. Time course study of dr change for CTP α S using hexokinase.

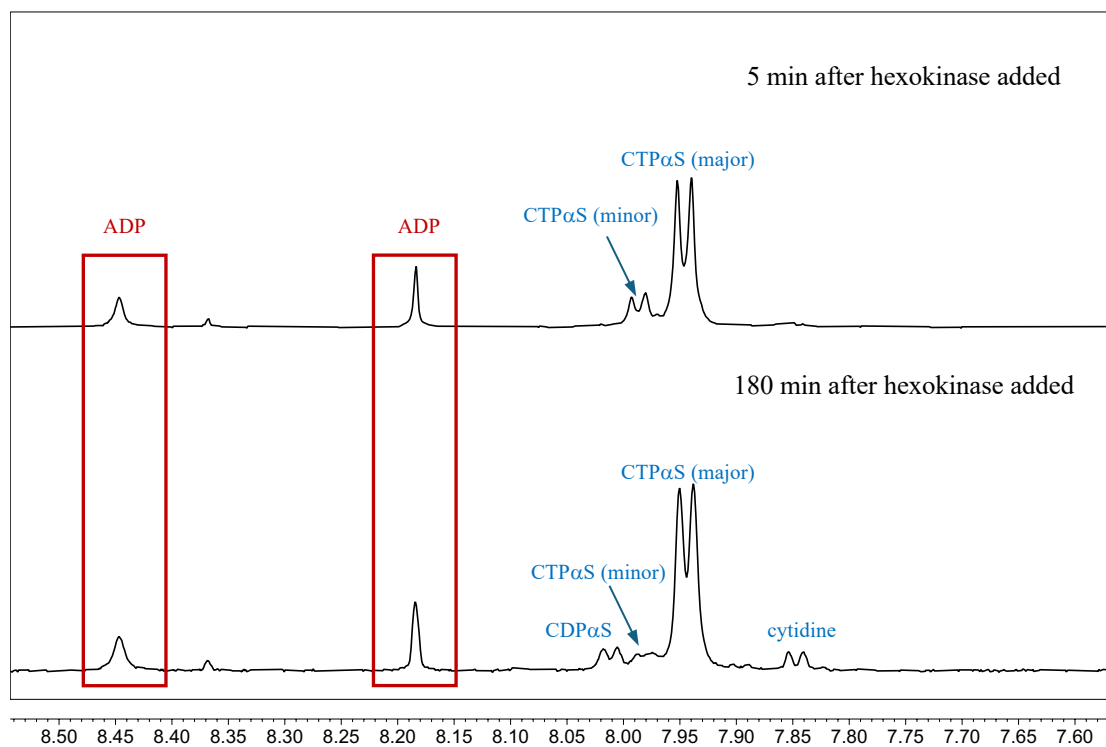
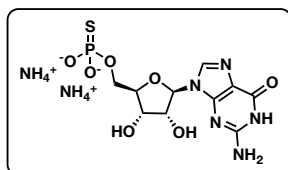


Figure S14. NMR of dr change of CTP α S after hexokinase was added t = 5 min (top) and t = 180 min (bottom).

9. Characterization of Products

9.1 Mono thiophosphate of nucleosides

Compound 2



5'-*O*-guanosine-diammonium monophosphorothioate

Compound **2** was obtained according to general procedure (**Method A**) from guanosine (0.05 mmol, 14.1 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) to afford 18.5 mg of the title compound after lyophilization (**Yield = 90%**).

Physical state: white solid

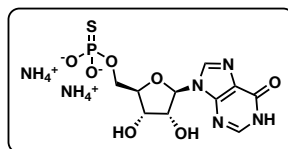
¹H NMR (600 MHz, D₂O): δ 8.20 (s, 1H), 5.93 (d, *J* = 6.0 Hz, 1H), 4.76 (t, *J* = 5.6 Hz, 1H), 4.50 (dd, *J* = 5.1, 3.4 Hz, 1H), 4.36 (dt, *J* = 4.7, 2.3 Hz, 1H), 4.18 – 4.09 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 158.9, 153.9, 151.7, 137.7, 116.0, 86.8, 84.1 (d, *J* = 9.4 Hz), 73.9, 70.6, 64.4 (d, *J* = 5.0 Hz).

³¹P NMR (243 MHz, D₂O): δ 48.4.

HRMS [ESI] C₁₀H₁₄N₅O₇PS ([M-H]⁻) calculated 378.0279 found 378.0279.

Compound 3



5'-*O*-inosine-diammonium monophosphorothioate

Compound **3** was obtained according to general procedure (**Method A**) from inosine (0.05 mmol, 13.4 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) to afford 14.1 mg of the title compound after lyophilization (**Yield = 71%**).

Physical state: white solid

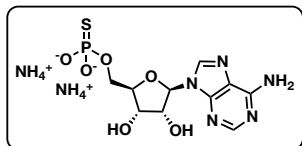
¹H NMR (600 MHz, D₂O): δ 8.59 (s, 1H), 8.22 (s, 1H), 6.15 (d, *J* = 5.8 Hz, 1H), 4.54 (dd, *J* = 5.1, 3.5 Hz, 1H), 4.41 (tt, *J* = 4.8, 2.3 Hz, 1H), 4.18 – 4.11 (m, 2H). (One signal overlaps with D₂O around δ 4.79 ppm).

¹³C NMR (151 MHz, D₂O): δ 153.5, 149.9, 148.8, 140.9, 118.5, 87.3, 84.2 (d, *J* = 9.7 Hz), 74.6, 70.6, 64.4 (d, *J* = 5.2 Hz).

³¹P NMR (243 MHz, D₂O): δ 48.0.

HRMS [ESI] C₁₀H₁₃N₄O₇PS ([M-H]⁻) calculated 363.0170 found 363.0169.

Compound 4



5'-O-adenosine-diammonium monophosphorothioate

Compound **4** was obtained according to general procedure (**Method A**) from adenosine (0.05 mmol, 13.4 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) to afford 15.3 mg of the title compound after lyophilization (**Yield = 77%**).

Physical state: white solid

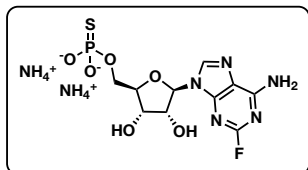
¹H NMR (600 MHz, D₂O): δ 8.63 (s, 1H), 8.31 (s, 1H), 6.16 (d, *J* = 5.6 Hz, 1H), 4.77 (t, *J* = 5.3 Hz, 1H, overlaps with D₂O), 4.54 (dd, *J* = 5.1, 3.7 Hz, 1H), 4.42 (dq, *J* = 5.0, 2.9 Hz, 1H), 4.23 – 4.14 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 158.6, 148.9, 146.1, 139.8, 123.7, 87.5, 84.2 (d, *J* = 9.4 Hz), 74.6, 70.5, 64.5 (d, *J* = 5.4 Hz).

³¹P NMR (243 MHz, D₂O): δ 48.0.

HRMS [ESI] C₁₀H₁₄N₅O₆PS ([M-H]⁻) calculated 362.0330 found 362.0330.

Compound 5



5'-O-2-fluoroadenosine-diammonium monophosphorothioate

Compound **5** was obtained according to general procedure (**Method A**) from 2-F-adenosine (0.05 mmol, 14.3 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) to afford 14.3 mg of the title compound after lyophilization (**Yield = 71%**).

Physical state: white solid

¹H NMR (600 MHz, D₂O): δ 8.54 (s, 1H), 6.02 (d, *J* = 5.8 Hz, 1H), 4.76 (t, *J* = 5.4 Hz, 1H), 4.52 (dd, *J* = 5.1, 3.5 Hz, 1H), 4.40 (dt, *J* = 4.9, 2.4 Hz, 1H), 4.14 (dd, *J* = 6.0, 3.0 Hz, 2H).

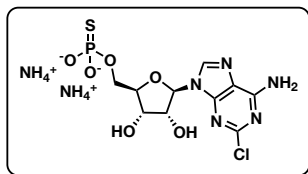
¹³C NMR (151 MHz, D₂O): δ 159.7 (d, *J* = 212.0 Hz), 157.2 (d, *J* = 19.8 Hz), 150.6, 140.2, 116.9, 87.0, 84.3 (d, *J* = 9.7 Hz), 74.5, 70.7, 64.2 (d, *J* = 4.9 Hz). *J*_{CF} in title compound is consistent with data of unphosphorylated compound reported in a previous report⁷.

³¹P NMR (243 MHz, D₂O): δ 47.9.

¹⁹F NMR (565 MHz, D₂O): δ -52.9.

HRMS [ESI] C₁₀H₁₃FN₅O₆PS ([M-H]⁻) calculated 380.0235 found 380.0223.

Compound 6



5'-O-2-chloroadenosine-diammonium monophosphorothioate

Compound **6** was obtained according to general procedure (**Method A**) from 2-Cl-adenosine (0.05 mmol, 15.1 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) to afford 19.8 mg of the title compound after lyophilization (**Yield = 92%**).

Physical state: white solid

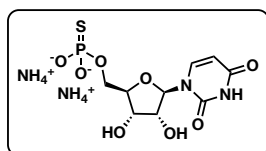
¹H NMR (600 MHz, D₂O): δ 8.60 (s, 1H), 6.02 (d, *J* = 5.7 Hz, 1H), 4.75 (t, *J* = 5.5 Hz, 1H), 4.55 – 4.50 (m, 1H), 4.43 – 4.37 (m, 1H), 4.13 – 4.06 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 156.1, 153.7, 150.1, 140.4, 117.5, 87.0, 84.5 (d, *J* = 9.4 Hz), 74.7, 70.7, 63.9 (d, *J* = 4.8 Hz).

³¹P NMR (243 MHz, D₂O): δ 45.2

HRMS [ESI] C₁₀H₁₃ClN₅O₆PS ([M-H]⁻) calculated 395.9940 found 395.9941.

Compound 7



5'-O-uridine-diammonium monophosphorothioate

Compound **7** was obtained according to general procedure (**Method A**) from uridine (0.05 mmol, 12.2 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) to afford 16.3 mg of the title compound after lyophilization (**Yield = 87%**).

Physical state: white solid

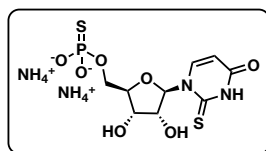
¹H NMR (600 MHz, D₂O): δ 8.19 (d, *J* = 8.1 Hz, 1H), 6.01 (d, *J* = 5.5 Hz, 1H), 5.99 (d, *J* = 8.1 Hz, 1H), 4.45 – 4.41 (m, 1H), 4.40 – 4.37 (m, 1H), 4.32 – 4.27 (m, 1H), 4.12 – 4.01 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 166.4, 160.2, 152.0, 142.3, 102.7, 88.1, 84.2 (d, *J* = 9.5 Hz), 74.0, 70.2, 63.5 (d, *J* = 5.2 Hz).

³¹P NMR (243 MHz, D₂O): δ 43.2.

HRMS [ESI] C₉H₁₃N₂O₈PS ([M-H]⁻) calculated 339.0058 found 339.0059.

Compound 8



5'-O-2-thiouridine-diammonium monophosphorothioate

Compound **8** was obtained according to general procedure (**Method A**) from 2-thiouridine (0.05 mmol, 13.0 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) to afford 18.3 mg of the title compound after lyophilization (**Yield = 93%**).

Physical state: white solid

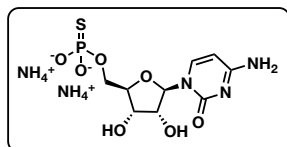
¹H NMR (600 MHz, D₂O): δ 8.29 (d, *J* = 8.2 Hz, 1H), 6.63 (d, *J* = 2.9 Hz, 1H), 6.23 (d, *J* = 8.2 Hz, 1H), 4.44 – 4.40 (m, 1H), 4.35 – 4.27 (m, 3H), 4.19 – 4.13 (m, 1H).

¹³C NMR (151 MHz, D₂O): δ 175.9, 162.7, 142.2, 106.8, 93.1, 82.9 (d, *J* = 9.5 Hz), 74.7, 68.6, 63.1 (d, *J* = 5.1 Hz).

³¹P NMR (243 MHz, D₂O): δ 49.8.

HRMS [ESI] C₉H₁₃N₂O₇PS₂ ([M-H]⁻) calculated 354.9829 found 354.9830.

Compound 9



5'-O-cytidine-diammonium monophosphorothioate

Compound **9** was obtained according to general procedure (**Method A**) from cytidine (0.05 mmol, 12.2 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) to afford 15.4 mg of the title compound after lyophilization (**Yield = 83%**). This compound contains 5% AMP impurity.

Physical state: white solid

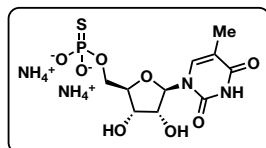
¹H NMR (600 MHz, D₂O): δ 8.29 (d, *J* = 8.2 Hz, 1H), 6.63 (d, *J* = 2.9 Hz, 1H), 6.23 (d, *J* = 8.2 Hz, 1H), 4.44 – 4.40 (m, 1H), 4.35 – 4.27 (m, 3H), 4.19 – 4.13 (m, 1H).

¹³C NMR (151 MHz, D₂O): δ 163.6, 154.4, 142.8, 96.1, 89.2, 83.3 (d, *J* = 9.7 Hz), 74.4, 69.5, 63.5 (d, *J* = 4.8 Hz).

³¹P NMR (243 MHz, D₂O): δ 47.3.

HRMS [ESI] C₉H₁₄N₃O₇PS ([M-H]⁻) calculated 338.0217 found 338.0219.

Compound 10



5'-O-5-methyluridine-diammonium monophosphorothioate

Compound **10** was obtained according to general procedure (**Method A**) from 5-methyluridine (0.05 mmol, 12.2 mg) using *Ta*-NK (5.0 mg, 0.32 mol%) as nucleoside kinase. The crude product

was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 20:80) to afford 8.3 mg of the title compound after lyophilization (**Yield = 43%**).

Physical state: white solid

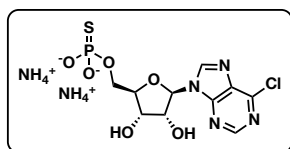
^1H NMR (600 MHz, D_2O): δ 7.93 (s, 1H), 6.02 (d, J = 6.0 Hz, 1H), 4.47 – 4.42 (m, 1H), 4.41 – 4.36 (m, 1H), 4.30 – 4.26 (m, 1H), 4.11 – 4.02 (m, 2H), 1.97 (s, 3H).

^{13}C NMR (151 MHz, D_2O): δ 166.6, 152.1, 137.5, 112.0, 87.7, 84.2 (d, J = 9.6 Hz), 73.6, 70.5, 63.8 (d, J = 4.6 Hz), 11.7.

^{31}P NMR (243 MHz, D_2O): δ 43.1.

HRMS [ESI] $\text{C}_{10}\text{H}_{15}\text{N}_2\text{O}_8\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 353.0214 found 353.0213.

Compound 11



5'-O-6-chloroadenosine-diammonium monophosphorothioate

Compound **11** was obtained according to general procedure (**Method A**) from 6-chloroadenosine (0.05 mmol, 14.3 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 25:75) to afford 13.1 mg of the title compound after lyophilization (**Yield = 63%**). This compound is unstable during lyophilization to decompose to afford small amount (~3%) unidentified impurity.

Physical state: white solid

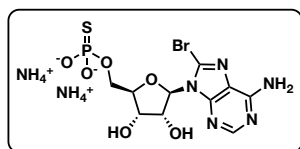
^1H NMR (600 MHz, D_2O): δ 9.05 (s, 1H), 8.77 (s, 1H), 6.28 (d, J = 5.4 Hz, 1H), 4.84 (dd, J = 5.2, 5.2 Hz, 1H), 4.57 (dd, J = 5.0, 3.7 Hz, 1H), 4.46 – 4.42 (m, 1H), 4.20 – 4.11 (m, 2H).

^{13}C NMR (151 MHz, D_2O): δ 151.9, 151.4, 150.1, 145.6, 131.2, 87.9, 84.6 (d, J = 9.3 Hz), 74.8, 70.7, 64.0 (d, J = 4.9 Hz).

^{31}P NMR (243 MHz, D_2O): δ 46.4.

HRMS [ESI] $\text{C}_{10}\text{H}_{12}\text{ClN}_4\text{O}_6\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 380.9831 found 380.9834.

Compound 12



5'-O-8-bromoadenosine-diammonium monophosphorothioate

Compound **12** was obtained according to general procedure (**Method A**) from 8-bromoadenosine (0.05 mmol, 17.3 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 30:70) to afford 20.0 mg of the title compound after lyophilization (**Yield = 84%**).

Physical state: white solid

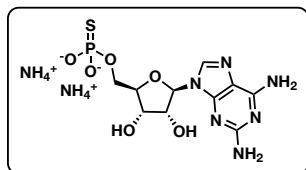
¹H NMR (600 MHz, D₂O): δ 8.17 (s, 1H), 6.09 (d, *J* = 5.8 Hz, 1H), 5.28 (dd, *J* = 5.9, 5.9 Hz, 1H), 4.66 (dd, *J* = 5.9, 4.6 Hz, 1H), 4.31 (dt, *J* = 5.8, 4.4 Hz, 1H), 4.26 – 4.14 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 153.2, 151.5, 150.0, 128.5, 119.0, 89.5, 83.6 (d, *J* = 9.1 Hz), 71.0, 69.7, 64.4 (d, *J* = 4.9 Hz).

³¹P NMR (243 MHz, D₂O): δ 48.9.

HRMS [ESI] C₁₀H₁₃BrN₅O₆PS ([M-H]⁻) calculated 439.9435 found 439.9432.

Compound 13



5'-O-2-aminoadenosine-diammonium monophosphorothioate

Compound **13** was obtained according to general procedure (**Method A**) from 2-aminoadenosine (0.05 mmol, 14.1 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 30:70) to afford 21.0 mg of the title compound after lyophilization (**Yield = 99%**).

Physical state: white solid

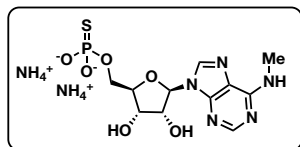
¹H NMR (600 MHz, D₂O): δ 8.25 (s, 1H), 5.92 (d, *J* = 5.9 Hz, 1H), 4.72 (dd, *J* = 5.5, 5.5 Hz, 1H), 4.57 – 4.51 (m, 1H), 4.40 – 4.33 (m, 1H), 4.17 – 4.07 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 157.0, 154.0, 150.1, 138.5, 112.3, 86.9, 84.3 (d, *J* = 9.3 Hz), 74.0, 70.7, 64.0 (d, *J* = 4.5 Hz).

³¹P NMR (243 MHz, D₂O): δ 45.2.

HRMS [ESI] C₁₀H₁₅N₆O₆PS ([M-H]⁻) calculated 377.0439 found 377.0438.

Compound 14



5'-O-6-methylaminoadenosine-diammonium monophosphorothioate

Compound **14** was obtained according to general procedure (**Method A**) from 6-methylaminoadenosine (0.05 mmol, 14.1 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 30:70) to afford 17.3 mg of the title compound after lyophilization (**Yield = 86%**).

Physical state: white solid

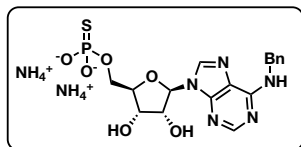
¹H NMR (600 MHz, D₂O): δ 8.53 (s, 1H), 8.14 (s, 1H), 6.07 (d, *J* = 5.8 Hz, 1H), 4.76 (dd, *J* = 5.5, 5.5 Hz, 1H), 4.52 (dd, *J* = 4.2, 4.2 Hz, 1H), 4.41 – 4.37 (m, 1H), 4.15 – 4.07 (m, 2H), 3.03 (s, 3H).

¹³C NMR (151 MHz, D₂O): δ 154.4, 152.1, 147.4, 139.5, 118.6, 86.9, 84.3 (d, *J* = 9.7 Hz), 74.6, 70.7, 64.1 (d, *J* = 4.7 Hz), 27.3.

³¹P NMR (243 MHz, D₂O): δ 46.3.

HRMS [ESI] C₁₁H₁₆N₅O₆PS ([M-H]⁻) calculated 376.0486 found 376.0488.

Compound 15



5'-O-6-benzylaminoadenosine-diammonium monophosphorothioate

Compound **15** was obtained according to general procedure (**Method A**) from 6-benzylaminoadenosine **S15** (0.05 mmol, 17.9 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 30:70) to afford 16.4 mg of the title compound after lyophilization (**Yield = 67%**).

Physical state: white solid

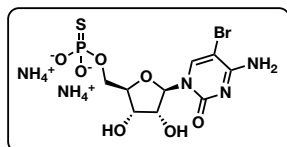
¹H NMR (600 MHz, D₂O): δ 8.58 (s, 1H), 8.16 (s, 1H), 7.37 – 7.30 (m, 4H), 7.29 – 7.24 (m, 1H), 6.10 (d, *J* = 5.7 Hz, 1H), 4.76 – 4.71 (m, 3H, partially overlaps with D₂O), 4.55 – 4.49 (m, 1H), 4.42 – 4.37 (m, 1H), 4.17 – 4.05 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 154.2, 152.5, 148.2 (weak), 139.6, 138.2, 128.6, 127.3, 126.9, 118.8 (weak), 86.9, 84.3 (d, *J* = 9.3 Hz), 74.6, 70.7, 64.1, 64.13 (d, *J* = 4.9 Hz), 43.9 (weak).

³¹P NMR (243 MHz, D₂O): δ 46.4.

HRMS [ESI] C₁₇H₂₀N₅O₆PS ([M-H]⁻) calculated 452.0799 found 452.0796.

Compound 16



5'-O-5-bromocytidine-diammonium monophosphorothioate

Compound **16** was obtained according to general procedure (**Method A**) from 5-bromocytidine (0.05 mmol, 16.1 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) to afford 12.2 mg of the title compound after lyophilization (**Yield = 56%**).

Physical state: white solid

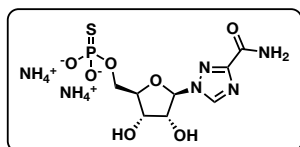
¹H NMR (600 MHz, D₂O): δ 8.29 (s, 1H), 5.96 (d, *J* = 4.6 Hz, 1H), 4.34 (dt, *J* = 14.4, 5.1 Hz, 2H), 4.30 – 4.24 (m, 1H), 4.13 – 4.03 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 163.0, 156.6, 142.1, 89.5, 89.4, 83.5 (d, *J* = 9.6 Hz), 74.3, 69.8, 63.6 (d, *J* = 4.7 Hz).

³¹P NMR (243 MHz, D₂O): δ 43.2.

HRMS [ESI] C₉H₁₃BrN₃O₇PS ([M-H]⁻) calculated 415.9322 found 415.9321.

Compound 17



5'-*O*-ribavirin-diammonium monophosphorothioate

Compound **17** was obtained according to general procedure (**Method A**) from ribavirin (0.05 mmol, 12.2 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) to afford 13.8 mg of the title compound after lyophilization (**Yield = 71%**). This compound contains 3% of AMP impurity.

Physical state: white solid

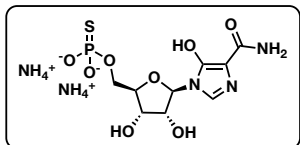
¹H NMR (600 MHz, D₂O): δ 8.91 (s, 1H), 6.04 (d, *J* = 4.2 Hz, 1H), 4.72 (dd, *J* = 4.6 Hz, 4.6 Hz, 1H), 4.55 (dd, *J* = 4.8 Hz, 4.8 Hz, 1H), 4.41 – 4.36 (m, 1H), 4.16 – 4.03 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 162.8, 156.2, 145.8, 92.1, 84.4 (d, *J* = 9.6 Hz), 74.8, 70.4, 64.0 (d, *J* = 4.9 Hz).

³¹P NMR (243 MHz, D₂O): δ 46.0.

HRMS [ESI] C₈H₁₃N₄O₇PS ([M-H]⁻) calculated 339.0170 found 339.0168.

Compound 18



5'-*O*-mizoribine-diammonium monophosphorothioate

Compound **18** was obtained according to general procedure (**Method A**) from mizoribine (0.05 mmol, 13.0 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) to afford 8.2 mg of the title compound after lyophilization (**Yield = 42%**).

Physical state: white solid

¹H NMR (600 MHz, D₂O): δ 8.55 (s, 1H), 5.82 (d, *J* = 4.6 Hz, 1H), 4.58 – 4.53 (m, 1H), 4.50 – 4.46 (m, 1H), 4.34 – 4.30 (m, 1H), 4.13 – 4.02 (m, 2H).

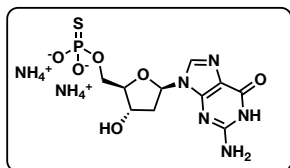
¹³C NMR (151 MHz, D₂O): δ 164.5, 155.7, 126.8, 100.8, 86.2, 83.9 (d, *J* = 9.5 Hz), 74.8, 70.0, 63.1 (d, *J* = 4.7 Hz).

³¹P NMR (243 MHz, D₂O): δ 43.3.

HRMS [ESI] C₉H₁₄N₃O₈PS ([M-H]⁻) calculated 354.0167 found 354.0165.

9.2 Mono thiophosphate of deoxynucleosides and derivatives

Compound 19



5'-O-deoxyguanosine-diammonium monophosphorothioate

Compound **19** was obtained according to general procedure (**Method A**) from deoxyguanosine (0.05 mmol, 13.4 mg) using *Ag*-dNK (2.5 mg, 0.18 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 30:70) to afford 15.5 mg of the title compound after lyophilization (**Yield** = **78%**).

Physical state: white solid

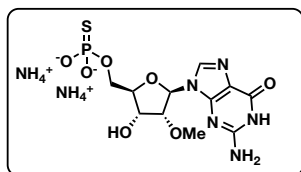
^1H NMR (600 MHz, D_2O): δ 8.18 (s, 1H), 6.30 (t, J = 6.9 Hz, 1H), 4.72 (dt, J = 6.1, 3.1 Hz, 1H), 4.30 – 4.24 (m, 1H), 4.10 (dd, J = 6.5, 3.9 Hz, 2H), 2.78 (ddd, J = 13.8, 7.5, 6.0 Hz, 1H), 2.53 (ddd, J = 14.0, 6.4, 3.3 Hz, 1H).

^{13}C NMR (151 MHz, D_2O): δ 158.6, 153.8, 151.1, 137.5, 115.7, 85.8 (d, J = 9.6 Hz), 83.7, 71.5, 64.9 (d, J = 5.2 Hz), 38.9.

^{31}P NMR (243 MHz, D_2O): δ 50.4.

HRMS [ESI] $\text{C}_{10}\text{H}_{14}\text{N}_5\text{O}_6\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 362.0330 found 362.0329.

Compound 20



2'-O-methylguanosine-5'-O-diammonium monophosphorothioate

Compound **20** was obtained according to general procedure (**Method A**) from 2'-O-methylguanosine (0.05 mmol, 14.9 mg) using *Ag*-dNK (2.5 mg, 0.18 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 30:70) to afford 8.9 mg of the title compound after lyophilization (**Yield** = **42%**).

Physical state: white solid

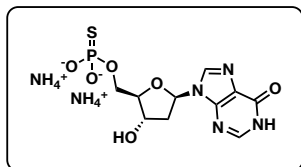
^1H NMR (600 MHz, D_2O): δ 8.23 (s, 1H), 6.01 (d, J = 6.0 Hz, 1H), 4.70 – 4.65 (m, 1H), 4.53 – 4.48 (m, 1H), 4.39 – 4.35 (m, 1H), 4.20 – 4.12 (m, 2H), 3.47 (s, 3H).

^{13}C NMR (151 MHz, D_2O): δ 158.9, 154.0, 151.6, 137.7, 116.0, 85.4, 84.58 (d, J = 9.3 Hz), 82.7, 69.1, 64.58 (d, J = 5.2 Hz), 58.1.

^{31}P NMR (243 MHz, D_2O): δ 50.2.

HRMS [ESI] $\text{C}_{11}\text{H}_{16}\text{N}_5\text{O}_7\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 392.0435 found 392.0435.

Compound 21



5'-*O*-deoxyinosine-diammonium monophosphorothioate

Compound **21** was obtained according to general procedure (**Method A**) from deoxyinosine (0.05 mmol, 12.6 mg) using *Ag*-dNK (2.5 mg, 0.18 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 30:70) to afford 14.7 mg of the title compound after lyophilization (**Yield** = 77%).

Physical state: white solid

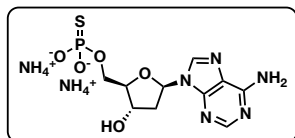
¹H NMR (600 MHz, D₂O): δ 8.49 (s, 1H), 8.19 (s, 1H), 6.50 (t, *J* = 6.8 Hz, 1H), 4.77 – 4.73 (m, 1H), 4.33 – 4.28 (m, 1H), 4.15 – 4.06 (m, 2H), 2.87 – 2.79 (m, 1H), 2.61 (ddd, *J* = 14.0, 6.4, 3.5 Hz, 1H).

¹³C NMR (151 MHz, D₂O): δ 158.5, 148.4, 145.9, 139.9, 123.5, 86.1 (d, *J* = 9.4 Hz), 84.2, 71.4, 64.7 (d, *J* = 5.0 Hz), 39.3.

³¹P NMR (243 MHz, D₂O): δ 49.5.

HRMS [ESI] C₁₀H₁₃N₄O₆PS ([M-H]⁻) calculated 347.0221 found 347.0222.

Compound 22



5'-*O*-deoxyadenosine-diammonium monophosphorothioate

Compound **22** was obtained according to general procedure (**Method A**) from deoxyinosine (0.05 mmol, 12.6 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) to afford 15.1 mg of the title compound after lyophilization (**Yield** = 79%).

Physical state: white solid

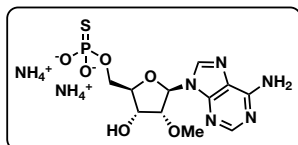
¹H NMR (600 MHz, D₂O): δ 8.59 (s, 1H), 8.25 (s, 1H), 6.52 (t, *J* = 6.9 Hz, 1H), 4.77 – 4.74 (m, 1H), 4.33 – 4.28 (m, 1H), 4.12 – 4.01 (m, 2H), 2.84 (ddd, *J* = 13.7, 6.8, 6.8 Hz, 1H), 2.60 (ddd, *J* = 13.9, 6.1, 3.1 Hz, 1H).

¹³C NMR (151 MHz, D₂O): δ 155.1, 152.0, 148.7, 140.4, 118.5, 86.2 (d, *J* = 9.4 Hz), 83.8, 71.6, 64.4 (d, *J* = 4.8 Hz), 39.3.

³¹P NMR (243 MHz, D₂O): δ 46.5.

HRMS [ESI] C₁₀H₁₄N₅O₆PS ([M-H]⁻) calculated 346.0381 found 346.0383.

Compound 23



2'-*O*-methyladenosine-5'-*O*-diammonium monophosphorothioate

Compound **23** was obtained according to general procedure (**Method A**) from 2'-*O*-methyladenosine (0.05 mmol, 14.1 mg) using *Ag*-dNK (2.5 mg, 0.18 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) to afford 8.6 mg of the title compound after lyophilization (**Yield = 41%**).

Physical state: white solid

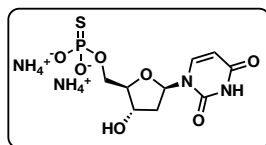
¹H NMR (600 MHz, D₂O): δ 8.65 (s, 1H), 8.30 (s, 1H), 6.21 (d, *J* = 5.6 Hz, 1H), 4.73 – 4.68 (m, 1H), 4.52 – 4.48 (m, 1H), 4.44 – 4.39 (m, 1H), 4.22 – 4.14 (m, 2H), 3.47 (s, 3H).

¹³C NMR (151 MHz, D₂O): δ 152.6, 149.0, 147.9, 140.3, 117.7, 85.0, 84.0 (d, *J* = 9.4 Hz), 82.7, 68.4, 63.6 (d, *J* = 5.1 Hz), 57.4.

³¹P NMR (243 MHz, D₂O): δ 50.1.

HRMS [ESI] C₁₀H₁₆N₅O₆PS ([M-H]⁻) calculated 376.0486 found 376.0488.

Compound 24



5'-*O*-deoxyuridine-diammonium monophosphorothioate

Compound **24** was obtained according to general procedure (**Method A**) from deoxyuridine (0.05 mmol, 11.4 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) to afford 10.7 mg of the title compound after lyophilization (**Yield = 60%**).

Physical state: white solid

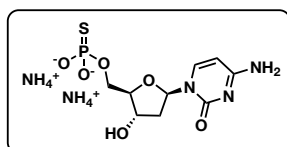
¹H NMR (600 MHz, D₂O): δ 8.09 (d, *J* = 8.1 Hz, 1H), 6.34 (t, *J* = 6.8 Hz, 1H), 5.95 (d, *J* = 8.1 Hz, 1H), 4.61 (dt, *J* = 7.0, 3.6 Hz, 1H), 4.24 – 4.20 (m, 1H), 4.12 – 4.03 (m, 2H), 2.42 – 2.36 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 166.3, 151.7, 142.3, 102.4, 85.9 (d, *J* = 9.7 Hz), 85.4, 71.2, 64.4 (d, *J* = 5.2 Hz), 39.0.

³¹P NMR (243 MHz, D₂O): δ 47.3.

HRMS [ESI] C₉H₁₃N₂O₇PS ([M-H]⁻) calculated 323.0108 found 323.0109.

Compound 25



5'-*O*-deoxycytidine-diammonium monophosphorothioate

Compound **25** was obtained according to general procedure (**Method A**) from deoxycytidine (0.05 mmol, 11.4 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) to afford 11.5 mg of the title compound after lyophilization (**Yield = 64%**).

Physical state: white solid

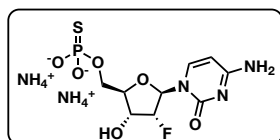
¹H NMR (600 MHz, D₂O): δ 8.21 (d, *J* = 7.8 Hz, 1H), 6.31 (t, *J* = 6.6 Hz, 1H), 6.19 (d, *J* = 7.8 Hz, 1H), 4.59 (dt, *J* = 6.5, 3.3 Hz, 1H), 4.24 (dt, *J* = 5.0, 2.3 Hz, 1H), 4.15 – 4.04 (m, 2H), 2.45 (ddd, *J* = 14.1, 6.3, 3.6 Hz, 1H), 2.38 – 2.30 (m, 1H).

¹³C NMR (151 MHz, D₂O): δ 162.7, 153.1, 143.3, 95.8, 86.3, 86.1 (d, *J* = 9.4 Hz), 71.0, 64.3 (d, *J* = 5.1 Hz), 39.6.

³¹P NMR (243 MHz, D₂O): δ 47.9.

HRMS [ESI] C₉H₁₄N₃O₆PS ([M-H]⁻) calculated 322.0268 found 322.0270.

Compound 26



2'-deoxy-2'-fluorocytidine-5'-*O*-diammonium monophosphorothioate

Compound **26** was obtained according to general procedure (**Method A**) from 2'-deoxy-2'-fluorocytidine (0.05 mmol, 12.3 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) to afford 12.8 mg of the title compound after lyophilization (**Yield = 65%**).

Physical state: white solid

¹H NMR (600 MHz, D₂O): δ 8.17 (d, *J* = 7.7 Hz, 1H), 6.15 (d, *J* = 7.8 Hz, 1H), 6.08 (dd, *J* = 17.1, 1.5 Hz, 1H), 5.13 (ddd, *J* = 52.4, 4.3, 1.5 Hz, 1H), 4.47 (ddd, *J* = 22.6, 7.9, 4.3 Hz, 1H), 4.34 – 4.27 (m, 2H), 4.12 (ddd, *J* = 12.7, 6.0, 3.1 Hz, 1H).

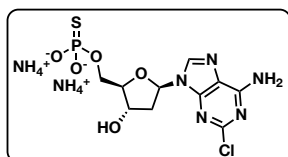
¹³C NMR (151 MHz, D₂O): δ 164.6, 155.0, 142.4, 96.0, 93.8 (d, *J* = 185.9 Hz), 88.5 (d, *J* = 34.2 Hz), 81.5 (d, *J* = 9.6 Hz), 67.5 (d, *J* = 16.1 Hz), 62.2 (d, *J* = 4.7 Hz).

³¹P NMR (243 MHz, D₂O): δ 47.1.

¹⁹F NMR (565 MHz, D₂O): δ -203.1 (ddd, *J* = 52.4, 22.7, 17.0 Hz).

HRMS [ESI] C₉H₁₃FN₃O₆PS ([M-H]⁻) calculated 340.0174 found 340.0173.

Compound 27



5'-*O*-cladribine-diammonium monophosphorothioate

Compound **27** was obtained according to general procedure (**Method A**) from cladribine (0.05 mmol, 12.3 mg) using *Ag*-dNK (2.5 mg, 0.18 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 30:70) to afford 17.3 mg of the title compound after lyophilization (**Yield** = **83%**).

Physical state: white solid

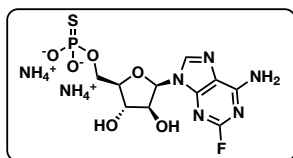
¹H NMR (600 MHz, D₂O): δ 8.48 (s, 1H), 6.36 (t, *J* = 6.8 Hz, 1H), 4.73 (dt, *J* = 6.1, 3.2 Hz, 1H), 4.33 – 4.26 (m, 1H), 4.13 – 4.01 (m, 2H), 2.78 (ddd, *J* = 13.6, 6.8, 6.8 Hz, 1H), 2.61 (ddd, *J* = 14.0, 6.3, 3.4 Hz, 1H).

¹³C NMR (151 MHz, D₂O): δ 155.9, 153.5, 149.6, 140.2, 117.3, 86.1 (d, *J* = 9.4 Hz), 83.8, 71.5, 64.6 (d, *J* = 4.9 Hz), 39.4.

³¹P NMR (243 MHz, D₂O): δ 48.2.

HRMS [ESI] C₁₀H₁₃ClN₅O₅PS ([M-H]⁻) calculated 379.9991 found 379.9989.

Compound 28



5'-*O*-fludarabine-diammonium monophosphorothioate

Compound **28** was obtained according to general procedure (**Method A**) from fludarabine (0.05 mmol, 14.3 mg) using *Ag*-dNK (2.5 mg, 0.18 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 30:70) to afford 15.7 mg of the title compound after lyophilization (**Yield** = **75%**).

Physical state: white solid

¹H NMR (600 MHz, D₂O): δ 8.52 (s, 1H), 6.29 (d, *J* = 5.7 Hz, 1H), 4.59 – 4.55 (m, 1H), 4.48 – 4.43 (m, 1H), 4.25 – 4.16 (m, 3H).

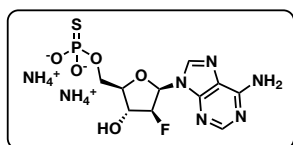
¹³C NMR (151 MHz, D₂O): δ 158.9 (d, *J* = 210.8 Hz), 157.0 (d, *J* = 19.4 Hz), 141.2 (d, *J* = 2.6 Hz), 116.5 (d, *J* = 3.5 Hz), 83.7, 81.4 (d, *J* = 9.3 Hz), 75.5, 73.2, 62.9 (d, *J* = 4.8 Hz).

³¹P NMR (243 MHz, D₂O): δ 46.4.

¹⁹F NMR (565 MHz, D₂O): δ 53.3.

HRMS [ESI] C₁₀H₁₄FN₅O₆PS ([M-H]⁻) calculated 380.0235 found 380.0244.

Compound 29



5'-*O*-9-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)adenine -diammonium monophosphorothioate

Compound **29** was obtained according to general procedure (**Method A**) from 9-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)adenine (0.05 mmol, 13.5 mg) using *Ag*-dNK (2.5 mg, 0.18 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE

Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 30:70) to afford 18.0 mg of the title compound after lyophilization (**Yield = 90%**).

Physical state: white solid

^1H NMR (600 MHz, D_2O): δ 8.46 (d, $J = 2.2$ Hz, 1H), 8.15 (s, 1H), 6.46 (dd, $J = 14.9, 4.3$ Hz, 1H), 5.33 (ddd, $J = 51.5, 3.9, 3.9$ Hz, 1H), 4.72 (ddd, $J = 18.0, 5.1, 3.6$ Hz, 1H), 4.30 – 4.25 (m, 1H), 4.22 – 4.12 (m, 2H).

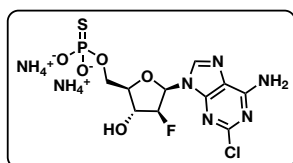
^{13}C NMR (151 MHz, D_2O): δ 154.8, 151.9, 148.3, 141.3 (d, $J = 5.0$ Hz), 117.8, 94.5 (d, $J = 192.7$ Hz), 82.3 (d, $J = 17.0$ Hz), 82.1 (dd, $J = 9.1, 4.6$ Hz), 73.0 (d, $J = 24.9$ Hz), 63.3 (d, $J = 4.4$ Hz).

^{31}P NMR (243 MHz, D_2O): δ 46.7.

^{19}F NMR (565 MHz, D_2O): δ -198.56 (dt, $J = 51.5, 16.2$ Hz).

HRMS [ESI] $\text{C}_{10}\text{H}_{13}\text{FN}_5\text{O}_5\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 364.0286 found 364.0287.

Compound 30



5'-*O*-clofarabine-diammonium monophosphorothioate

Compound **30** was obtained according to general procedure (**Method A**) from clofarabine (0.05 mmol, 13.5 mg) using *Ag*-dNK (2.5 mg, 0.18 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 30:70) to afford 18.8 mg of the title compound after lyophilization (**Yield = 87%**).

Physical state: white solid

^1H NMR (600 MHz, D_2O): δ 8.43 (d, $J = 2.2$ Hz, 1H), 6.38 (dd, $J = 14.8, 4.3$ Hz, 1H), 5.33 (ddd, $J = 51.4, 3.9, 3.9$ Hz, 1H), 4.71 (ddd, $J = 17.8, 5.0, 3.6$ Hz, 1H), 4.29 (q, $J = 4.8$ Hz, 1H), 4.25 – 4.14 (m, 2H).

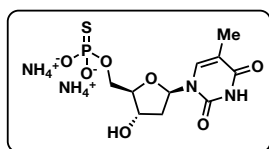
^{13}C NMR (151 MHz, D_2O): δ 156.0, 153.7, 149.5, 141.3 (d, $J = 4.9$ Hz), 94.4 (d, $J = 192.9$ Hz), 82.4 (d, $J = 17.0$ Hz), 82.2 (dd, $J = 9.2, 4.4$ Hz), 72.9 (d, $J = 25.1$ Hz), 63.4 (d, $J = 5.0$ Hz).

^{31}P NMR (243 MHz, D_2O): δ 48.0.

^{19}F NMR (565 MHz, D_2O): δ -198.5 (dt, $J = 51.3, 16.2$ Hz).

HRMS [ESI] $\text{C}_{10}\text{H}_{12}\text{ClFN}_5\text{O}_5\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 397.9897 found 397.9896.

Compound 31



5'-*O*-thymidine-diammonium monophosphorothioate

Compound **31** was obtained according to general procedure (**Method A**) from thymidine (0.05 mmol, 12.1 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase. The crude product was

purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 20:80) to afford 13.6 mg of the title compound after lyophilization (**Yield = 73%**).

Physical state: white solid

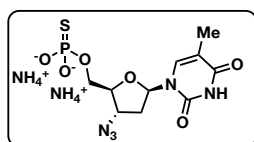
^1H NMR (600 MHz, D_2O): δ 7.83 (s, 1H), 6.36 (dd, $J = 7.7, 6.3$ Hz, 1H), 4.61 (dt, $J = 5.9, 3.1$ Hz, 1H), 4.23 – 4.18 (m, 1H), 4.10 (dd, $J = 6.0, 3.4$ Hz, 2H), 2.42 – 2.31 (m, 2H), 1.95 (s, 3H).

^{13}C NMR (151 MHz, D_2O): δ 166.6, 151.8, 137.5, 111.8, 85.6 (d, $J = 9.7$ Hz), 85.0, 71.3, 64.7 (d, $J = 5.1$ Hz), 38.7, 11.6.

^{31}P NMR (243 MHz, D_2O): δ 48.4.

HRMS [ESI] $\text{C}_{10}\text{H}_{15}\text{N}_2\text{O}_7\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 337.0265 found 337.0265.

Compound 32



5'-*O*-zidovudine-diammonium monophosphorothioate

Compound **32** was obtained according to general procedure (**Method A**) from zidovudine (0.05 mmol, 13.4 mg) using *Tm*-TK (2.5 mg, 0.24 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 25:75) to afford 13.3 mg of the title compound after lyophilization (**Yield = 67%**).

Physical state: white solid

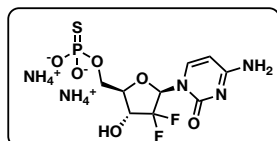
^1H NMR (600 MHz, D_2O): δ 7.84 (s, 1H), 6.29 (t, $J = 6.8$ Hz, 1H), 4.56 (dt, $J = 6.4, 4.2$ Hz, 1H), 4.25 – 4.22 (m, 1H), 4.18 – 4.09 (m, 2H), 2.55 – 2.45 (m, 2H), 1.95 (s, 3H).

^{13}C NMR (151 MHz, D_2O): δ 166.6, 151.7, 137.4, 111.8, 84.9, 83.2 (d, $J = 9.4$ Hz), 64.6 (d, $J = 4.9$ Hz), 60.8, 36.4, 11.7.

^{31}P NMR (243 MHz, D_2O): δ 48.5.

HRMS [ESI] $\text{C}_{10}\text{H}_{14}\text{N}_5\text{O}_6\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 362.0330 found 362.0329.

Compound 33



5'-*O*-gemcitabine-diammonium monophosphorothioate

Compound **33** was obtained according to general procedure (**Method A**) from gemcitabine (0.05 mmol, 13.2 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 30:70) to afford 12.2 mg of the title compound after lyophilization (**Yield = 62%**).

Physical state: white solid

¹H NMR (600 MHz, D₂O): δ 8.04 (d, *J* = 7.6 Hz, 1H), 6.25 (t, *J* = 7.1 Hz, 1H), 6.16 (d, *J* = 7.6 Hz, 1H), 4.52 (td, *J* = 12.4, 8.7 Hz, 1H), 4.29 (ddd, *J* = 12.6, 6.0, 1.9 Hz, 1H), 4.24 – 4.19 (m, 1H), 4.15 (ddd, *J* = 12.2, 6.6, 3.0 Hz, 1H).

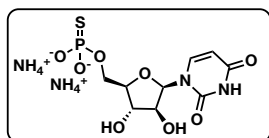
¹³C NMR (151 MHz, D₂O): δ 165.6, 156.4, 141.6, 122.3 (t, *J* = 258.3 Hz), 96.5, 84.8 – 84.1 (m), 79.4 – 79.0 (m), 68.4 (t, *J* = 22.7 Hz), 61.7 (d, *J* = 4.6 Hz).

³¹P NMR (243 MHz, D₂O): δ 46.5.

¹⁹F NMR (565 MHz, D₂O): δ -118.4 (m).

HRMS [ESI] C₉H₁₂F₂N₃O₆PS ([M-H]⁻) calculated 358.0080 found 358.0079.

Compound 34



5'-O-2'-β-D-arabinouridine-diammonium monophosphorothioate

Compound **34** was obtained according to general procedure (**Method A**) from 2'-β-D-arabinouridine (AraU, 0.05 mmol, 12.2 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) to afford 14.8 mg of the title compound after lyophilization (**Yield = 79%**).

Physical state: white solid

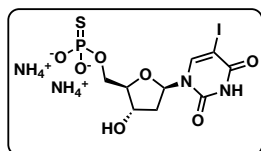
¹H NMR (600 MHz, D₂O): δ 8.07 (d, *J* = 8.1 Hz, 1H), 6.22 (d, *J* = 5.5 Hz, 1H), 5.93 (d, *J* = 8.1 Hz, 1H), 4.45 (dd, *J* = 5.5, 5.5 Hz, 1H), 4.27 (dd, *J* = 5.6, 5.6 Hz, 1H), 4.21 – 4.16 (m, 1H), 4.15 – 4.10 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 166.32, 151.49, 143.20, 101.42, 84.91, 81.61 (d, *J* = 9.1 Hz), 75.37, 73.86, 62.89 (d, *J* = 4.8 Hz).

³¹P NMR (243 MHz, D₂O): δ 46.5.

HRMS [ESI] C₉H₁₃N₂O₈PS ([M-H]⁻) calculated 339.0058 found 339.0059.

Compound 35



5'-O-idoxuridine-diammonium monophosphorothioate

Compound **35** was obtained according to general procedure (**Method A**) from idoxuridine (0.05 mmol, 12.2 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 30:70) to afford 20.6 mg of the title compound after lyophilization (**Yield = 85%**).

Physical state: white solid

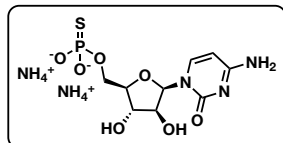
¹H NMR (600 MHz, D₂O): δ 8.31 (s, 1H), 6.28 (t, *J* = 6.9 Hz, 1H), 4.61 – 4.55 (m, 1H), 4.24 – 4.19 (m, 1H), 4.12 – 4.03 (m, 2H), 2.43 – 2.34 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 163.2, 151.4, 146.2, 85.9 (d, *J* = 9.2 Hz), 85.7, 71.2, 68.4, 64.6 (d, *J* = 4.8 Hz), 38.8.

³¹P NMR (243 MHz, D₂O): δ 46.8.

HRMS [ESI] C₉H₁₂IN₂O₇PS ([M-H]⁻) calculated 448.9075 found 448.9078.

Compound 36



5'-*O*-cytarabine-diammonium monophosphorothioate

Compound **36** was obtained according to general procedure (**Method A**) from cytarabine (0.05 mmol, 12.2 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) to afford 12.3 mg of the title compound after lyophilization (**Yield = 66%**).

Physical state: white solid

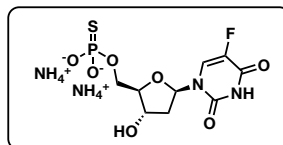
¹H NMR (600 MHz, D₂O): δ 8.13 (d, *J* = 7.7 Hz, 1H), 6.24 (d, *J* = 5.3 Hz, 1H), 6.16 (d, *J* = 7.7 Hz, 1H), 4.46 (dd, *J* = 5.3, 5.3 Hz, 1H), 4.27 (dd, *J* = 5.3, 5.3 Hz, 1H), 4.23 – 4.18 (m, 1H), 4.17 – 4.11 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 163.7, 154.3, 143.8, 95.2, 85.6, 81.7 (d, *J* = 9.2 Hz), 75.3, 73.9, 63.0 (d, *J* = 4.9 Hz).

³¹P NMR (243 MHz, D₂O): δ 47.3.

HRMS [ESI] C₉H₁₄N₃O₇PS ([M-H]⁻) calculated 338.0217 found 338.0219.

Compound 37



5'-*O*-floxuridine-diammonium monophosphorothioate

Compound **37** was obtained according to general procedure (**Method A**) from floxuridine (0.05 mmol, 12.3 mg) using *Tm*-TK (2.5 mg, 0.24 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) to afford 16.2 mg of the title compound after lyophilization (**Yield = 95%**).

Physical state: white solid

¹H NMR (600 MHz, D₂O): δ 8.21 (d, *J* = 6.3 Hz, 1H), 6.32 (t, *J* = 6.9 Hz, 1H), 4.60 (dt, *J* = 6.3, 3.2 Hz, 1H), 4.24 – 4.19 (m, 1H), 4.13 – 4.05 (m, 2H), 2.45 – 2.33 (m, 2H).

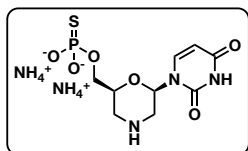
¹³C NMR (151 MHz, D₂O): δ 159.5 (d, *J* = 26.0 Hz), 150.1, 140.8 (d, *J* = 232.8 Hz), 126.0 (d, *J* = 34.3 Hz), 86.0 (d, *J* = 9.3 Hz), 85.7, 71.1, 64.4 (d, *J* = 4.9 Hz), 38.9.

^{31}P NMR (243 MHz, D_2O): δ 47.3.

^{19}F NMR (565 MHz, D_2O): δ 165.3 (d, J = 6.4 Hz).

HRMS [ESI] $\text{C}_9\text{H}_{12}\text{FN}_2\text{O}_7\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 341.0014 found 341.0017.

Compound 38



5'-*O*-morpholino-uridine-diammonium monophosphorothioate

Compound **38** was obtained according to general procedure (**Method A**) from 1-(6-(hydroxymethyl)morpholin-2-yl)uracil (morpholino-uridine, 0.05 mmol, 11.4 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 20:80) to afford 8.8 mg of the title compound after lyophilization (**Yield = 49%**).

Physical state: white solid

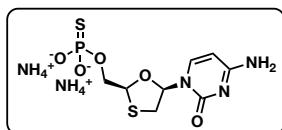
^1H NMR (600 MHz, D_2O): δ 7.88 (d, J = 8.1 Hz, 1H), 6.10 (dd, J = 10.8, 2.6 Hz, 1H), 5.93 (d, J = 8.1 Hz, 1H), 4.47 – 4.41 (m, 1H), 4.09 – 3.99 (m, 2H), 3.61 – 3.52 (m, 2H), 3.31 (d, J = 12.2 Hz, 1H), 3.22 (t, J = 12.3 Hz, 1H).

^{13}C NMR (151 MHz, D_2O): δ 165.9, 150.8, 141.5, 102.8, 77.7, 73.5 (d, J = 8.8 Hz), 63.8 (d, J = 4.4 Hz), 43.7, 43.0.

^{31}P NMR (243 MHz, D_2O): δ 45.3

HRMS [ESI] $\text{C}_9\text{H}_{14}\text{N}_3\text{O}_6\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 322.0268 found 322.0268.

Compound 39



5'-*O*-lamivudine-diammonium monophosphorothioate

Compound **39** was obtained according to general procedure (**Method A**) from lamivudine (0.05 mmol, 11.5 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 20:80) to afford 13.0 mg of the title compound after lyophilization (**Yield = 72%**).

Physical state: white solid

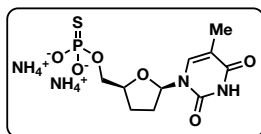
^1H NMR (600 MHz, D_2O): δ 8.30 (d, J = 7.7 Hz, 1H), 6.33 (dd, J = 5.4, 3.5 Hz, 1H), 6.15 (d, J = 7.7 Hz, 1H), 5.50 (t, J = 4.1 Hz, 1H), 4.35 (ddd, J = 11.9, 6.9, 3.0 Hz, 1H), 4.19 (ddd, J = 11.6, 6.9, 4.2 Hz, 1H), 3.59 (dd, J = 12.5, 5.4 Hz, 1H), 3.30 (dd, J = 12.5, 3.5 Hz, 1H).

^{13}C NMR (151 MHz, D_2O): δ 163.0, 153.1, 143.5, 95.2, 87.4, 85.6 (d, J = 9.3 Hz), 64.9 (d, J = 4.7 Hz), 37.3.

^{31}P NMR (243 MHz, D_2O): δ 48.3.

HRMS [ESI] C₈H₁₂N₃O₅PS₂ ([M-H]⁻) calculated 323.9883 found 323.9882.

Compound 40



5'-O-3'-deoxythymidine-diammonium monophosphorothioate

Compound **40** was obtained according to general procedure (Method A) from 3'-deoxythymidine (ddT, 0.05 mmol, 11.3 mg) using *Tm*-TK (2.5 mg, 0.24 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) to afford 14.5 mg of the title compound after lyophilization (**Yield = 81%**).

Physical state: white solid

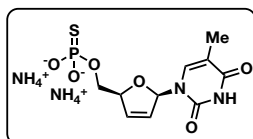
¹H NMR (600 MHz, D₂O): δ 7.82 (s, 1H), 6.13 (dd, *J* = 6.9, 3.9 Hz, 1H), 4.42 – 4.35 (m, 1H), 4.19 (ddd, *J* = 11.6, 5.9, 2.7 Hz, 1H), 4.05 (ddd, *J* = 11.5, 7.0, 4.5 Hz, 1H), 2.48 – 2.39 (m, 1H), 2.20 – 2.10 (m, 2H), 2.08 – 1.99 (m, 1H), 1.95 (s, 3H).

¹³C NMR (151 MHz, D₂O): δ 166.7, 151.7, 137.7, 111.1, 86.1, 80.3 (d, *J* = 9.3 Hz), 66.1 (d, *J* = 5.1 Hz), 31.1, 25.0, 11.7.

³¹P NMR (243 MHz, D₂O): δ 49.8

HRMS [ESI] C₁₀H₁₅N₂O₆PS ([M-H]⁻) calculated 321.0316 found 321.0313.

Compound 41



5'-O-stavudine-diammonium-monophosphorothioate

Compound **41** was obtained according to general procedure (Method A) from stavudine (d4T, 0.05 mmol, 11.2 mg) using *Tm*-TK (2.5 mg, 0.24 mol%) as nucleoside kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) to afford 9.0 mg of the title compound after lyophilization (**Yield = 51%**). This compound contains small amount of adenosine monophosphate impurity after purification.

Physical state: white solid

¹H NMR (600 MHz, D₂O): δ 7.63 (d, *J* = 1.3 Hz, 1H), 6.96 (dt, *J* = 3.3, 1.7 Hz, 1H), 6.51 (dt, *J* = 6.1, 1.8 Hz, 1H), 5.95 (dt, *J* = 6.2, 1.9 Hz, 1H), 5.15 – 5.09 (m, 1H), 4.08 (td, *J* = 6.8, 3.5 Hz, 2H), 1.91 (s, 3H).

¹³C NMR (151 MHz, D₂O): δ 166.8, 152.3, 138.3, 134.4, 125.0, 111.4, 90.0, 86.0 (d, *J* = 9.3 Hz), 65.6 (d, *J* = 4.9 Hz), 11.5.

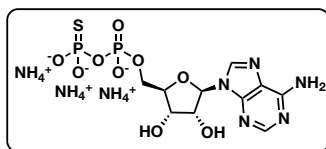
³¹P NMR (243 MHz, D₂O): δ 48.9.

HRMS [ESI] C₁₀H₁₃N₂O₆PS ([M-H]⁻) calculated 319.0159 found 319.0157.

9.3 β -thio nucleoside diphosphates

Note: We observed that β -thio nucleoside diphosphates are unstable during lyophilization and often decompose to the corresponding diphosphate products under various conditions. Small amount of NH_4HCO_3 keeps the product relatively stable and prevents the loss of sulfur. To obtain spectroscopic data for this class of products, ^1H NMR and ^{31}P NMR were collected directly after evaporation of most of NH_4HCO_3 buffer, ^{13}C NMR was collected after lyophilization to suppress the signal of HCO_3^- . As such, diphosphate impurity often shows up in ^{13}C NMR spectra, but the level is low enough to not significantly affect the yield calculation.

Compound 42



5'-*O*-adenosine-triammonium-diphosphoro- β -thioate

Compound **42** was obtained according to general procedure (**Method B**) from adenosine monophosphate (0.05 mmol, 17.4 mg) using *Sc*-AK (2.5 mg, 0.21 mol%) as monophosphate kinase. Reaction time was 6 hours. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 35:65) to afford 18.6 mg of the title compound after lyophilization (**Yield = 75%**).

Physical state: white solid

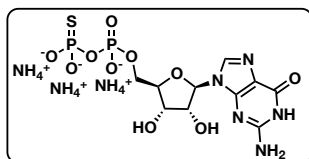
^1H NMR (600 MHz, D_2O): δ 8.26 (s, 1H), 7.97 (s, 1H), 5.86 (d, $J = 5.3$ Hz, 1H), 4.51 (dd, $J = 5.3$, 5.3 Hz, 1H), 4.34 (dd, 1H), 4.16 – 4.10 (m, 1H), 4.02 – 3.92 (m, 2H).

^{13}C NMR (151 MHz, D_2O): δ 155.2, 152.3, 149.0, 118.5, 86.9, 84.0 (d, $J = 9.2$ Hz), 74.4, 70.3, 64.9 (d, $J = 5.3$ Hz).

^{31}P NMR (243 MHz, D_2O): δ 33.3 (d, $J = 29.7$ Hz), -11.7 (d, $J = 30.2$ Hz).

HRMS [ESI] $\text{C}_{10}\text{H}_{15}\text{N}_5\text{O}_9\text{P}_2\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 441.9993 found 441.9992.

Compound 43



5'-*O*-guanosine-triammonium-diphosphoro- β -thioate

Compound **43** was obtained according to general procedure (**Method B**) from guanosine monophosphate sodium salts (0.05 mmol, 20.4 mg) using *Hs*-GMPK (2.5 mg, 0.23 mol%) as monophosphate kinase. The reaction was carried out at 30 °C for 6 hours. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 35:65) to afford 16.1 mg of the title compound after lyophilization (**Yield = 63%**).

Physical state: white solid

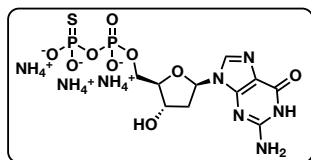
^1H NMR (600 MHz, D_2O): δ 8.06 (s, 1H), 5.84 (d, $J = 5.6$ Hz, 1H), 4.69 – 4.65 (m, 1H), 4.54 – 4.49 (m, 1H), 4.29 – 4.23 (m, 1H), 4.18 – 4.08 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 158.92, 153.93, 151.76, 116.10, 86.74, 83.93 (d, *J* = 9.4 Hz), 73.68, 70.44, 65.04 (d, *J* = 5.5 Hz).

³¹P NMR (243 MHz, D₂O): δ 33.3 (d, *J* = 30.8 Hz), -11.7 (d, *J* = 30.8 Hz).

HRMS [ESI] C₁₀H₁₅N₅O₁₀P₂S ([M-H]⁻) calculated 457.9942 found 457.9940.

Compound 44



5'-*O*-deoxyguanosine-triammonium-diphosphoro-β-thioate

Compound **44** was obtained according to general procedure (**Method B**) from deoxyguanosine monophosphate sodium salts (0.05 mmol, 19.6 mg) using *Ec*-GMPK (2.5 mg, 0.21 mol%) as monophosphate kinase. The reaction was carried out at 30 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 35:65) to afford 16.7 mg of the title compound after lyophilization (**Yield = 67%**).

Physical state: white solid

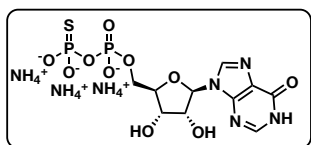
¹H NMR (600 MHz, D₂O): δ 7.86 (s, 1H), 6.04 (t, *J* = 6.7 Hz, 1H), 4.52 (dt, *J* = 6.2, 3.8 Hz, 1H), 4.02 – 3.97 (m, 1H), 3.93 – 3.86 (m, 2H), 2.53 (ddd, *J* = 13.6, 6.6, 6.6 Hz, 1H), 2.27 (ddd, *J* = 14.0, 6.5, 4.0 Hz, 1H).

¹³C NMR (151 MHz, D₂O): δ 158.7, 153.9, 151.2, 137.6, 115.8, 85.7 (d, *J* = 9.4 Hz), 83.7, 71.4, 65.3 (d, *J* = 5.6 Hz), 38.7.

³¹P NMR (243 MHz, D₂O): δ 33.2 (br, supposed to be doublet), -11.7 (d, *J* = 30.4 Hz).

HRMS [ESI] C₁₀H₁₅N₅O₉P₂S ([M-H]⁻) calculated 441.9993 found 441.9992.

Compound 45



5'-*O*-inosine-triammonium-diphosphoro-β-thioate

Compound **45** was obtained according to general procedure (**Method B**) from inosine monophosphate sodium salts (0.05 mmol, 19.6 mg) using *Hs*-GMPK (2.5 mg, 0.23 mol%) as monophosphate kinase. The reaction was carried out at 30 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 35:65) to afford 10.4 mg of the title compound after lyophilization (**Yield = 42%**).

Physical state: white solid

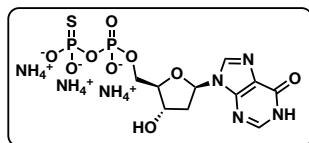
¹H NMR (600 MHz, D₂O): δ 8.34 (s, 1H), 8.05 (s, 1H), 5.97 (d, *J* = 5.1 Hz, 1H), 4.60 (t, *J* = 5.1 Hz, 1H), 4.45 (t, *J* = 4.7 Hz, 1H), 4.25 – 4.20 (m, 1H), 4.12 – 4.03 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 158.6, 148.9, 146.1, 139.9, 123.7, 87.3, 84.2 (d, *J* = 9.3 Hz), 74.4, 70.4, 64.9 (d, *J* = 5.4 Hz).

³¹P NMR (243 MHz, D₂O): δ 33.3 (d, *J* = 30.4 Hz, manually determined), -11.7 (d, *J* = 30.5 Hz).

HRMS [ESI] C₁₀H₁₄N₄O₁₀P₂S ([M-H]⁻) calculated 442.9833 found 442.9841.

Compound 46



5'-*O*-deoxyinosine-triammonium-diphosphoro-β-thioate

Compound **46** was obtained according to general procedure (**Method B**) from deoxyinosine monophosphate sodium salts (0.05 mmol, 18.6 mg) using *Hs*-GMPK (2.5 mg, 0.23 mol%) as monophosphate kinase. The reaction was carried out at 30 °C. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 35:65) to afford 9.6 mg of the title compound after lyophilization (**Yield = 40%**).

Physical state: white solid

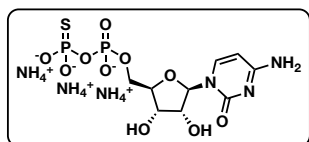
¹H NMR (600 MHz, D₂O): δ 8.44 (s, 1H), 8.18 (s, 1H), 6.48 (t, *J* = 6.6 Hz, 1H), 4.28 – 4.23 (m, 1H), 4.21 – 4.15 (m, 1H), 4.15 – 4.10 (m, 1H), 2.82 (ddd, *J* = 13.5, 6.5, 6.5 Hz, 1H), 2.57 (ddd, *J* = 14.0, 6.5, 4.2 Hz, 1H).

¹³C NMR (151 MHz, D₂O): δ 158.8, 148.6, 146.1, 123.6, 85.9 (d, *J* = 8.9 Hz), 84.0, 70.8, 64.8 (d, *J* = 5.5 Hz), 38.8.

³¹P NMR (243 MHz, D₂O): δ 33.2 (d, *J* = 31.6 Hz), -11.7 (d, *J* = 31.1 Hz).

HRMS [ESI] C₁₀H₁₄N₄O₉P₂S ([M-H]⁻) calculated 426.9884 found 426.9886.

Compound 47



5'-*O*-cytidine-triammonium-diphosphoro-β-thioate

Compound **47** was obtained according to general procedure (**Method B**) from cytidine monophosphate (0.05 mmol, 16.2 mg) using *Hs*-UMP-CMPK (2.5 mg, 0.23 mol%) as monophosphate kinase. The concentration of substrate is 2.5 mM (using 20 mL buffer) and DTT was used as additive (final concentration 10 mM). The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 30:70) to afford 14.0 mg of the title compound after lyophilization (**Yield = 60%**).

Physical state: white solid

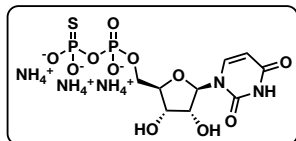
¹H NMR (600 MHz, D₂O): δ 7.89 (d, *J* = 8.1 Hz, 1H), 5.87 (br, 1H), 5.86 (d, *J* = 4.2 Hz, 1H), 4.35 (dd, *J* = 5.0, 5.0 Hz, 1H), 4.31 (dd, *J* = 5.1, 5.1 Hz, 1H), 4.20 – 4.15 (m, 1H), 4.15 – 4.10 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 163.4, 154.2, 142.7, 96.2, 89.2, 83.1 (d, *J* = 9.5 Hz), 74.3, 69.3, 64.4 (d, *J* = 5.2 Hz).

³¹P NMR (243 MHz, D₂O): δ 33.2 (d, *J* = 30.6 Hz), -11.8 (d, *J* = 30.8 Hz).

HRMS [ESI] C₉H₁₅N₃O₁₀P₂S ([M-H]⁻) calculated 417.9881 found 417.9879.

Compound 48



5'-*O*-uridine-triammonium-diphosphoro-β-thioate

Compound **48** was obtained according to general procedure (**Method B**) from uridine monophosphate sodium salts (0.05 mmol, 18.5 mg) using *Hs*-UMP-CMPK (2.5 mg, 0.23 mol%) as monophosphate kinase. The concentration of substrate is 2.5 mM (using 20 mL buffer) DTT was used as additive (final concentration 10 mM). The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 30:70) to afford 13.5 mg of the title compound after lyophilization (**Yield = 56%**).

Physical state: white solid

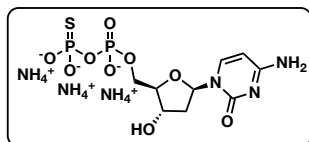
¹H NMR (600 MHz, D₂O): δ 7.73 (d, *J* = 8.1 Hz, 1H), 5.74 (br, 1H), 5.72 (d, *J* = 4.6 Hz, 1H), 4.18 (ddd, *J* = 10.0, 5.1, 5.1 Hz, 2H), 4.07 – 4.03 (m, 1H), 4.02 – 3.97 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 166.3, 151.9, 141.8, 102.7, 88.2, 83.4 (d, *J* = 9.4 Hz), 73.8, 69.7, 64.7 (d, *J* = 5.5 Hz).

³¹P NMR (243 MHz, D₂O): δ 33.3 (d, *J* = 30.5 Hz), -11.8 (d, *J* = 30.6 Hz).

HRMS [ESI] C₉H₁₄N₂O₁₁P₂S ([M-H]⁻) calculated 418.9721 found 418.9723.

Compound 49



5'-*O*-deoxycytidine-triammonium-diphosphoro-β-thioate

Compound **49** was obtained according to general procedure (**Method B**) from deoxycytidine monophosphate (0.05 mmol, 16.2 mg) using *Bs*-CMPK (2.5 mg, 0.20 mol%) as monophosphate kinase. DTT was used as additive (final concentration 10 mM). The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 30:70) to afford 11.4 mg of the title compound after lyophilization (**Yield = 50%**).

Physical state: white solid

¹H NMR (600 MHz, D₂O): δ 8.01 (d, *J* = 7.6 Hz, 1H), 6.33 (t, *J* = 6.5 Hz, 1H), 6.13 (d, *J* = 7.5 Hz, 1H), 4.69 (dt, *J* = 6.4, 3.9 Hz, 1H), 4.27 – 4.22 (m, 1H), 4.22 – 4.14 (m, 2H), 2.41 (ddd, *J* = 14.0, 6.4, 4.3 Hz, 1H), 2.33 (ddd, *J* = 13.7, 6.6, 6.6 Hz, 1H).

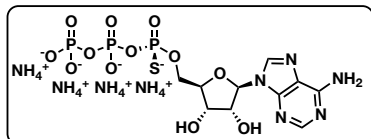
^{13}C NMR (151 MHz, D_2O): δ 165.8, 157.1, 142.0, 96.5, 85.8, 85.5 (d, J = 9.0 Hz), 70.4, 64.8 (d, J = 5.5 Hz), 39.2.

^{31}P NMR (243 MHz, D_2O): δ 33.1 (d, J = 31.0 Hz), -11.8 (d, J = 31.0 Hz).

HRMS [ESI] $\text{C}_9\text{H}_{15}\text{N}_3\text{O}_9\text{P}_2\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 401.9932 found 401.9927.

9.4 Nucleosides α -thio triphosphate

Compound 50



5'-*O*-adenosine-tetrammonium (*S*)-triphosphoro- α -thioate

Compound **50** was obtained according to general procedure (**Method C**) from adenosine (0.05 mmol, 13.4 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase, *Sc*-AK (2.5 mg, 0.21 mol%) as monophosphate kinase, *Tm*-AcK (2.5 mg, 0.16 mol%) as acetate kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 40:60) to afford 17.3 mg of the title compound after lyophilization (**Yield** = **58%**, **dr** = **12:1**). The absolute configuration at phosphorus was determined by comparison with standard compounds using HPLC.

Physical state: white solid

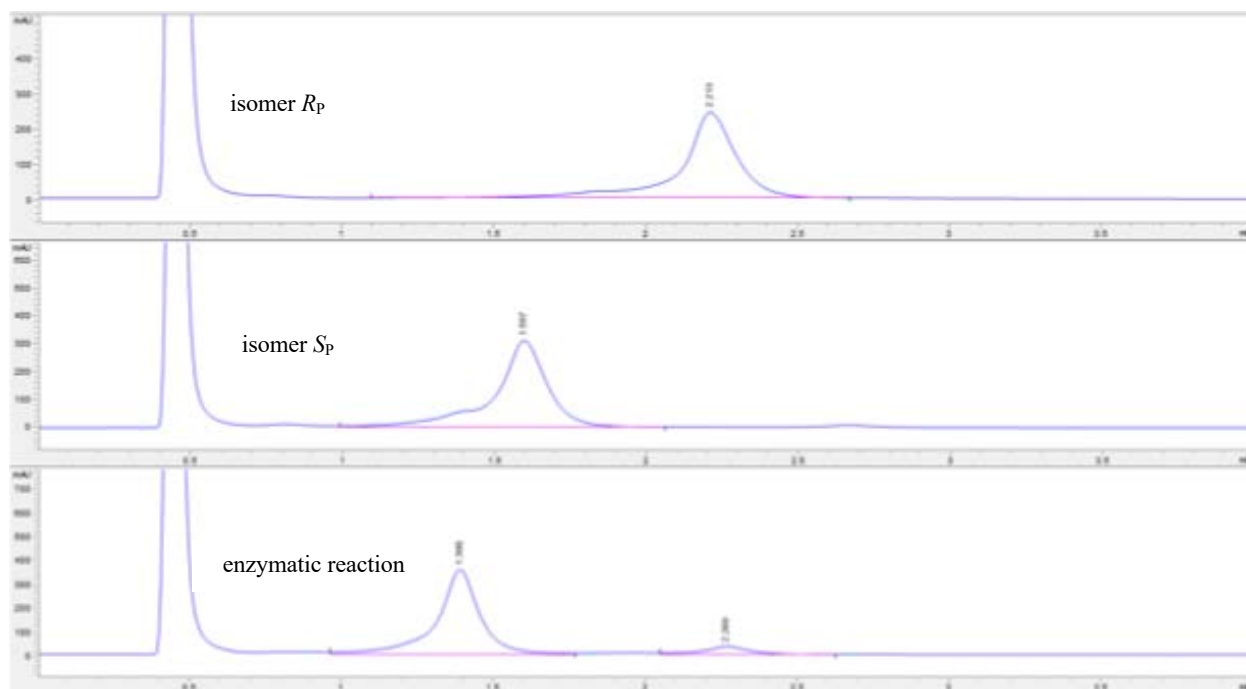
^1H NMR (600 MHz, D_2O): δ 8.46 (s, 1H), 8.07 (s, 1H), 5.98 (d, J = 5.4 Hz, 1H), 4.63 (dd, J = 5.3, 5.3 Hz, 1H), 4.44 (dd, J = 4.6, 4.6 Hz, 1H), 4.28 – 4.24 (m, 1H), 4.22 – 4.09 (m, 2H).

^{13}C NMR (151 MHz, D_2O): δ 154.6, 151.5, 148.9, 140.5, 118.4, 87.0, 83.9 (d, J = 9.7 Hz), 74.5, 70.5, 65.2 (d, J = 6.5 Hz).

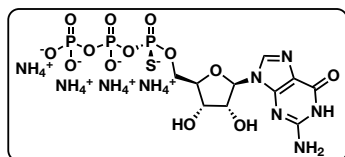
^{31}P NMR (243 MHz, D_2O): δ 43.3 (d, J = 27.0 Hz), -6.3 (d, J = 20.5 Hz), -22.7 (dd, J = 23.8, 23.8 Hz).

HRMS [ESI] $\text{C}_{10}\text{H}_{16}\text{N}_5\text{O}_{12}\text{P}_3\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 521.9656 found 521.9658.

Retention time: 1.39 min (HPLC method 1)



Compound 51



5'-*O*-guanosine-tetrammonium (*S*)-triphosphoro- α -thioate

Compound **51** was obtained according to general procedure (**Method C**) from guanosine (0.05 mmol, 14.2 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase, *Bf*-GMPK (2.5 mg, 0.20 mol%) as monophosphate kinase, *Tm*-AcK (2.5 mg, 0.16 mol%) as acetate kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 50:50) to afford 20.0 mg of the title compound after lyophilization (**Yield = 66%**, **dr > 19:1**). The absolute configuration at phosphorus was determined by comparison with standard compounds using ^1H NMR.

Physical state: white solid

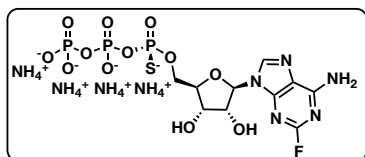
^1H NMR (600 MHz, D_2O): δ 8.17 (s, 1H), 5.86 (d, $J = 6.2$ Hz, 1H), 4.74 (dd, $J = 6.2, 5.2$ Hz, 1H), 4.51 (dd, $J = 5.2, 3.2$ Hz, 1H), 4.33 – 4.28 (m, 1H), 4.24 (ddd, $J = 11.2, 7.6, 3.3$ Hz, 1H), 4.19 (ddd, $J = 11.6, 5.9, 3.6$ Hz, 1H).

^{13}C NMR (151 MHz, D_2O): δ 158.9, 153.9, 151.7, 137.9, 116.1, 86.7, 83.7 (d, $J = 9.4$ Hz), 73.7, 70.3, 65.1 (d, $J = 6.5$ Hz).

^{31}P NMR (243 MHz, D_2O): δ 43.5 (d, $J = 26.8$ Hz), -10.1 (d, $J = 19.5$ Hz), -23.9 (dd, $J = 23.3, 23.3$ Hz).

HRMS [ESI] $\text{C}_{10}\text{H}_{16}\text{N}_5\text{O}_{13}\text{P}_3\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 537.9605 found 537.9601.

Compound 52



5'-O-2-fluoroadenosine-tetrammonium (*S*)-triphosphoro- α -thioate

Compound **52** was obtained according to general procedure (**Method C**) from 2-F-adenosine (0.05 mmol, 14.3 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase, *Sc*-AK (2.5 mg, 0.21 mol%) as monophosphate kinase, *Tm*-AcK (2.5 mg, 0.16 mol%) as acetate kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 40:60) to afford 15.2 mg of the title compound after lyophilization (**Yield = 50%, dr = 12:1**). The absolute configuration at phosphorus was determined by comparison with standard compounds using HPLC.

Physical state: white solid

^1H NMR (600 MHz, D_2O): δ 8.60 (s, 1H), 6.02 (d, $J = 5.6$ Hz, 1H), 4.61 (dd, $J = 5.1, 3.6$ Hz, 1H), 4.44 – 4.40 (m, 1H), 4.35 (ddd, $J = 10.9, 7.7, 2.9$ Hz, 1H), 4.27 (ddd, $J = 11.7, 5.4, 3.0$ Hz, 1H).

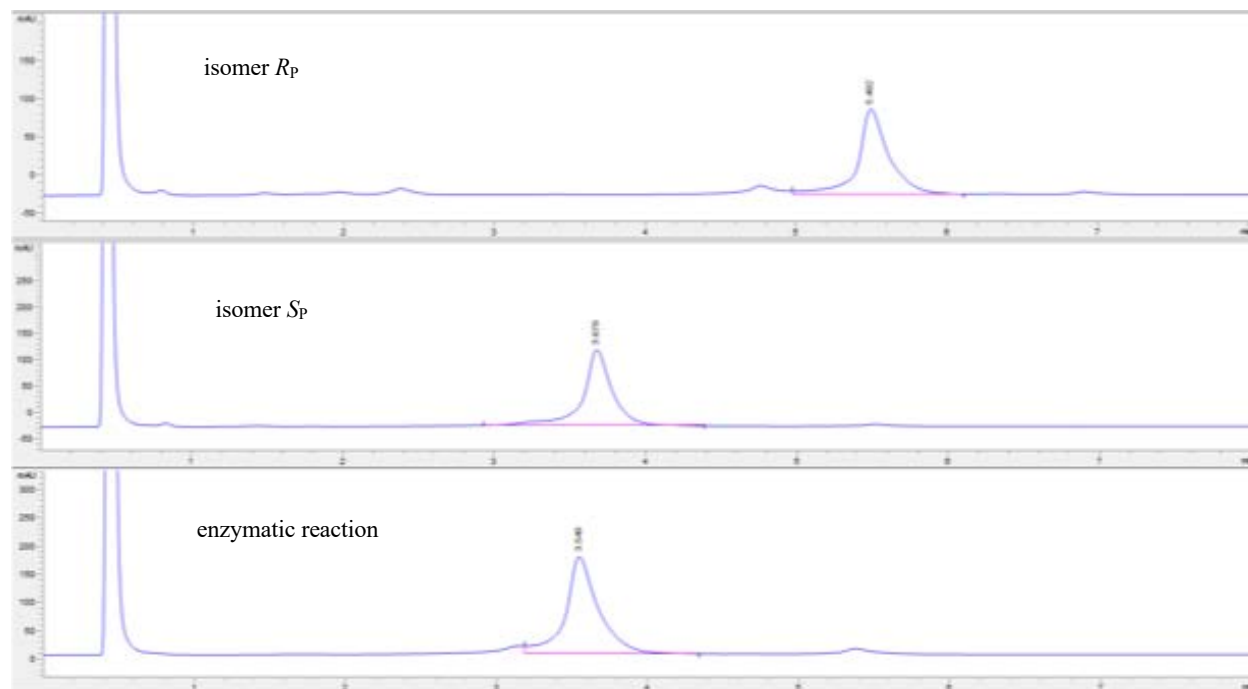
^{13}C NMR (151 MHz, D_2O): δ 159.7, 158.3, 157.1 (d, $J = 19.6$ Hz), 150.5 (d, $J = 18.6$ Hz), 140.3, 116.9, 86.9, 83.9 (d, $J = 9.4$ Hz), 74.3, 70.4, 65.1 (d, $J = 6.5$ Hz).

^{31}P NMR (243 MHz, D_2O): δ 43.32 (d, $J = 27.0$ Hz), -6.27 (d, $J = 19.8$ Hz), -22.73 (dd, $J = 22.6, 22.6$ Hz).

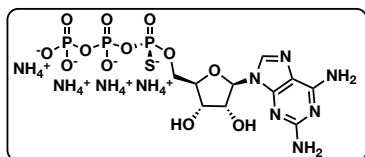
^{19}F NMR (565 MHz, D_2O): δ -53.0.

HRMS [ESI] $\text{C}_{10}\text{H}_{15}\text{FN}_5\text{O}_{12}\text{P}_3\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 539.9562 found 539.9560.

Retention time: 3.55 min (HPLC method 2)



Compound 53



5'-O-2-aminoadenosine-tetrammonium (*S*)-triphosphoro- α -thioate

Compound **53** was obtained according to general procedure (**Method C**) from 2-aminoadenosine (0.05 mmol, 14.1 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase, *Sc*-AK (2.5 mg, 0.21 mol%) as monophosphate kinase, *Tm*-AcK (2.5 mg, 0.16 mol%) as acetate kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 50:50) to afford 4.6 mg of the title compound after lyophilization (**Yield = 16%, dr = 9:1**). The absolute configuration at phosphorus was determined by comparison with standard compounds using HPLC.

Physical state: white solid

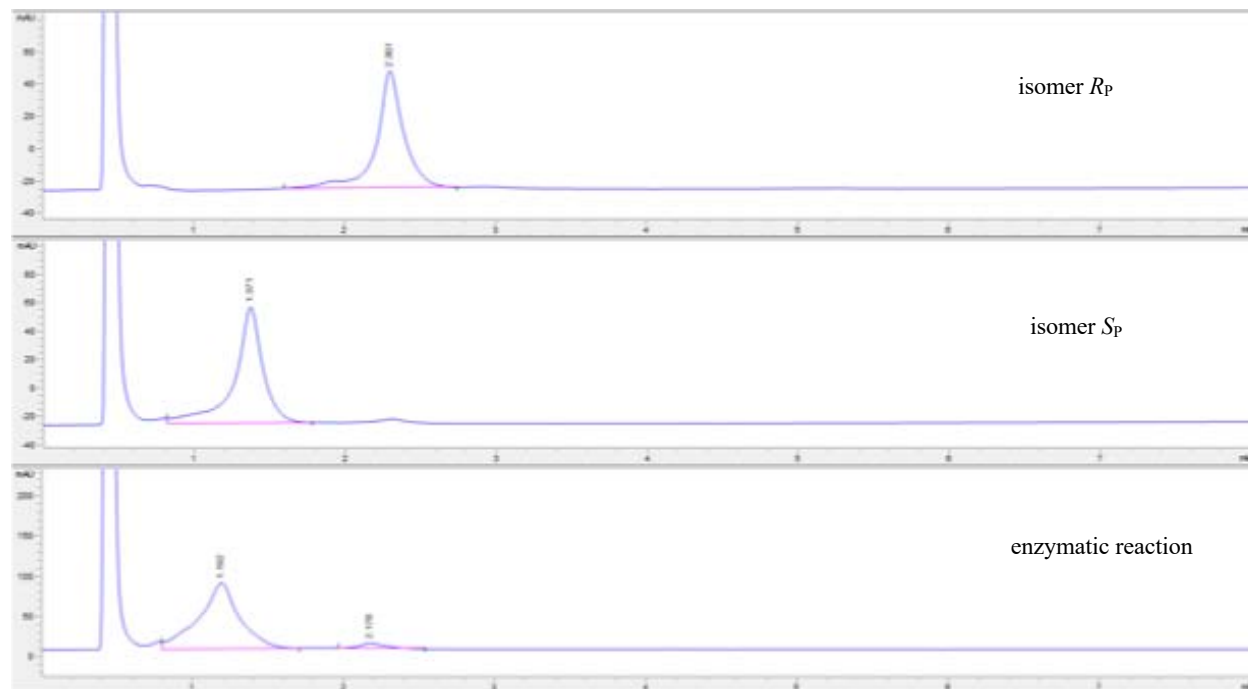
^1H NMR (600 MHz, D_2O): δ 8.29 (s, 1H), 5.91 (d, $J = 5.8$ Hz, 1H), 4.71 – 4.66 (m, 1H), 4.58 – 4.52 (m, 1H), 4.40 – 4.37 (m, 1H), 4.36 – 4.26 (m, 2H).

^{13}C NMR (151 MHz, D_2O): δ 156.4, 152.9, 150.9, 138.4, 111.8, 86.6, 83.5 (d, $J = 9.6$ Hz), 74.0, 70.2, 65.1 (d, $J = 6.4$ Hz).

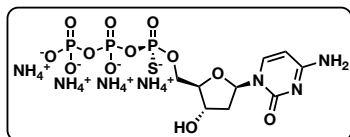
^{31}P NMR (243 MHz, D_2O): δ 43.8 (d, $J = 27.5$ Hz), -10.0 (br), -23.5 (br).

HRMS [ESI] $\text{C}_{10}\text{H}_{17}\text{N}_6\text{O}_{12}\text{P}_3\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 536.9765 found 536.9766.

Retention time: 1.19 min (HPLC method 2)



Compound 54



5'-O-deoxycytidine-tetrammonium (*R*)-triphosphoro- α -thioate

Compound **54** was obtained according to general procedure (**Method D**) from deoxycytidine (0.05 mmol, 11.4 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase, *Bs*-CMPK (2.5 mg, 0.20 mol%) as monophosphate kinase, *Ec*-AcK (2.5 mg, 0.20 mol%) as acetate kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 35:65) to afford 16.1 mg of the title compound after lyophilization (**Yield = 58%**, **dr = 11:1**). The absolute configuration at phosphorus was determined by comparison with standard compounds using HPLC.

Physical state: white solid

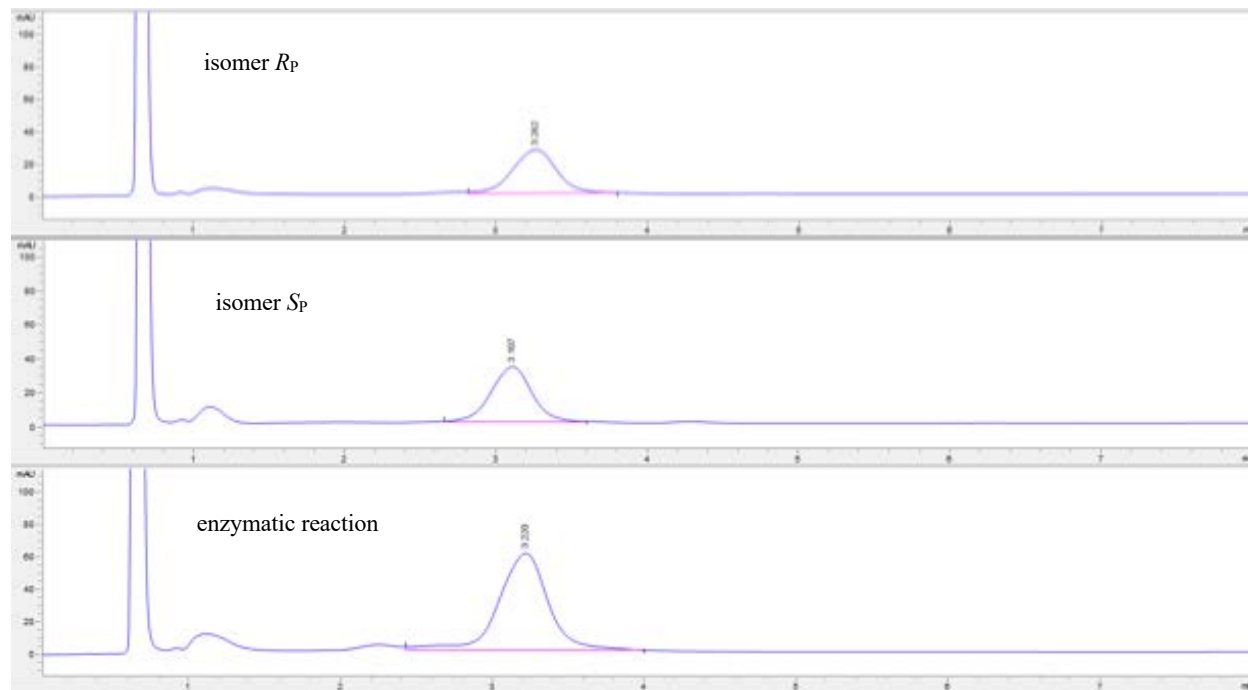
^1H NMR (600 MHz, D_2O): δ 8.18 (d, $J = 7.8$ Hz, 1H), 6.30 (t, $J = 6.5$ Hz, 1H), 6.26 (d, $J = 7.8$ Hz, 1H), 4.65 (dt, $J = 6.8, 3.7$ Hz, 1H), 4.33 – 4.22 (m, 2H), 2.46 (ddd, $J = 14.1, 6.3, 3.9$ Hz, 1H), 2.38 (ddd, $J = 14.1, 6.5, 6.5$ Hz, 1H).

^{13}C NMR (151 MHz, D_2O): δ 161.9, 152.0, 143.5, 95.8, 86.3, 85.8 (d, $J = 9.7$ Hz), 70.6, 65.4 (d, $J = 6.1$ Hz), 39.4.

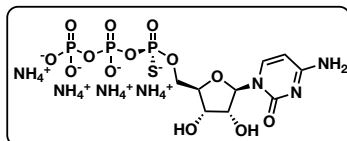
^{31}P NMR (243 MHz, D_2O): δ 43.4 (d, $J = 27.3$ Hz), -10.8 (d, $J = 19.2$ Hz), -24.0 (dd, $J = 27.0, 19.1$ Hz).

HRMS [ESI] $\text{C}_9\text{H}_{16}\text{N}_3\text{O}_{12}\text{P}_3\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 481.9595 found 481.9593.

Retention time: 3.22 min (HPLC method 2)



Compound 55



5'-O-cytidine-tetrammonium (*R*)-triphosphoro- α -thioate

Compound **55** was obtained according to general procedure (**Method D**) from cytidine (0.05 mmol, 12.2 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase, *Bs*-CMPK (2.5 mg, 0.20 mol%) as monophosphate kinase, *Ec*-AcK (2.5 mg, 0.20 mol%) as acetate kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 35:65) to afford 12.0 mg of the title compound after lyophilization (**Yield = 42%, dr = 10:1**). The absolute configuration at phosphorus was determined by comparison with standard compounds using HPLC.

Physical state: white solid

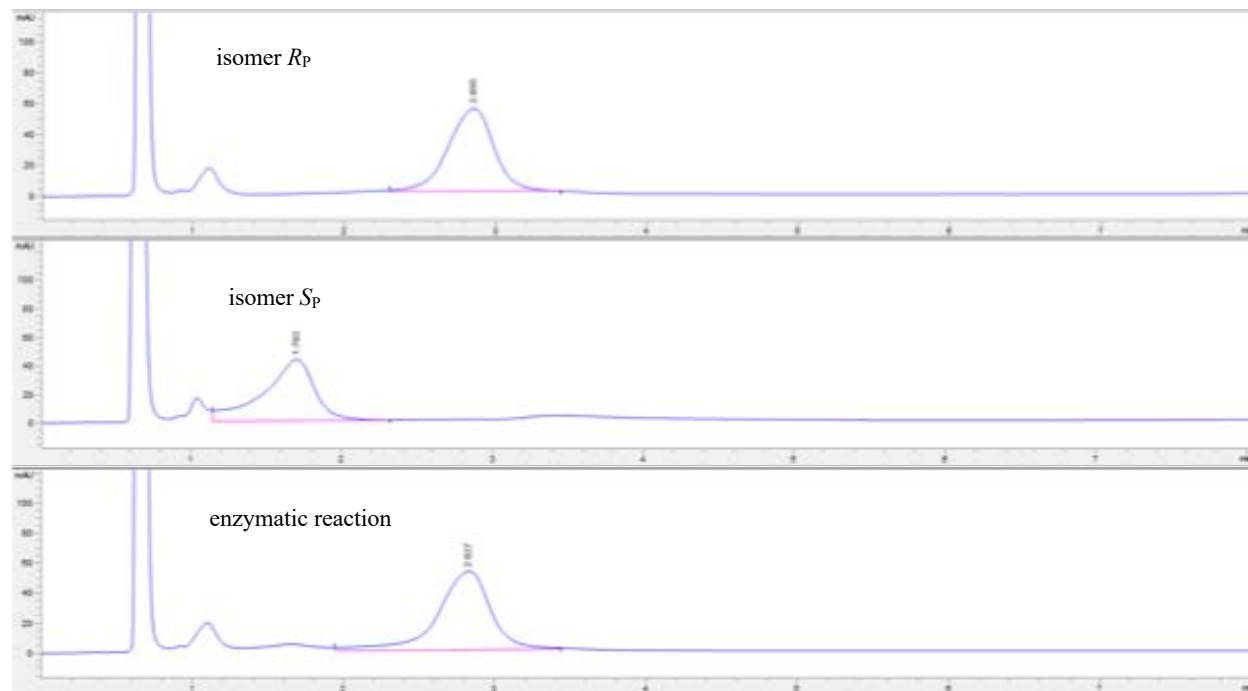
^1H NMR (600 MHz, D_2O): δ 8.07 (d, J = 7.6 Hz, 1H), 6.18 (d, J = 7.7 Hz, 1H), 6.01 (d, J = 4.4 Hz, 1H), 4.43 (dd, J = 4.9, 4.9 Hz, 1H), 4.38 – 4.33 (m, 2H), 4.33 – 4.28 (m, 2H).

^{13}C NMR (151 MHz, D_2O): δ 165.7, 157.1, 141.8, 96.6, 89.1, 82.7 (d, J = 9.7 Hz), 74.2, 69.3, 64.9 (d, J = 5.5 Hz).

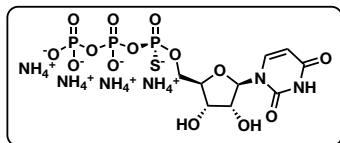
^{31}P NMR (243 MHz, D_2O): δ 43.4 (d, J = 27.8 Hz), -10.0 (d, J = 20.7 Hz), -23.8 (dd, J = 28.0, 20.5 Hz). After hexokinase purification, ^{31}P NMR data collected was less resolved, some J values were collected from sample before hexokinase purification.

HRMS [ESI] $\text{C}_9\text{H}_{16}\text{N}_3\text{O}_{13}\text{P}_3\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 497.9544 found 497.9593.

Retention time: 2.83 min (HPLC method 2)



Compound 56



5'-*O*-uridine-tetrammonium (*R*)-triphospho- α -thioate

Compound **56** was obtained according to general procedure (**Method D**) from uridine (0.05 mmol, 12.2 mg) using *Ta*-NK (2.5 mg, 0.16 mol%) as nucleoside kinase, *Bs*-CMPK (2.5 mg, 0.20 mol%) as monophosphate kinase, *Ec*-AcK (2.5 mg, 0.20 mol%) as acetate kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 35:65) to afford 4.6 mg of the title compound after lyophilization (**Yield = 16%, dr > 19:1**). The absolute configuration at phosphorus was determined by comparison with standard compounds using HPLC.

Physical state: white solid

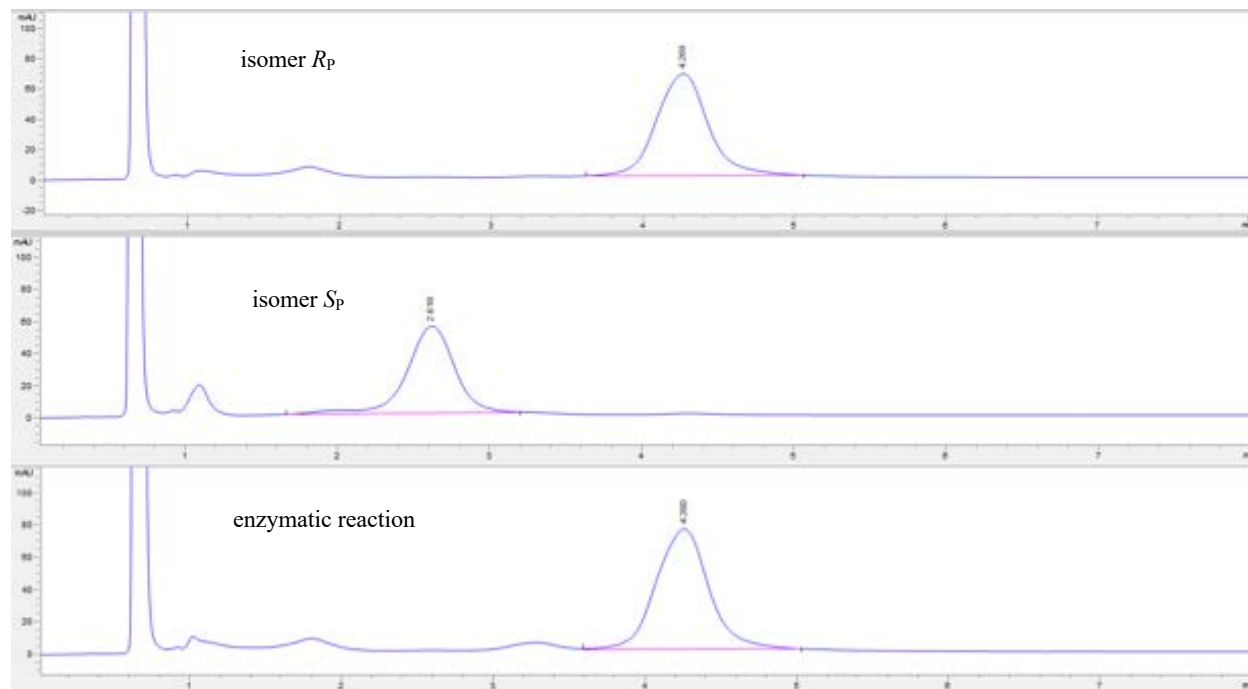
^1H NMR (600 MHz, D_2O): δ 8.04 (d, J = 7.9 Hz, 1H), 6.00 (dd, J = 10.4, 6.6 Hz, 2H), 4.49 – 4.40 (m, 2H), 4.35 – 4.28 (m, 3H).

^{13}C NMR (151 MHz, D_2O): δ 166.3, 151.9, 141.9, 102.6, 88.1, 83.2 (d, J = 9.7 Hz), 73.7, 69.7, 65.3 (d, J = 5.9 Hz).

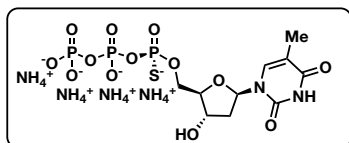
^{31}P NMR (243 MHz, D_2O): δ 43.4 (d, J = 29.8 Hz), -8.9 (d, J = 19.9 Hz), -23.2 (dd, J = 30.0, 20.0 Hz). After hexokinase purification, ^{31}P NMR data collected was less resolved, J values were collected from sample before hexokinase purification.

HRMS [ESI] $\text{C}_9\text{H}_{15}\text{N}_2\text{O}_{14}\text{P}_3\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 498.9384 found 498.9389.

Retention time: 4.26 min (HPLC method 2)



Compound 57



5'-O-thymidine-tetrammonium (*R*)-triphosphoro- α -thioate

Compound **57** was obtained according to general procedure (**Method D**) from thymidine (0.05 mmol, 12.1 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase, *Ec*-TmK (2.5 mg, 0.21 mol%) as monophosphate kinase, *Ec*-AcK (2.5 mg, 0.20 mol%) as acetate kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 35:65) to afford 11.9 mg of the title compound after lyophilization (**Yield = 42%**, **dr > 19:1**). The absolute configuration at phosphorus was determined by comparison with standard compounds using HPLC.

Physical state: white solid

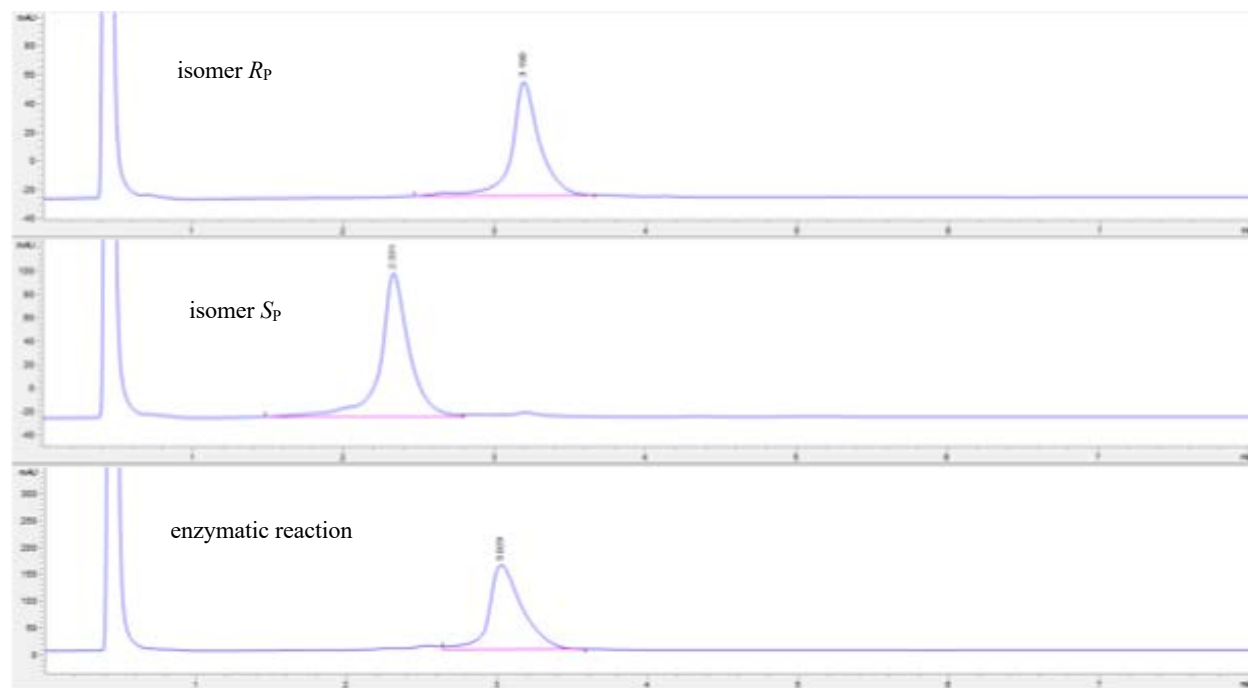
^1H NMR (600 MHz, D_2O): δ 7.80 (s, 1H), 6.37 (dd, $J = 7.7, 6.3$ Hz, 1H), 4.69 (dt, $J = 6.1, 3.1$ Hz, 1H), 4.30 – 4.25 (m, 2H), 4.25 – 4.20 (m, 1H), 2.41 (ddd, $J = 14.0, 7.8, 6.1$ Hz, 1H), 2.35 (ddd, $J = 14.1, 6.3, 3.2$ Hz, 1H), 1.96 (s, 3H).

^{13}C NMR (151 MHz, D_2O): δ 166.6, 151.8, 137.4, 111.8, 85.4 (d, $J = 9.8$ Hz), 84.9, 71.1, 65.9 (d, $J = 6.2$ Hz), 38.6, 11.7.

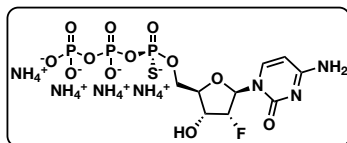
^{31}P NMR (243 MHz, D_2O): δ 43.0 (d, $J = 28.5$ Hz), -10.7 (d, $J = 19.8$ Hz), -23.9 (dd, $J = 28.5, 19.8$ Hz). After hexokinase purification, ^{31}P NMR data collected was less resolved, J values were collected from sample before hexokinase purification.

HRMS [ESI] $\text{C}_{10}\text{H}_{17}\text{N}_2\text{O}_{13}\text{P}_3\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 496.9591 found 496.9590.

Retention time: 3.03 min (HPLC method 2)



Compound 58



5'-O-2'-deoxy-2'-fluorocytidine-tetrammonium (*R*)-triphosphoro- α -thioate

Compound **58** was obtained according to general procedure (**Method D**) from 2'-deoxy-2'-fluorocytidine (0.05 mmol, 12.3 mg)) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase, *Bs*-CMPK (2.5 mg, 0.20 mol%) as monophosphate kinase, *Ec*-AcK (2.5 mg, 0.20 mol%) as acetate kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 35:65) to afford 15.0 mg of the title compound after lyophilization (**Yield** = **53%**, **dr** = **9:1**). The absolute configuration at phosphorus was determined by comparison with standard compounds using HPLC.

Physical state: white solid

^1H NMR (600 MHz, D_2O): δ 8.15 (d, J = 7.3 Hz, 1H), 6.30 (brs, 1H), 6.10 (d, J = 16.7 Hz, 1H), 5.16 (dd, J = 52.1, 4.0 Hz, 1H), 4.59 – 4.44 (m, 2H), 4.38 – 4.29 (m, 2H).

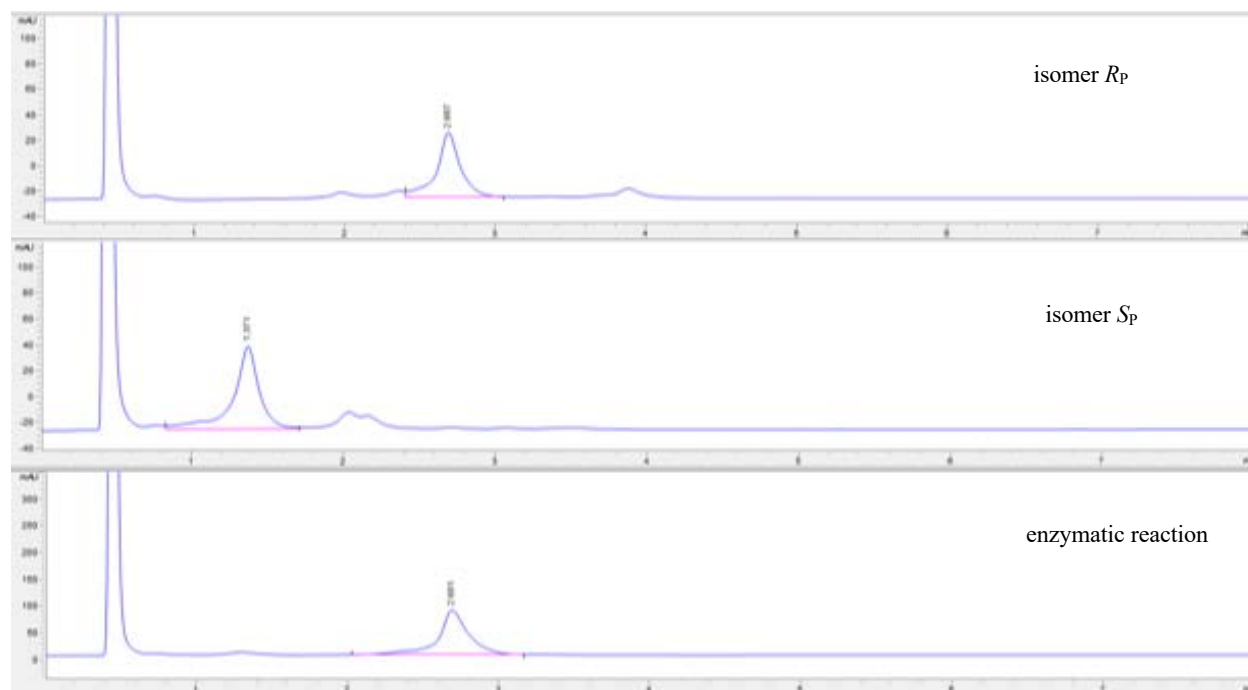
^{13}C NMR (151 MHz, D_2O): δ 163.3, 153.3, 142.6, 94.4, 93.1, 88.5 (d, J = 34.5 Hz), 81.2 (d, J = 9.6 Hz), 67.2 (d, J = 16.1 Hz), 63.4 (d, J = 4.7 Hz).

^{31}P NMR (243 MHz, D_2O): δ 43.4 (d, J = 27.0 Hz), -10.3 (d, J = 19.3 Hz), -23.4 (dd, J = 27.2, 19.6 Hz). After hexokinase purification, ^{31}P NMR data collected was less resolved, J values were collected from sample before hexokinase purification.

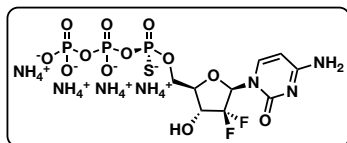
^{19}F NMR (565 MHz, D_2O): δ -203.3 (ddd, J = 52.1, 23.6, 16.6 Hz).

HRMS [ESI] $\text{C}_9\text{H}_{15}\text{FN}_3\text{O}_{12}\text{P}_3\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 499.9501 found 499.9501.

Retention time: 2.69 min (HPLC method 2)



Compound 59



5'-*O*-gemcitabine-tetrammonium (*R*)-triphosphoro- α -thioate

Compound **59** was obtained according to general procedure (**Method C**) from gemcitabine (0.05 mmol, 13.2 mg) using *Dm*-dNK (2.5 mg, 0.17 mol%) as nucleoside kinase, *Bs*-CMPK (2.5 mg, 0.20 mol%) as monophosphate kinase, *Ec*-AcK (2.5 mg, 0.20 mol%) as acetate kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 40:60) to afford 15.6 mg of the title compound after lyophilization (**Yield = 53%**, **dr = 9:1**). The absolute configuration at phosphorus was determined by comparison with standard compounds using HPLC.

Physical state: white solid

^1H NMR (600 MHz, D_2O): δ 7.92 (d, $J = 7.6$ Hz, 1H), 6.27 – 6.21 (m, 1H), 6.13 (d, $J = 7.6$ Hz, 1H), 4.63 – 4.52 (m, 1H), 4.49 – 4.32 (m, 2H), 4.24 – 4.14 (m, 1H).

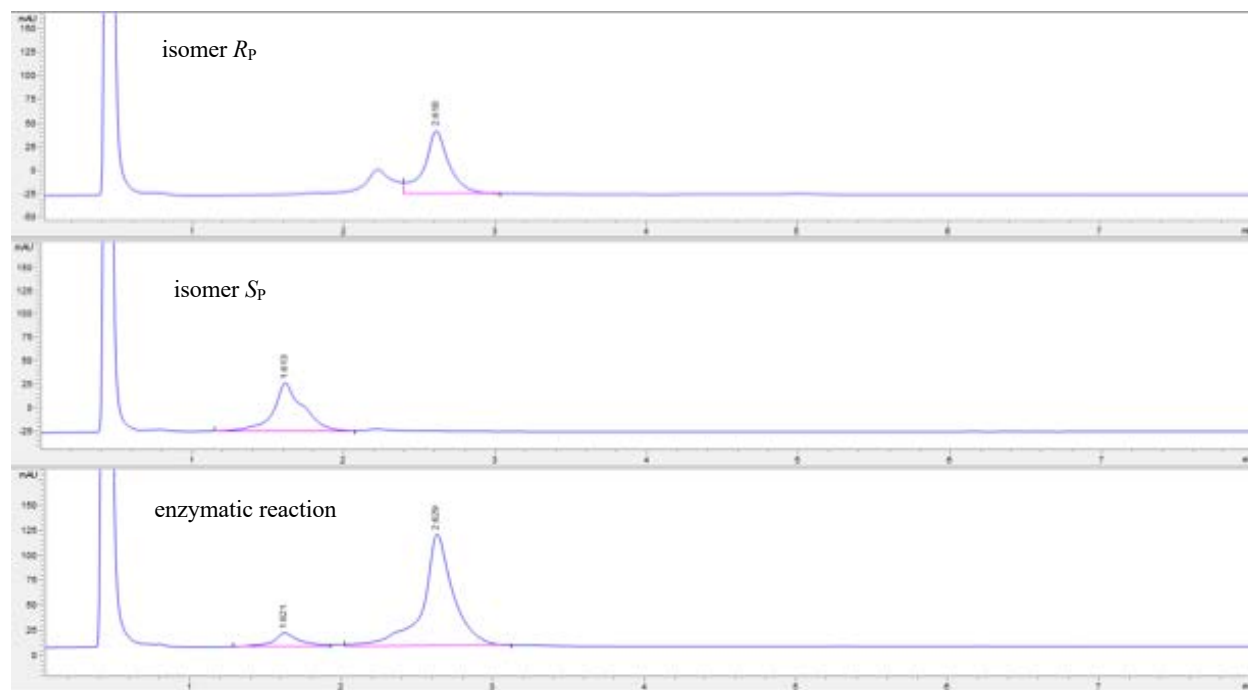
^{13}C NMR (151 MHz, D_2O): δ 166.3, 157.3, 141.2, 124.9 – 120.4 (m), 96.8, 85.0 – 84.1 (m), 78.7 (t, $J = 9.2$ Hz), 68.3 – 67.7 (m), 63.1 (d, $J = 5.3$ Hz).

^{31}P NMR (243 MHz, D_2O): δ 43.1 (d, $J = 28.6$ Hz), -6.3, -22.9. Coupling constants for the latter two multiplets are not well resolved but are likely to have similar values as its other derivatives reported in this work.

^{19}F NMR (565 MHz, D_2O): δ -117.3 – -119.0 (m).

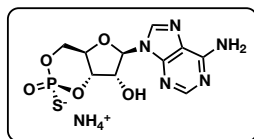
HRMS [ESI] $\text{C}_9\text{H}_{14}\text{F}_2\text{N}_3\text{O}_{12}\text{P}_3\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 517.9406 found 517.9408.

Retention time: 2.63 min (HPLC method 2)



9.5 Cyclic nucleosides thiotriphosphate

Compound 60



R_P-adenosine-3',5'-cyclic monophosphorothioate

Compound **60** was obtained according to general procedure (**Method H**) from adenosine (0.05 mmol, 13.2 mg) using *Ta*-NK (2.5 mg, 0.17 mol%) as nucleoside kinase, *Sc*-AK (2.5 mg, 0.20 mol%) as monophosphate kinase, *Ec*-AcK (2.5 mg, 0.20 mol%) as acetate kinase, *Ar*-Ac (2.5 mg, 0.13 mol%) as nucleoside cyclase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 40:60) to afford 6.7 mg of the title compound after lyophilization (**Yield = 37%, dr = 10:1**). The absolute configuration at phosphorus should be opposite to *S_P*-ATPαS based on *S_N2* mechanism.

Physical state: white solid

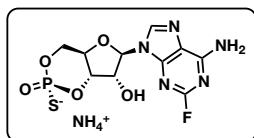
¹H NMR (600 MHz, D₂O): δ 8.27 (s, 1H), 8.26 (s, 1H), 6.19 (s, 1H), 4.88 – 4.84 (m, 1H), 4.54 (ddd, *J* = 20.3, 8.7, 4.1 Hz, 1H), 4.42 (dtd, *J* = 19.7, 10.5, 4.6 Hz, 2H). There is one H buried underneath D₂O.

¹³C NMR (151 MHz, D₂O): δ 155.6, 153.0, 148.5, 139.9, 118.9, 91.2, 76.0 (d, *J* = 6.7 Hz), 72.2 (d, *J* = 7.5 Hz), 71.4 (d, *J* = 5.4 Hz), 67.7 (d, *J* = 9.6 Hz).

³¹P NMR (243 MHz, D₂O): δ 55.9

HRMS [ESI] C₁₀H₁₂N₅O₅PS ([*M*-H]⁻) calculated 344.0224 found 344.0223.

Compound 61



R_P-2-fluoroadenosine-3',5'-cyclic monophosphorothioate

Compound **61** was obtained according to general procedure (**Method H**) from adenosine (0.05 mmol, 13.2 mg) using *Ta*-NK (2.5 mg, 0.17 mol%) as nucleoside kinase, *Sc*-AK (2.5 mg, 0.20 mol%) as monophosphate kinase, *Ec*-AcK (2.5 mg, 0.20 mol%) as acetate kinase, *Ar*-Ac (2.5 mg, 0.13 mol%) as nucleoside cyclase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 40:60) to afford 4.4 mg of the title compound after lyophilization (**Yield = 23%, dr = 10:1**). The absolute configuration at phosphorus should be opposite to *S_P*-ATPαS based on *S_N2* mechanism.

Physical state: white solid

¹H NMR (600 MHz, D₂O): δ 8.18 (s, 1H), 6.08 (s, 1H), 4.86 – 4.80 (m, 1H), 4.74 (d, *J* = 5.3 Hz, 1H), 4.53 (ddd, *J* = 20.5, 9.3, 4.7 Hz, 1H), 4.41 (dtt, *J* = 25.0, 10.2, 4.1 Hz, 2H). There is one H mixed with D₂O.

¹³C NMR (151 MHz, D₂O): δ 158.9 (d, *J* = 211.2 Hz), 157.1 (d, *J* = 19.5 Hz), 149.6, 140.0, 117.1, 91.1, 75.9 (d, *J* = 6.6 Hz), 72.1 (d, *J* = 7.5 Hz), 71.4 (d, *J* = 5.5 Hz), 67.6 (d, *J* = 9.5 Hz).

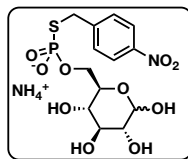
¹⁹F NMR (565 MHz, D₂O): δ -52.9.

³¹P NMR (243 MHz, D₂O): δ 55.8

HRMS [ESI] C₁₀H₁₁FN₅O₅PS ([M-H]⁻) calculated 362.0130 found 362.0130.

9.6 Other biomolecules thiophosphate and derivatives

Compound 62



p-nitrobenzyl protected-6-*O*-glucose-ammonium monophosphorothioate

Compound **62** was obtained according to general procedure (**Method E**) from D-glucose (0.05 mmol, 9.0 mg) using *Sc*-hexokinase (75U) as sugar kinase. NMR yield of thiophosphorylation is 86%. After one-pot protection, the crude product was first purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) and then further purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford 12.8 mg of the title compound after lyophilization (**Yield = 60%, α : β = 9:11**). Isomers were assigned based on coupling constants of the anomeric proton.

Physical state: white solid

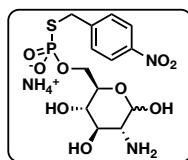
¹H NMR (600 MHz, D₂O): ¹H NMR (600 MHz, D₂O) δ 8.26 – 8.21 (m, 2H), 7.67 – 7.62 (m, 2H), 5.14 (d, J = 3.8 Hz, 1H, α isomer), 4.55 (d, J = 8.0 Hz, 0.55H, β isomer), 4.06 (d, J = 13.8 Hz, 2H), 4.00 – 3.95 (m, 1H, β isomer), 3.93 – 3.81 (m, 1H, α + β isomer), 3.66 – 3.58 (m, 1H, α + β isomer), 3.49 – 3.46 (m, 1H, α isomer), 3.44 – 3.38 (m, 1H, β isomer), 3.38 – 3.27 (m, 2H, α + β isomer), 3.24 – 3.19 (m, 1H, β isomer).

¹³C NMR (151 MHz, D₂O): δ 146.7(α + β isomer), 146.6(α + β isomer), 129.9 (β isomer), 129.8 (α isomer), 123.9 (α isomer), 123.9 (β isomer), 95.9 (β isomer), 92.0 (α isomer), 75.5(β isomer), 74.5 (α + β isomer), 74.0 (β isomer), 72.5 (α isomer), 71.4 (α isomer), 70.1 (α + β isomer), 69.5 (α isomer), 69.3 (β isomer), 64.6 (α + β isomer), 33.5 (α + β isomer).

³¹P NMR (243 MHz, D₂O): δ 20.1.

HRMS [ESI] C₁₃H₁₈NO₁₀PS ([M-H]⁻) calculated 410.0316 found 410.0315.

Compound 63



p-nitrobenzyl protected-6-*O*-2-glucosamine-ammonium monophosphorothioate

Compound **63** was obtained according to general procedure (**Method E**) from D-glucosamine hydrochloride (0.05 mmol, 10.8 mg) using *Sc*-hexokinase (75U) as sugar kinase. NMR yield of thiophosphorylation is 68%. After one-pot protection, the crude product was first purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) and then further purified by reverse phase chromatography on C18 column (methanol/water, from

0:100 to 100:0) to afford 4.1 mg of the title compound after lyophilization (**Yield = 19%, $\alpha:\beta = 3:2$**). Isomers were assigned based on coupling constants of the anomeric proton.

Physical state: white solid

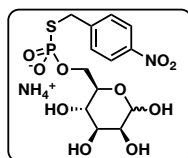
^1H NMR (600 MHz, D_2O): δ 8.22 (d, $J = 8.5$ Hz, 2H), 7.63 (dd, $J = 8.5, 6.3$ Hz, 2H), 5.29 (d, $J = 3.5$ Hz, 1H, α isomer), 4.69 (d, $J = 8.3$ Hz, 1H, β isomer), 4.06 (d, $J = 10.5$ Hz, 2H, $\alpha+\beta$ isomer), 3.99 – 3.93 (m, 1H, β isomer), 3.91 – 3.85 (m, 1H, $\alpha+\beta$ isomer), 3.85 – 3.79 (m, 1H, α isomer), 3.71 (dd, $J = 10.5, 9.1$ Hz, 1H, α isomer), 3.59 – 3.53 (m, 1H, α isomer), 3.50 – 3.45 (m, 1H, β isomer), 3.42 – 3.30 (m, 2H, mixtures of two isomers), 3.10 (dd, $J = 10.5, 3.6$ Hz, 1H, α isomer), 2.79 (dd, $J = 10.3, 8.3$ Hz, 1H, β isomer).

^{13}C NMR (151 MHz, D_2O): δ 147.3 (d, $J = 3.9$ Hz, α isomer), 147.1 (d, $J = 3.9$ Hz, β isomer), 146.7 (β isomer), 146.6 (α isomer), 129.8, 123.9 (α isomer), 123.8 (β isomer), 94.5 (β isomer), 89.8 (α isomer), 74.7 (d, $J = 9.2$ Hz, β isomer), 73.5 (β isomer), 70.3 ($\alpha+\beta$ isomer), 70.2 (α isomer), 69.5 ($\alpha+\beta$ isomer), 69.4 (β isomer), 56.9 (β isomer), 54.4 (α isomer), 33.5 (d, $J = 3.6$ Hz, $\alpha+\beta$ isomer).

^{31}P NMR (243 MHz, D_2O): δ 20.1 (α isomer), 20.0 (β isomer).

HRMS [ESI] $\text{C}_{13}\text{H}_{18}\text{N}_2\text{O}_9\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 409.0476 found 409.0475.

Compound 64



p-nitrobenzyl protected-6-*O*-mannose-ammonium monophosphorothioate

Compound **64** was obtained according to general procedure (**Method E**) from D-mannose (0.05 mmol, 9.0 mg) using *Sc*-hexokinase (75U) as sugar kinase. NMR yield of thiophosphorylation is 72%. After one-pot protection, the crude product was first purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 20:80) and then further purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford 12.4 mg of the title compound after lyophilization (**Yield = 58%, $\alpha:\beta = 3:1$**). Isomers were assigned based on chemical shifts and coupling constants of the anomeric proton and related reference⁸.

Physical state: white solid

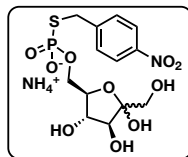
^1H NMR (600 MHz, D_2O): δ 8.25 – 8.19 (m, 2H), 7.67 – 7.61 (m, 2H), 5.08 (d, $J = 1.8$ Hz, 1H, α isomer), 4.79 (overlaps with D_2O , 1H, β isomer), 4.06 (d, $J = 10.8$ Hz, 2H, $\alpha+\beta$ isomer), 4.02 – 3.89 (m, 1H, $\alpha+\beta$ isomer), 3.88 – 3.85 (m, α isomer), 3.85 – 3.76 (m, 1H, $\alpha+\beta$ isomer), 3.73 (dd, $J = 9.6, 3.4$ Hz, 1H, α isomer), 3.59 – 3.47 (m, 1H, $\alpha+\beta$ isomer), 3.46 – 3.39 (m, 1H, α isomer), 3.20 (ddd, $J = 9.1, 6.4, 2.1$ Hz, 1H, β isomer).

^{13}C NMR (151 MHz, D_2O): δ 147.2 (d, $J = 3.6$ Hz, $\alpha+\beta$ isomer), 146.6 ($\alpha+\beta$ isomer), 129.9 (β isomer), 129.8 (α isomer), 123.9 (α isomer), 123.8 (α isomer), 93.9 (α isomer), 93.7 (α isomer), 74.8 (d, $J = 8.4$ Hz, α isomer), 72.9 (β isomer), 71.1 (α isomer), 71.0 (β isomer), 70.5 (α isomer), 70.0 (α isomer), 66.6 (α isomer), 66.3 (β isomer), 64.9 ($\alpha+\beta$ isomer), 33.5 (d, $J = 3.5$ Hz, $\alpha+\beta$ isomer).

^{31}P NMR (243 MHz, D_2O): δ 20.2 (α isomer), 20.1 (β isomer).

HRMS [ESI] $\text{C}_{13}\text{H}_{18}\text{NO}_{10}\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 410.0316 found 410.0314.

Compound 65



p-nitrobenzyl protected-6-*O*-fructose-ammonium monophosphorothioate

Compound **65** was obtained according to general procedure (**Method E**) from D-fructose (0.05 mmol, 9.0 mg) using *Sc*-hexokinase (75U) as sugar kinase. NMR yield of thiophosphorylation is 80%. After one-pot protection, the crude product was first purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 20:80) and then further purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford 16.2 mg of the title compound after lyophilization (**Yield = 76%**, **dr = 4:1**).

Physical state: white solid

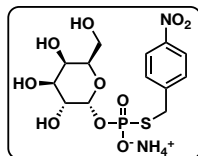
^1H NMR (600 MHz, D_2O): δ 8.23 (m, 2H), 7.67 – 7.61 (m, 2H), 4.09 – 3.97 (m, 4H), 3.85 – 3.65 (m, 2H), 3.60 – 3.47 (m, 3H).

^{13}C NMR (151 MHz, D_2O): δ 147.3, 147.2, 146.6, 129.8, 123.8, 104.5, 101.6, 81.6, 78.9, 78.9, 76.1, 75.0, 74.4, 66.2, 66.1, 62.7, 62.5, 33.5, 33.5.

^{31}P NMR (243 MHz, D_2O): δ 19.9 (minor), 19.8 (major)

HRMS [ESI] $\text{C}_{13}\text{H}_{18}\text{NO}_{10}\text{PS}$ ($[\text{M}-\text{H}]^-$) calculated 410.0316 found 410.0314.

Compound 66



p-nitrobenzyl protected-1-*O*-galactose-ammonium monophosphorothioate

Compound **66** was obtained according to general procedure (**Method E**) from D-galactose (0.05 mmol, 9.0 mg) using *Ec*-GalK Y371H (5.0 mg, 0.24 mol%) as sugar kinase. NMR yield of thiophosphorylation is 83%. After one-pot protection, the crude product was first purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 20:80) and then further purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford 8.7 mg of the title compound after lyophilization (**Yield = 41%**). The configuration of the anomeric carbon was assigned based on chemical shift of the anomeric proton and coupling constant⁹.

Physical state: white solid

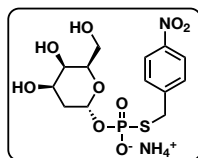
¹H NMR (600 MHz, D₂O): δ 8.25 – 8.19 (m, 2H), 7.68 – 7.62 (m, 2H), 5.59 (dd, *J* = 8.7, 3.7 Hz, 1H), 4.16 – 4.14 (m, 1H), 4.13 – 4.11 (m, 1H), 3.92 – 3.88 (m, 1H), 3.86 – 3.78 (m, 2H), 3.71 (dd, *J* = 10.3, 3.4 Hz, 1H), 3.69 – 3.66 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 147.1 (d, *J* = 5.1 Hz), 146.6, 129.9, 123.8, 95.6 (d, *J* = 7.2 Hz), 71.8, 69.0, 69.0, 68.0 (d, *J* = 7.8 Hz), 60.7, 33.7 (d, *J* = 3.7 Hz).

³¹P NMR (243 MHz, D₂O): δ 18.2.

HRMS [ESI] C₁₃H₁₈NO₁₀PS ([M-H]⁻) calculated 410.0316 found 410.0316.

Compound 67



p-nitrobenzyl protected-1-*O*-galactose-ammonium monophosphorothioate

Compound **67** was obtained according to general procedure (**Method E**) from 2-deoxy-D-galactose (0.05 mmol, 8.4 mg) using *Ec*-GalK Y371H (5.0 mg, 0.24 mol%) as sugar kinase. NMR yield of thiophosphorylation is 60%. After one-pot protection, the crude product was first purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) and then further purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford 7.4 mg of the title compound after lyophilization (**Yield = 38%**).

Physical state: white solid

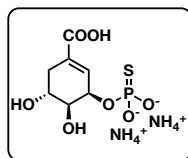
¹H NMR (600 MHz, D₂O): 8.27 – 8.22 (m, 2H), 7.68 – 7.63 (m, 2H), 5.59 – 5.54 (m, 1H), 4.12 – 4.09 (m, 1H), 4.09 – 4.07 (m, 1H), 3.84 (t, *J* = 6.2 Hz, 1H), 3.79 – 3.73 (m, 2H), 3.68 – 3.64 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 147.5 (d, *J* = 3.5 Hz), 146.7, 129.9, 123.8, 94.6 (d, *J* = 6.0 Hz), 72.2, 67.5, 64.4, 61.3, 33.8 (d, *J* = 3.9 Hz), 31.8 (d, *J* = 6.5 Hz).

³¹P NMR (243 MHz, D₂O): δ 17.4.

HRMS [ESI] C₁₃H₁₈NO₉PS ([M-H]⁻) calculated 394.0367 found 394.0366.

Compound 68



1-*O*-shikimate-ammonium monophosphorothioate

Compound **68** was obtained according to general procedure (**Method E**) from (-) shikimic acid (0.05 mmol, 8.7 mg) using *Ec*-AroL (2.5 mg, 0.26 mol%) as sugar kinase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 30:70) to afford 14.4 mg of the title compound after lyophilization (**Yield = 95%**).

Physical state: white solid

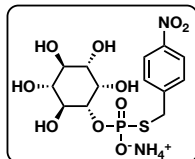
¹H NMR (600 MHz, D₂O): δ 6.59 – 6.54 (m, 1H), 5.07 – 5.00 (m, 1H), 4.13 – 4.05 (m, 1H), 3.91 (dd, *J* = 7.9, 4.1 Hz, 1H), 2.76 – 2.68 (m, 1H), 2.27 – 2.19 (m, 1H).

¹³C NMR (151 MHz, D₂O): δ 174.36, 134.66, 131.32 (d, *J* = 3.7 Hz), 70.64 (d, *J* = 4.1 Hz), 70.48 (d, *J* = 5.5 Hz), 67.25.

³¹P NMR (243 MHz, D₂O): δ 48.1.

HRMS [ESI] C₇H₁₁O₇PS ([M-H]⁻) calculated 268.9890 found 268.9893.

Compound 69



p-nitrobenzyl protected-3-*O*-inositol-ammonium monophosphorothioate

Compound **69** was obtained according to general procedure (**Method E**) from myo-inositol (0.05 mmol, 9.0 mg) using *Tk*-I3P (5.0 mg, 0.33 mol%) as sugar kinase. NMR yield of thiophosphorylation is 62%. After one-pot protection, the crude product was first purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) and then further purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford 9.1 mg of the title compound after lyophilization (**Yield = 43%**).

Physical state: white solid

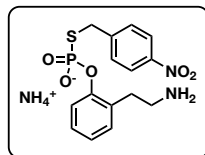
¹H NMR (600 MHz, D₂O): δ 8.25 – 8.20 (m, 2H), 7.67 – 7.62 (m, 2H), 4.17 (t, *J* = 2.9 Hz, 1H), 4.13 (dd, *J* = 12.0, 5.5 Hz, 2H), 4.00 (td, *J* = 10.3, 2.8 Hz, 1H), 3.76 (t, *J* = 9.7 Hz, 1H), 3.65 (t, *J* = 9.7 Hz, 1H), 3.50 (dd, *J* = 10.0, 2.8 Hz, 1H), 3.32 (t, *J* = 9.4 Hz, 1H).

¹³C NMR (151 MHz, D₂O): δ 146.9 (d, *J* = 6.0 Hz), 146.7, 129.8, 123.9, 76.2 (d, *J* = 6.9 Hz), 74.0, 72.2, 71.4, 71.2 (d, *J* = 6.0 Hz), 70.7, 33.5 (d, *J* = 3.6 Hz).

³¹P NMR (243 MHz, D₂O): δ 19.4.

HRMS [ESI] C₁₃H₁₈NO₁₀PS ([M-H]⁻) calculated 410.0316 found 410.0316.

Compound 70



p-nitrobenzyl protected-*O*-2-(2-aminoethyl)phenol-ammonium monophosphorothioate

Compound **70** was obtained according to general procedure (**Method E**) from 2-(2-aminoethyl)phenol (0.05 mmol, 6.9 mg) using PsiK (2.5 mg, 0.11 mol%) as sugar kinase. NMR yield of thiophosphorylation is 70%. After one-pot protection, the crude product was first purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) and then further purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford 14.2 mg of the title compound after lyophilization (**Yield = 74%**).

Physical state: white solid

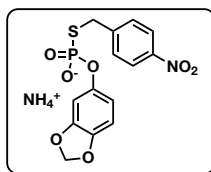
¹H NMR (600 MHz, CD₃OD): δ 8.09 – 8.04 (m, 2H), 7.55 – 7.50 (m, 2H), 7.41 – 7.37 (m, 1H), 7.21 – 7.15 (m, 2H), 7.08 – 7.02 (m, 1H), 4.11 (d, *J* = 13.3 Hz, 2H), 3.12 (t, *J* = 7.7 Hz, 2H), 2.92 (t, *J* = 7.6 Hz, 2H).

¹³C NMR (151 MHz, D₂O): δ 152.5 (d, *J* = 8.3 Hz), 148.5 (d, *J* = 4.8 Hz), 148.2, 131.5, 130.9, 129.4 (d, *J* = 5.8 Hz), 129.3, 125.2, 124.4, 121.7 (d, *J* = 3.2 Hz), 40.8, 35.6 (d, *J* = 3.9 Hz), 29.7.

³¹P NMR (243 MHz, D₂O): δ 13.8.

HRMS [ESI] C₁₅H₁₇N₂O₅PS ([M-H]⁻) calculated 367.0523 found 367.0524.

Compound 71



p-nitrobenzyl protected-*O*-(benzo[*d*][1,3]dioxol-5-yl)-ammonium monophosphorothioate

Compound **71** was obtained according to general procedure (**Method E**) from sesamol (0.05 mmol, 6.9 mg) using PsiK (2.5 mg, 0.11 mol%) as sugar kinase. NMR yield of thiophosphorylation is 22%. After one-pot protection, the crude product was first purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 20:80) and then further purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford 4.8 mg of the title compound after lyophilization (**Yield = 25%**).

Physical state: white solid

¹H NMR (600 MHz, D₂O): δ 8.09 (d, *J* = 8.3 Hz, 2H), 7.47 (d, *J* = 8.3 Hz, 2H), 6.68 – 6.64 (m, 1H), 6.44 – 6.38 (m, 1H), 6.37 – 6.33 (m, 1H), 5.87 (s, 2H), 4.03 (d, *J* = 16.1 Hz, 2H).

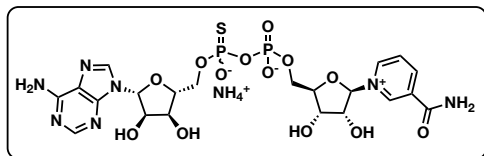
¹³C NMR (151 MHz, D₂O): δ 147.1, 147.1, 146.4, 145.7 (d, *J* = 8.4 Hz), 143.4, 129.7, 123.6, 113.0 (d, *J* = 4.6 Hz), 107.9, 102.8 (d, *J* = 4.8 Hz), 101.5, 34.1 (d, *J* = 4.3 Hz).

³¹P NMR (243 MHz, D₂O): δ 16.3

HRMS [ESI] C₁₄H₁₂NO₇PS ([M-H]⁻) calculated 367.9999 found 367.9998.

9.7 NAD and NADP thiophosphate analogues

Compound 72



Nicotinamide adenine dinucleotide ammonium phosphoro-α-thioate

Compound **72** was obtained according to general procedure (**Method F**) from adenosine (0.05 mmol, 13.4 mg) using *Ta*-NK as nucleoside kinase, then with *Ec*-NAD mutant as dinucleotide synthase and *Ec*-IPYR as phosphatase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) and then further

purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford 10.7 mg of the title compound after lyophilization (**Yield = 40%, single diastereomer**).

Physical state: white solid

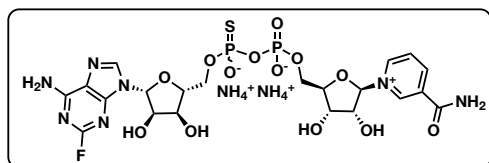
¹H NMR (600 MHz, D₂O): δ 9.36 (s, 1H), 9.22 (d, *J* = 6.3 Hz, 1H), 8.85 (d, *J* = 8.1 Hz, 1H), 8.55 (s, 1H), 8.23 (dd, *J* = 8.1, 6.3 Hz, 1H), 8.18 (d, *J* = 0.8 Hz, 1H), 6.12 (d, *J* = 5.5 Hz, 1H), 6.07 (d, *J* = 5.9 Hz, 1H), 4.82 – 4.80 (m, 1H), 4.60 – 4.55 (m, 2H), 4.53 (dd, *J* = 5.1, 3.5 Hz, 1H), 4.49 (dd, *J* = 5.0, 2.7 Hz, 1H), 4.45 (ddd, *J* = 12.0, 4.3, 2.5 Hz, 1H), 4.43 – 4.40 (m, 1H), 4.33 – 4.24 (m, 3H).

¹³C NMR (151 MHz, D₂O): δ 165.3, 155.2, 152.5, 149.0, 145.7, 142.4, 140.1, 139.9, 133.6, 128.7, 118.4, 100.0, 87.1 (d, *J* = 9.2 Hz), 86.8, 83.9 (d, *J* = 9.5 Hz), 77.5, 74.1, 70.8, 70.6, 65.4 (d, *J* = 6.5 Hz), 65.0 (d, *J* = 5.5 Hz).

³¹P NMR (243 MHz, D₂O): δ 43.4 (d, *J* = 27.7 Hz), -12.3 (d, *J* = 27.7 Hz).

HRMS [ESI] C₂₁H₂₇N₇O₁₃P₂S ([M-H]⁻) calculated 678.0790 found 678.0787.

Compound 73



Nicotinamide 2-fluoro-adenine dinucleotide ammonium phosphoro-α-thioate

Compound **73** was obtained according to general procedure (**Method F**) from 2-fluoroadenosine (0.05 mmol, 14.3 mg) using *Ta*-NK as nucleoside kinase, then with *Ec*-NAD mutant as dinucleotide synthase and *Ec*-IPYR as phosphatase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 25:75) and then further purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford 6.6 mg of the title compound after lyophilization (**Yield = 28%, dr = 13:1**).

Physical state: white solid

¹H NMR (600 MHz, D₂O): δ 9.39 (s, 1H), 9.25 (dt, *J* = 6.3, 1.4 Hz, 1H), 8.89 (dt, *J* = 8.1, 1.5 Hz, 1H), 8.48 (s, 1H), 8.27 (dd, *J* = 8.1, 6.3 Hz, 1H), 6.14 (d, *J* = 5.6 Hz, 1H), 5.95 (d, *J* = 5.8 Hz, 1H), 4.76 (d, *J* = 5.5 Hz, 1H), 4.61 – 4.57 (m, 2H), 4.53 – 4.49 (m, 2H), 4.43 (ddd, *J* = 11.9, 4.3, 2.4 Hz, 1H), 4.40 (p, *J* = 3.0 Hz, 1H), 4.33 – 4.21 (m, 3H).

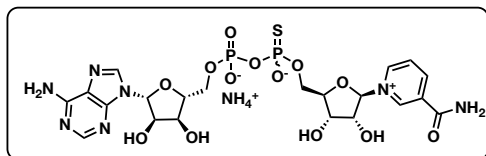
¹³C NMR (151 MHz, D₂O): δ 165.3, 159.0 (d, *J* = 211.4 Hz), 157.0 (d, *J* = 19.7 Hz), 150.5 (d, *J* = 19.2 Hz), 145.8, 142.5, 140.1 (d, *J* = 2.7 Hz), 139.9, 133.7, 128.7, 116.7 (d, *J* = 3.7 Hz), 100.1, 87.2 (d, *J* = 9.4 Hz), 86.9, 83.9 (d, *J* = 9.5 Hz), 77.5, 74.1, 70.8, 70.6, 65.4 (d, *J* = 6.4 Hz), 65.0 (d, *J* = 5.3 Hz).

³¹P NMR (243 MHz, D₂O): δ 43.3 (d, *J* = 27.9 Hz), -12.4 (d, *J* = 27.8 Hz).

¹⁹F NMR (565 MHz, D₂O): δ -52.6.

HRMS [ESI] C₂₁H₂₆FN₇O₁₃P₂S ([M-H]⁻) calculated 696.0696 found 696.0696.

Compound 74



Nicotinamide adenine dinucleotide ammonium phosphor- β -thioate

Compound **74** was obtained according to general procedure (**Method G**) from nicotinamide riboside (0.05 mmol, 12.8 mg) using *Km*-NRK as nucleoside kinase, then with *Hs*-NMNAT as dinucleotide synthase and *Ec*-IPYR as phosphatase. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH_4HCO_3 /water, from 0:100 to 25:75) and then further purified by reverse phase chromatography on C18 column (methanol/water, from 0:100 to 100:0) to afford 15.3 mg of two diastereoisomers of the title compound after lyophilization (Yield = 46%, dr = 1:1).

Physical state: white solid

Diastereomer 1

^1H NMR (600 MHz, D_2O): δ 9.33 (s, 1H), 9.19 (dd, J = 6.3, 1.4 Hz, 1H), 8.83 (dd, J = 8.1, 1.5 Hz, 1H), 8.46 (s, 1H), 8.23 – 8.18 (m, 1H), 8.16 (s, 1H), 6.08 (d, J = 5.5 Hz, 1H), 6.04 (d, J = 6.1 Hz, 1H), 4.84 – 4.81 (m, 1H), 4.59 – 4.55 (m, 2H), 4.52 (dd, J = 5.3, 5.3 Hz, 1H), 4.48 – 4.45 (m, 1H), 4.41 – 4.36 (m, 2H), 4.35 – 4.30 (m, 1H), 4.27 – 4.19 (m, 2H).

^{13}C NMR (151 MHz, D_2O): δ 165.2, 155.4, 152.8, 149.1, 145.7, 142.4, 140.0, 139.8, 133.6, 128.6, 118.4, 100.1, 87.1 (d, J = 9.9 Hz), 86.5, 83.9 (d, J = 9.4 Hz), 77.6, 73.9, 70.8, 70.5, 65.4 (d, J = 5.4 Hz), 65.2 (d, J = 5.6 Hz).

^{31}P NMR (243 MHz, D_2O): δ 43.3 (d, J = 27.2 Hz), -12.1 (d, J = 27.6 Hz).

HRMS [ESI] $\text{C}_{21}\text{H}_{27}\text{N}_7\text{O}_{13}\text{P}_2\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 678.0790 found 678.0785.

Diastereomer 2

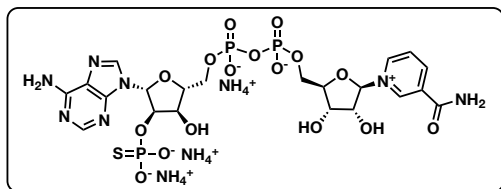
^1H NMR (600 MHz, D_2O): δ 9.35 (s, 1H), 9.26 (d, J = 6.3 Hz, 1H), 8.85 (d, J = 8.1 Hz, 1H), 8.46 (s, 1H), 8.23 (dd, J = 8.1, 6.3 Hz, 1H), 8.19 (s, 1H), 6.12 (d, J = 5.4 Hz, 1H), 6.06 (d, J = 6.1 Hz, 1H), 4.61 – 4.59 (m, 1H), 4.57 (dd, J = 5.2, 5.2 Hz, 1H), 4.55 – 4.50 (m, 1H), 4.49 – 4.45 (m, 1H), 4.44 – 4.37 (m, 2H), 4.33 – 4.27 (m, 2H), 4.26 – 4.17 (m, 2H).

^{13}C NMR (151 MHz, D_2O): δ 165.3, 155.5, 152.8, 149.1, 145.6, 142.5, 140.0, 139.8, 133.6, 128.7, 118.5, 100.1, 87.0 (d, J = 10.0 Hz), 86.6, 83.9 (d, J = 9.3 Hz), 77.6, 74.0, 70.8, 70.5, 65.4 (d, J = 5.6 Hz), 64.9 (d, J = 6.2 Hz).

^{31}P NMR (243 MHz, D_2O): δ 43.2 (d, J = 28.7 Hz), -12.1 (d, J = 28.1 Hz).

HRMS [ESI] $\text{C}_{21}\text{H}_{27}\text{N}_7\text{O}_{13}\text{P}_2\text{S}$ ($[\text{M}-\text{H}]^-$) calculated 678.0790 found 678.0785.

Compound 75



Nicotinamide adenine dinucleotide triammonium-2'-phosphorothioate

Compound **75** was obtained according to general procedure (**Method A**) from NAD⁺ (0.05 mmol, 33.1 mg) using *Ec*-NADK (5.0 mg, 0.30 mol%) as nucleoside kinase and Mn²⁺ (40 mol%, from MnSO₄·xH₂O) as cofactor. The crude product was purified by ion-exchange chromatography on DEAE Sephadex (1 M NH₄HCO₃/water, from 0:100 to 30:70) to afford 27.5 mg of the title compound after lyophilization (**Yield = 70%**).

Physical state: white solid

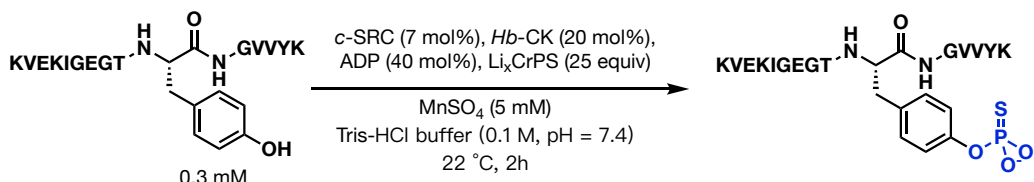
¹H NMR (600 MHz, D₂O): δ 9.30 (s, 1H), 9.12 (d, *J* = 6.2 Hz, 1H), 8.84 – 8.80 (m, 1H), 8.46 (s, 1H), 8.20 (dd, *J* = 8.1, 6.2 Hz, 1H), 8.14 (s, 1H), 6.16 (d, *J* = 5.6 Hz, 1H), 6.05 (d, *J* = 5.6 Hz, 1H), 5.19 (ddd, *J* = 10.7, 5.5, 5.5 Hz, 1H), 4.68 (dd, *J* = 4.8, 4.8 Hz, 1H), 4.52 (t, *J* = 2.6 Hz, 1H), 4.47 (t, *J* = 5.3 Hz, 1H), 4.42 (dd, *J* = 5.1, 2.7 Hz, 1H), 4.39 (dt, *J* = 4.6, 2.4 Hz, 1H), 4.34 (ddd, *J* = 12.0, 4.3, 2.4 Hz, 1H), 4.29 (ddd, *J* = 11.7, 5.0, 2.7 Hz, 1H), 4.26 – 4.18 (m, 2H).

¹³C NMR (151 MHz, D₂O): δ 165.1, 155.1, 152.3, 149.0, 145.7, 142.3, 140.6, 139.8, 133.6, 128.6, 118.4, 100.0, 87.1 (d, *J* = 8.9 Hz), 86.2 (d, *J* = 7.1 Hz), 83.3 (d, *J* = 9.0 Hz), 77.6, 76.3 (d, *J* = 4.6 Hz), 70.7, 70.0 (d, *J* = 3.0 Hz), 65.6 (d, *J* = 5.3 Hz), 64.9 (d, *J* = 5.1 Hz).

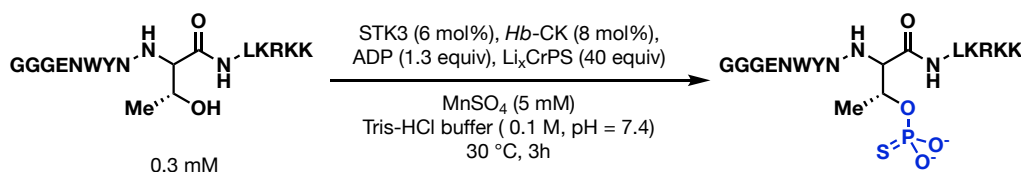
³¹P NMR (243 MHz, D₂O): δ 45.7, -11.3 (d, *J* = 20.6 Hz), -11.7 (d, *J* = 20.2 Hz).

HRMS [ESI] C₂₁H₂₈N₇O₁₆P₃S ([M-H]⁻) calculated 758.0453 found 758.0446.

10. Enzymatic Thiophosphorylation of Peptide and Characterization



Peptide sequence of KK-15: KVEKIGEGTYGVVYK-NH₂



Peptide sequence of GK-14: GGGENWYNTLKRKK-NH₂

10.1 General procedure for peptide thiophosphorylation and analysis

A 1-dram vial equipped with a magnetic stir bar was charged with peptide KK-15 or GK-14 (0.5 mg, final concentration is roughly 0.3 mM) then dissolved in a buffer solution of Tris-HCl (0.1 M, pH 7.4) containing MnSO₄ (final concentration is 5 mM for both peptides). To this solution, ADP (final concentration are 0.12 mM, 0.4 equiv for KK-15 and 0.4 mM, 1.3 equiv for GK-14), and Li_xCrPS (final concentration is 7.5 mM or 12 mM) were added sequentially from the corresponding stock solutions. The reaction mixture was stirred at room temperature for 1 min to mix, then SRC (final concentration is 21 μM, 7 mol%) or STK3 (final concentration is 18 μM, 6 mol%) and Hb-CK (final concentration are 60 μM, 20 mol% for KK-15 or 24 μM, 8 mol% for GK-14) were added sequentially. The total volume is 1 mL (for KK-15) or 500 μL (for GK-14) and the reaction was stirred at 22 °C or 30 °C for 2-3 hours. After completion, 50 μL of the reaction mixture was diluted with H₂O/MeOH solution (1:1, 950 μL) and centrifuged (12,000 rpm, 10 min). The supernatant was filtered through a syringe filter (0.22 μm) and analyzed by LC-MS and LC-MS/MS.

LC-MS was conducted on an Agilent Quadrupole-TOF LC/MS spectrometer with an Ascentis® Express Peptide ES-C18 column (15 cm × 2.1 mm, 2.7 μm). The flow rate was 0.4 mL/min and column temperature is 35 °C. A gradient of water with 0.1% formic acid (A)/acetonitrile (B) was employed. Mass spectra were acquired from m/z 200-2000 under negative mode.

Time (min)	Solvent A (%)	Solvent B (%)
0	95	5
10	50	50
13	5	95
16	5	95

LC-MS/MS was conducted on an Agilent Quadrupole-TOF LC/MS spectrometer with an Ascentis® Express Peptide ES-C18 column (15 cm × 2.1 mm, 2.7 μm). The flow rate was 0.4 mL/min. A gradient of water with 0.1% formic acid (A)/acetonitrile (B) was employed. Mass spectra were acquired from m/z 100-3000 under negative mode. MS/MS spectra were acquired

from identical m/z range using optimal MS/MS mode. The data was analyzed by mMass v.5.5.088¹⁰ with error threshold setting as 25 ppm.

Time (min)	Solvent A (%)	Solvent B (%)
0	95	5
10	65	35
13	5	95
20	5	95

10.2 Results for peptide thiophosphorylation

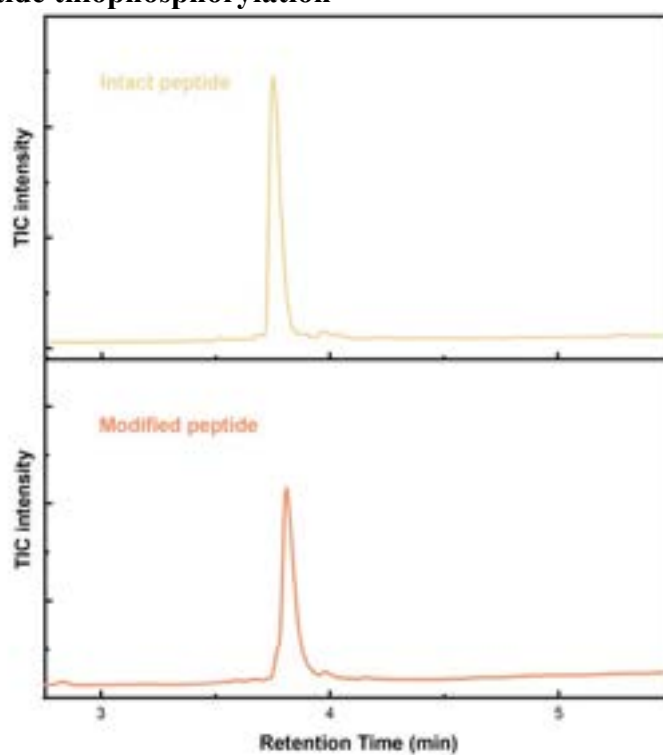


Figure S15. Chromatogram of intact peptide and modified peptide of KK-15.

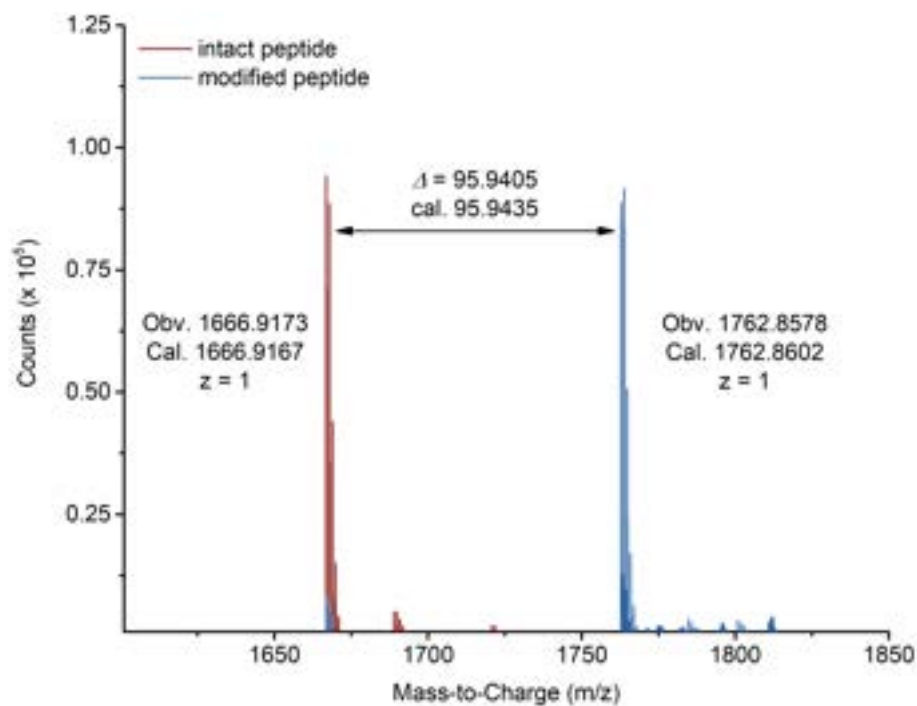


Figure S16. Comparison of intact peptide and modified peptide KK-15.

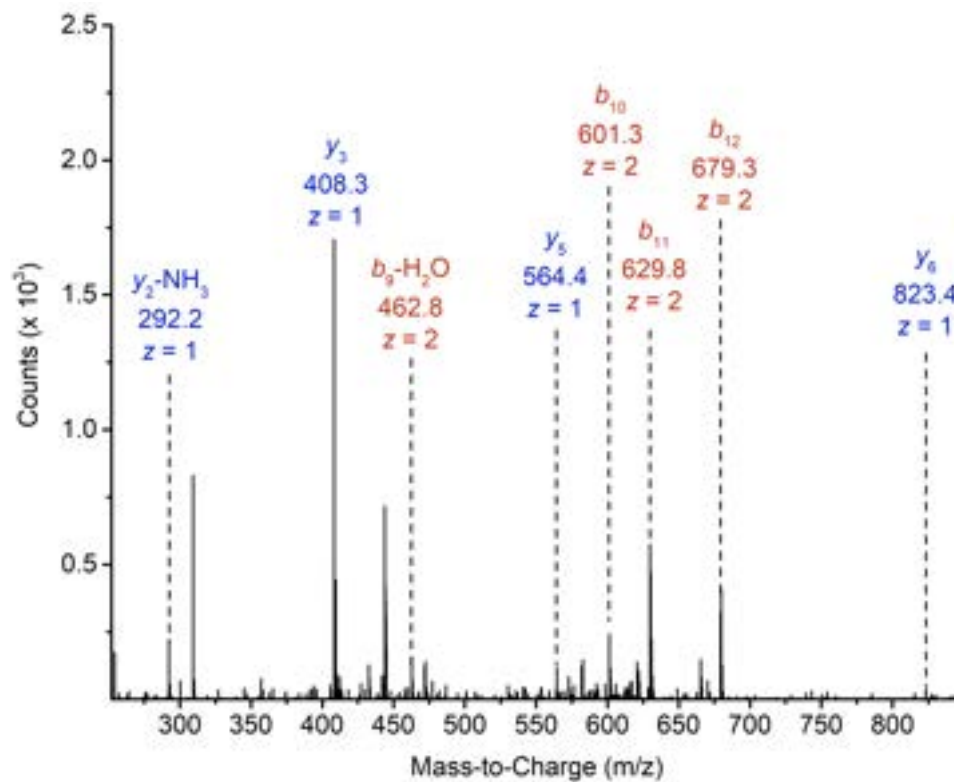


Figure S17. Selected MS/MS fragmentation of modified peptide KK-15.

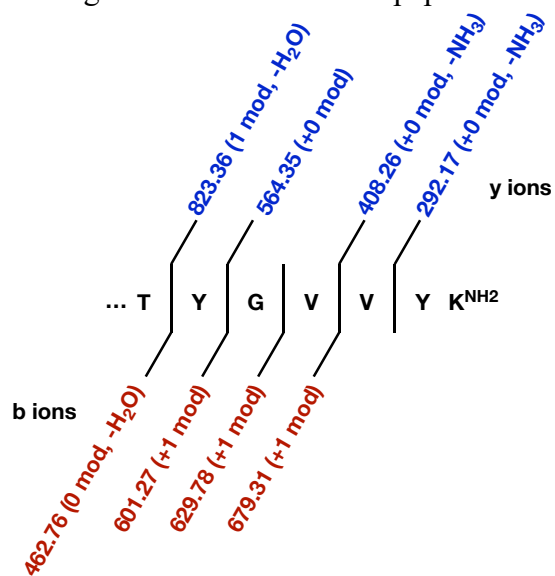


Figure S18. Selected b and y ions of modified peptide of KK-15.

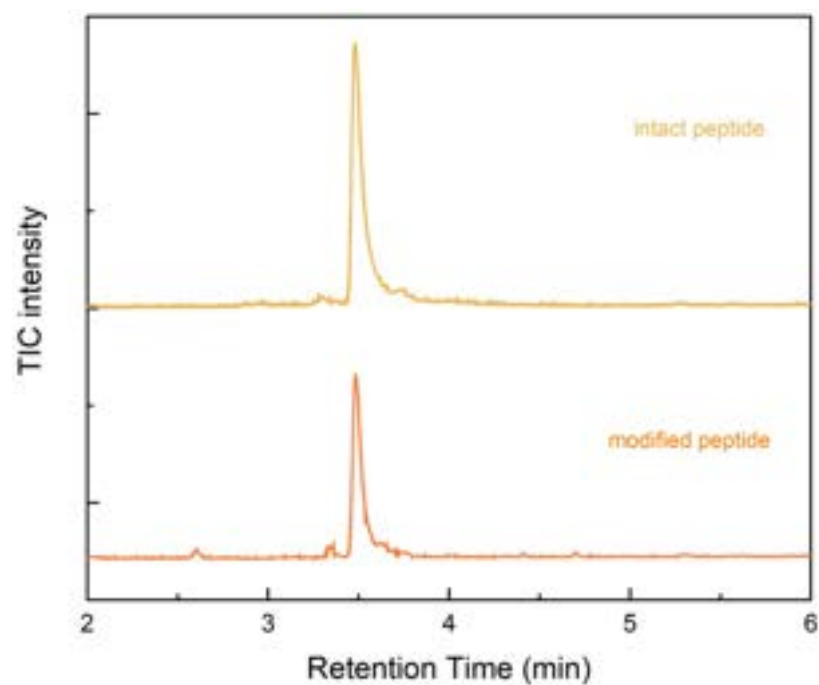


Figure S19. Chromatogram of intact peptide and modified peptide of GK-14.

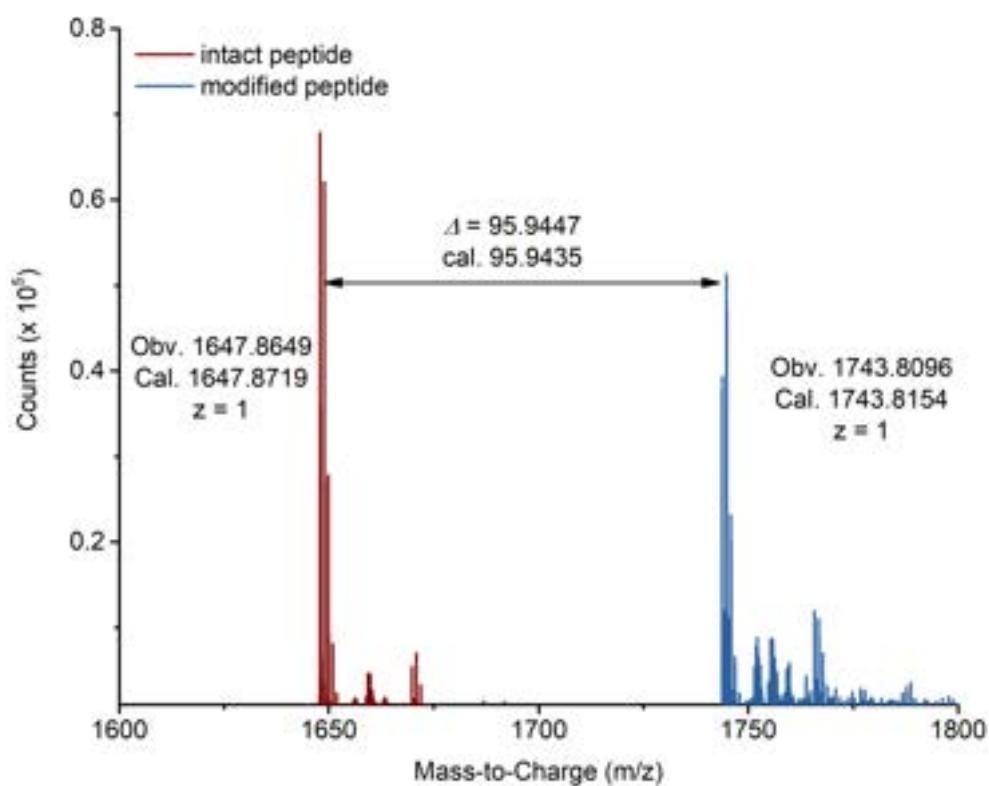


Figure S20. Comparison of intact peptide and modified peptide GK-14.

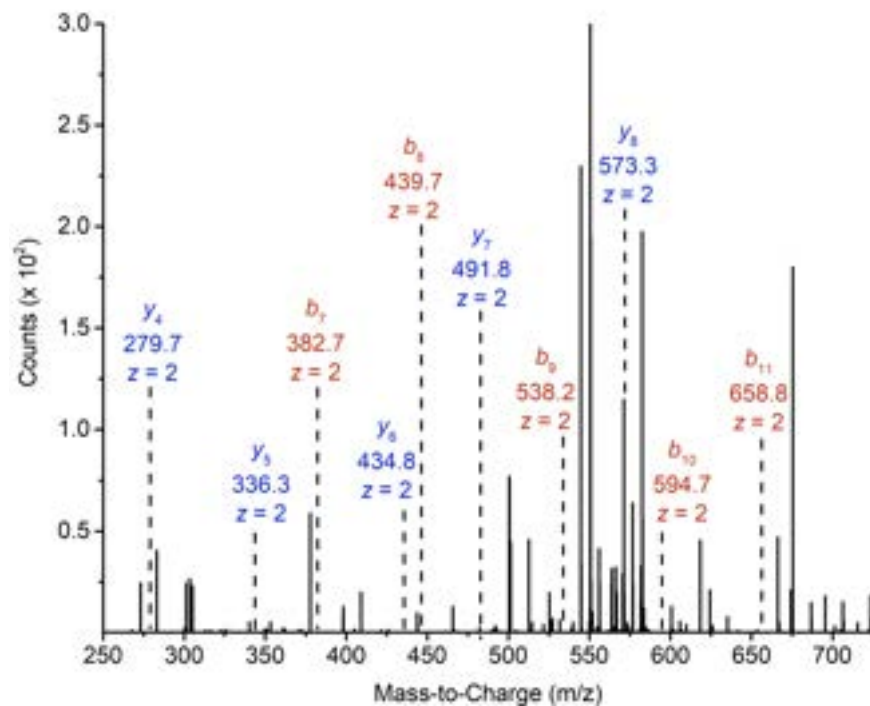


Figure S21. Selected MS/MS fragmentation of modified peptide GK-14.

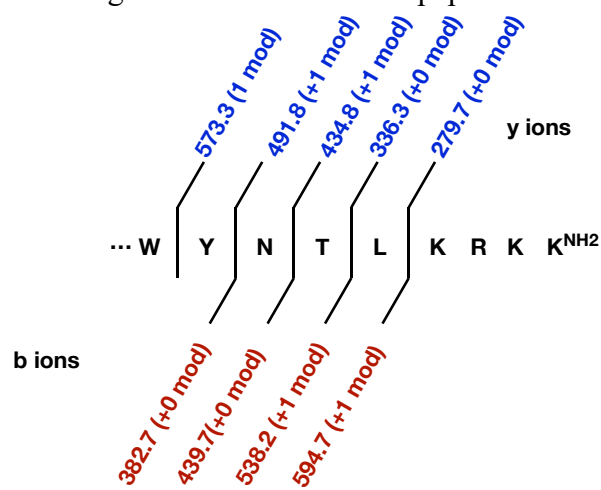


Figure S22. Selected b and y ions of modified peptide of GK-14.

Table S15. Mmodified peptide KK-15 fragments obtained from LC-MS/MS.

fragmentation	link	m/z	charge	peptide	Error (ppm)
b5	[1-5]	598.3923	1	.KVEKI.g	15.7
b9 -H2O	[1-9]	462.7611	2	.KVEKIGEGT.y	15.5
b10	[1-10]	601.2698	2	.KVEKIGEGTY.g [1x Thiophos]	14.7
b10	[1-10]	553.298	2	.KVEKIGEGTY.g	14.2
b10 -H2O	[1-10]	592.2645	2	.KVEKIGEGTY.g [1xThiophos]	17.1
b11	[1-11]	629.7805	2	.KVEKIGEGTYG.v [1xThiophos]	13.4
b11	[1-11]	581.8088	2	.KVEKIGEGTYG.v	18.1
b11 -H2O	[1-11]	620.7752	2	.KVEKIGEGTYG.v [1xThiophos]	12.3
b11 -H2O	[1-11]	572.8035	2	.KVEKIGEGTYG.v	16.8
b11 -NH3	[1-11]	573.2955	2	.KVEKIGEGTYG.v	8.3
b12	[1-12]	679.3147	2	.KVEKIGEGTYGV.v[1xThiophos]	14.1
b12 -H2O	[1-12]	670.3094	2	.KVEKIGEGTYGV.v [1xThiophos]	15.3
int-b	[3-14]	648.8272	2	v.EKIGEGTYGVVY.k	14.2
int-b	[4-11]	902.3478	1	e.KIGEGTYG.v [1xThiophos]	-1.9
int-b	[8-10]	418.0832	1	e.GTY.g [1xThiophos]	1.9
int-b	[9-11]	418.0832	1	g.TYG.v [1xThiophos]	1.9
y6	[10-15]	823.3572	1	t.YGVVYK. [1xAmide;1xThiophos]	8.6
y5	[11-15]	564.3504	1	y.GVVYK. [1xAmide]	-7.9
y3	[13-15]	408.2605	1	v.VYK. [1xAmide]	14.7
y3 -NH3	[13-15]	196.1206	2	v.VYK. [1xAmide]	16
y2 -NH3	[14-15]	292.1656	1	v.YK. [1xAmide]	12.2

Table S16. Modified peptide GK-14 fragments obtained from LC-MS/MS

fragmentation	link	m/z	charge	peptide	Error (ppm)
b4	[1-4]	101.0429	3	.GGGE.n	8.5
b5	[1-5]	139.0572	3	.GGGEN.w	9
b6	[1-6]	201.0837	3	.GGGENW.y	12
b7	[1-7]	255.4381	3	.GGGENWY.n	4.6
b8	[1-8]	293.4524	3	.GGGENWYN.t	2.3
b9	[1-9]	359.1162	3	.GGGENWYNT.l [1xThiophos]	8.5
b9	[1-9]	327.135	3	.GGGENWYNT.l	1.2
b10	[1-10]	396.8108	3	.GGGENWYNTL.k [1xThiophos]	5.4
b10	[1-10]	364.8297	3	.GGGENWYNTL.k	2
b11	[1-11]	439.5092	3	.GGGENWYNTLK.r [1xThiophos]	-4.9
b11	[1-11]	407.528	3	.GGGENWYNTLK.r	0.2
b12	[1-12]	491.5429	3	.GGGENWYNTLKR.k [1xThiophos]	-5.8
b12	[1-12]	459.5617	3	.GGGENWYNTLKR.k	-3.5
b13	[1-13]	534.2412	3	.GGGENWYNTLKRK.k	-2.8
b13	[1-13]	502.26	3	.GGGENWYNTLKRK.k	6.5
y13	[2-14]	563.6079	3	g.GGENWYNTLKRKK. [1xAmide; 1xThiophos]	2.4
y13	[2-14]	531.6267	3	g.GGENWYNTLKRKK. [1xAmide]	4.3
y12	[3-14]	544.6007	3	g.GENWYNTLKRKK. [1xAmide; 1xThiophos]	0.7
y12	[3-14]	512.6196	3	g.GENWYNTLKRKK. [1xAmide]	-5.6
y11	[4-14]	525.5936	3	g.ENWYNTLKRKK. [1xAmide; 1xThiophos]	0.8
y11	[4-14]	493.6124	3	g.ENWYNTLKRKK. [1xAmide]	0
y10	[5-14]	482.5794	3	e.NWYNTLKRKK. [1xAmide; 1xThiophos]	0.5
y10	[5-14]	450.5982	3	e.NWYNTLKRKK. [1xAmide]	-3.8
y9	[6-14]	444.5651	3	n.WYNTLKRKK. [1xAmide; 1xThiophos]	5.7
y9	[6-14]	412.5839	3	n.WYNTLKRKK. [1xAmide]	1.2
y8	[7-14]	382.5386	3	w.YNTLKRKK. [1xAmide; 1xThiophos]	-0.1
y8	[7-14]	350.5575	3	w.YNTLKRKK. [1xAmide]	5
y7	[8-14]	328.1842	3	y.NTLKRKK. [1xAmide; 1xThiophos]	8.9
y7	[8-14]	296.203	3	y.NTLKRKK. [1xAmide]	-1.7
y6	[9-14]	290.1699	3	n.TLKRKK. [1xAmide; 1xThiophos]	-5.1
y6	[9-14]	258.1887	3	n.TLKRKK. [1xAmide]	-2
y5	[10-14]	224.5061	3	t.LKRKK. [1xAmide]	-1.5
y4	[11-14]	186.8115	3	l.KRKK. [1xAmide]	8.2
y3	[12-14]	144.1131	3	k.RKK. [1xAmide]	-10.7

10.3 Comparison of conditions for peptide thiophosphorylation

Two conditions with lower enzyme/reagent loading were tested for peptide thiophosphorylation using GK-14 as the substrate.

Condition B (lower loading of enzymes): loading of STK3 was reduced to 0.25 mol%, *Hb*-CK was reduced to 0.35 mol%, other conditions remained unchanged.

Condition C (lower loading of reagents and enzymes): loading of STK3 was reduced to 0.25 mol%, *Hb*-CK was reduced to 0.35 mol%, ADP was reduced to 5 mol%, LixCrPS was reduced to 1.2 equiv, the concentration of Mn(II) and Tris-HCl buffer remained unchanged.

The reaction was stirred at 30 °C for 3 hours. After completion, 50 µL of the reaction mixture was diluted with H₂O/MeOH solution (1:1, 950 µL) and centrifuged (12,000 rpm, 10 min). The supernatant was filter through a syringe filter (0.22 µm) and analyzed by LC-MS.

Both condition B and condition C showed that incomplete conversion of peptide substrate. The results suggest original higher loading of enzymes and reagents is necessary, presumably due to slower kinetics of these kinases with peptide substrate compared with nucleoside substrates.

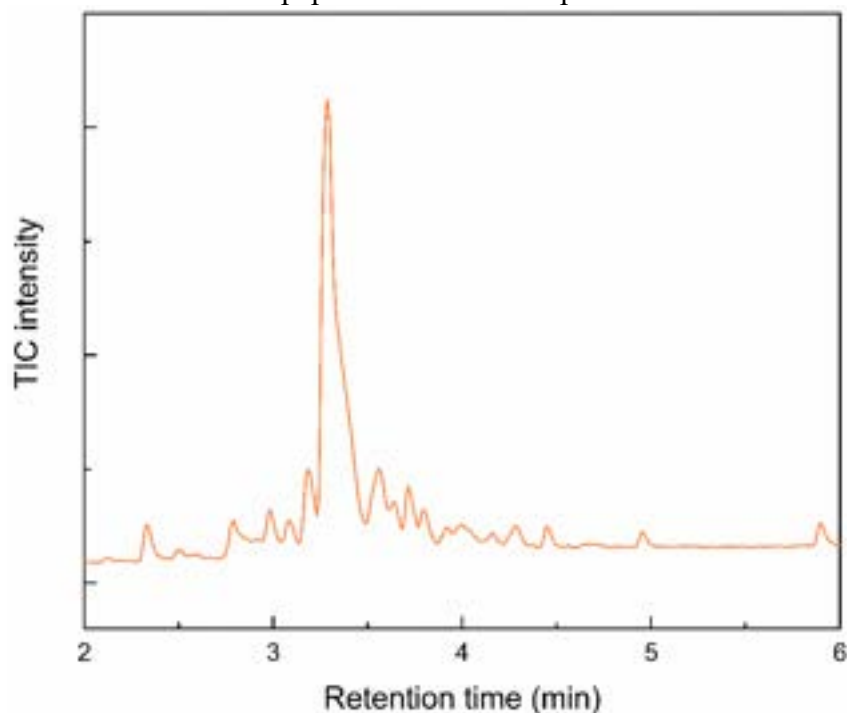


Figure S23. Chromatogram of modified peptide of GK-14 using condition B.

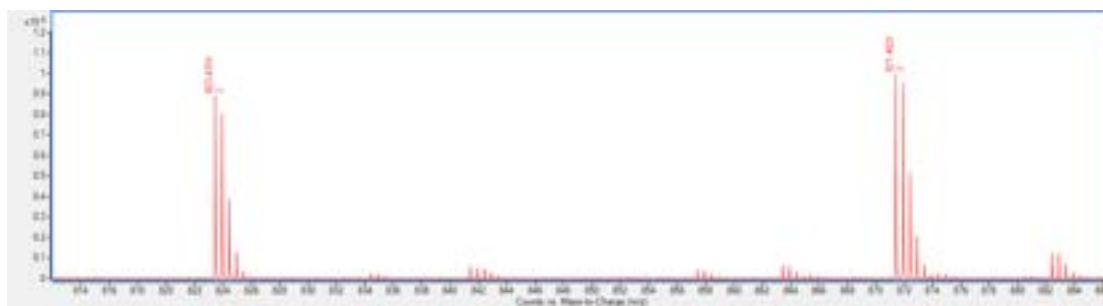


Figure S24. Mass extraction of GK-14 using condition B. Only doubly charged peptide was shown because the singly charged species has weak signals. Thiophosphorylated peptide ($m/z = 871.4031$,

two negative charges) and unreacted peptide ($m/z = 823.4316$, two negative charges) can be observed.

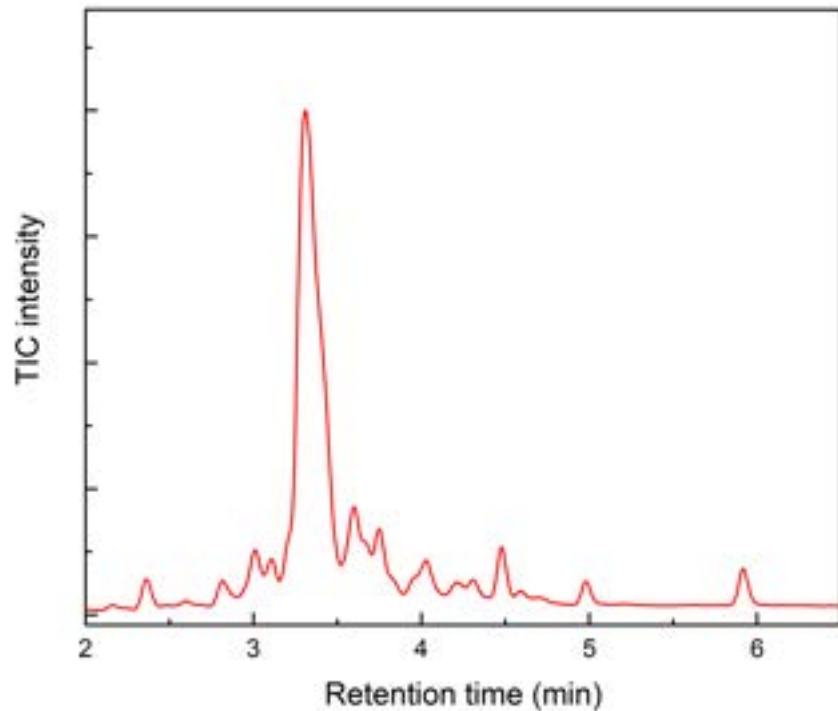


Figure S25. Chromatogram of modified peptide of GK-14 using condition C.

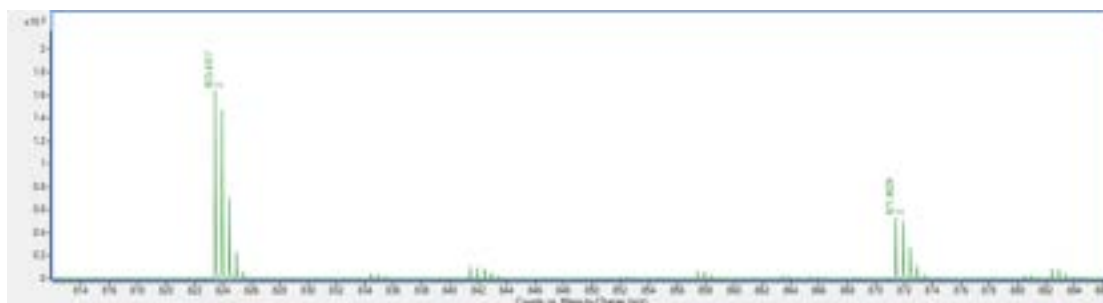


Figure S26. Mass extraction of GK-14 using condition C. Only doubly charged peptide was shown because the singly charged species has weak signals. Thiophosphorylated peptide ($m/z = 871.4029$, two negative charges) and unreacted peptide ($m/z = 823.4317$, two negative charges) can be observed.

11. Enzymatic Thiophosphorylation of Protein STK3 and Characterization

11.1 Sample preparation for native gel and results

1.A 1.5 mL low protein binding microcentrifuge tube was charged with following solutions and proteins.

Phosphorylation (with stoichiometric ATP):

Components	Amount
STK3 (25 mg/mL)	20 μ L
Mg ²⁺ solution (50 mM)	50 μ L
ATP	2.8 mg
Tris-HCl (0.1M, pH 7.4)	430 μ L

Thiophosphorylation (with stoichiometric ATP γ S):

Components	Amount
STK3 (25 mg/mL)	20 μ L
Mg ²⁺ solution (50 mM)	50 μ L
ATP γ S	3 mg
Tris-HCl (0.1M, pH 7.4)	430 μ L

Thiophosphorylation (with ATP γ S regeneration system):

Components	Amount
STK3 (25 mg/mL)	20 μ L
Mg ²⁺ solution (50 mM)	50 μ L
ADP (20 mM)	50 μ L
Li _x CrPS (600 mM)	10 μ L
Hb-CK (50 mg/mL)	10 μ L
Tris-HCl (0.1M, pH 7.4)	360 μ L

2. The reactions were incubated at 30°C for 12 h.

3. Reaction was quenched by addition of 100 μ L EDTA solution (50 mM)
4. 15 μ L of each reaction mixture was diluted with 30 μ L native gel loading buffer (Bio-Rad #1610738) to afford the native gel sample. The rest of thiophosphorylated STK3 generated from the enzymatic reaction was concentrated and purified with size exclusion chromatography. Fractions containing STK3 were concentrated and used for LC-MS and LC-MS/MS.
5. Native gel samples were analyzed by native PAGE using 4-20 % Tris- HCl gels (Bio-Rad #3450032). The phosphorylated, thiophosphorylated forms of STK3, intact STK3 and *Hb-CK* were visualized by staining with Coomassie blue.

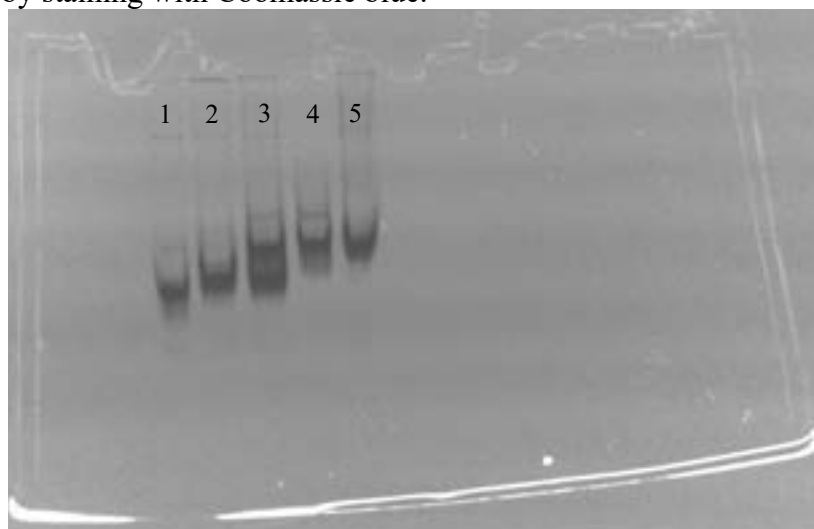


Figure S27. Native gel of phosphorylated and thiophosphorylated STK3. 1: phosphorylation with stoichiometric ATP; 2: thiophosphorylation with stoichiometric ATP γ S; 3: thiophosphorylation with ATP γ S regeneration system; 4: intake *Hb-CK*; 5: intact STK3.

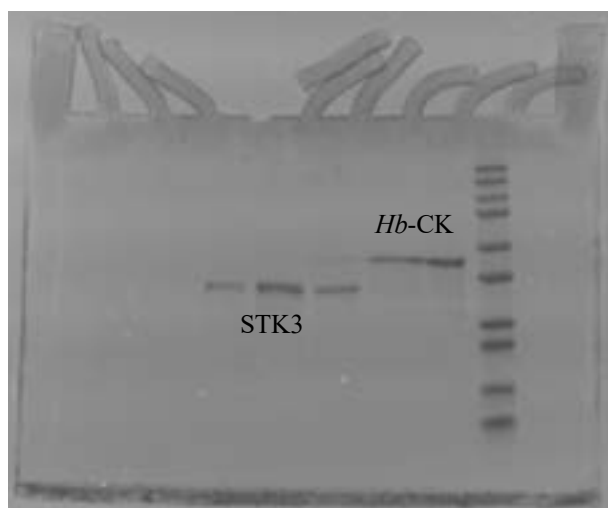


Figure S28. Size exclusion chromatography purified thiophosphorylated STK3 (SDS-PAGE gel).

11.2 Intact Protein Mass Analysis

Liquid Chromatography tandem mass spectrometry experiments were performed on a Thermo Scientific Orbitrap Ascend equipped with a Thermo Scientific Vanquish Neo chromatography system. Protein samples were injected onto a Waters NanoEase m/z BEH C4 column (300 Å pore size, 1.7 µm particle size, 150 µm I.D.×100 mm). Proteins were eluted over a gradient of 3-65% acetonitrile over 10 minutes at a constant flow rate of 2 µL/min. Spectra were acquired from m/z 500-2000 at a resolving power of 120000.

Spectral Deconvolution

Data was processed in Thermo Scientific Freestyle. Scans from 6.5-9 minutes were averaged, then deconvoluted using the Xtract deconvolution algorithm with the following parameters: m/z range 500-2000; charge range 5-50; relative abundance threshold 0%; Isotope Table Protein; minimum number of detected charge states 3.

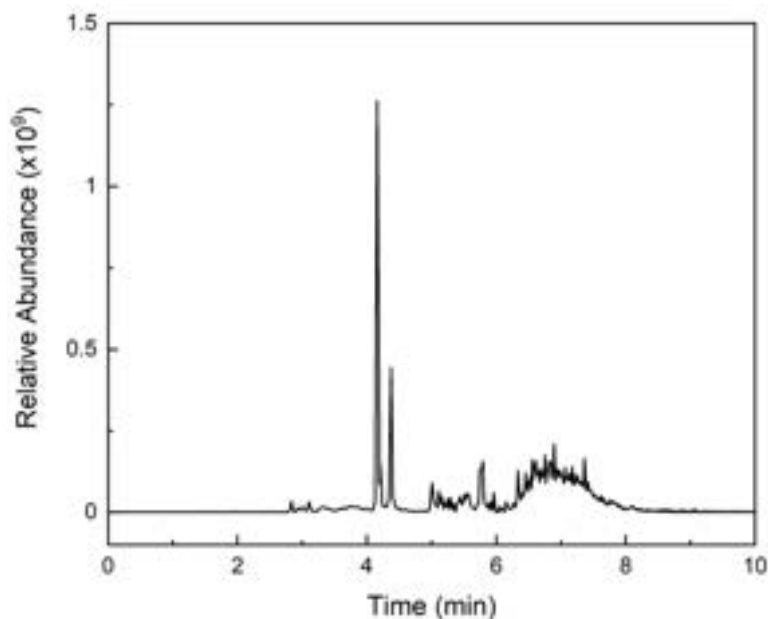


Figure S29. Chromatogram of intact STK3.

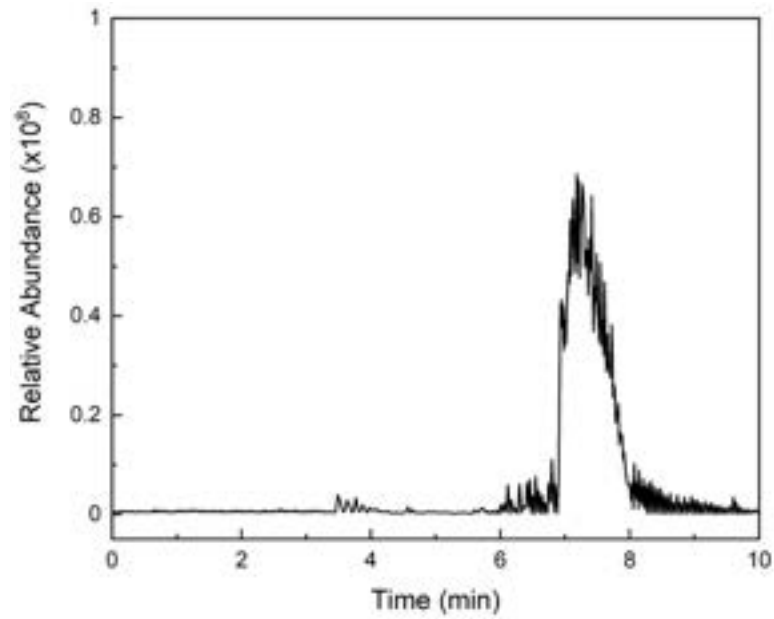


Figure S30. Chromatogram of modified STK3.

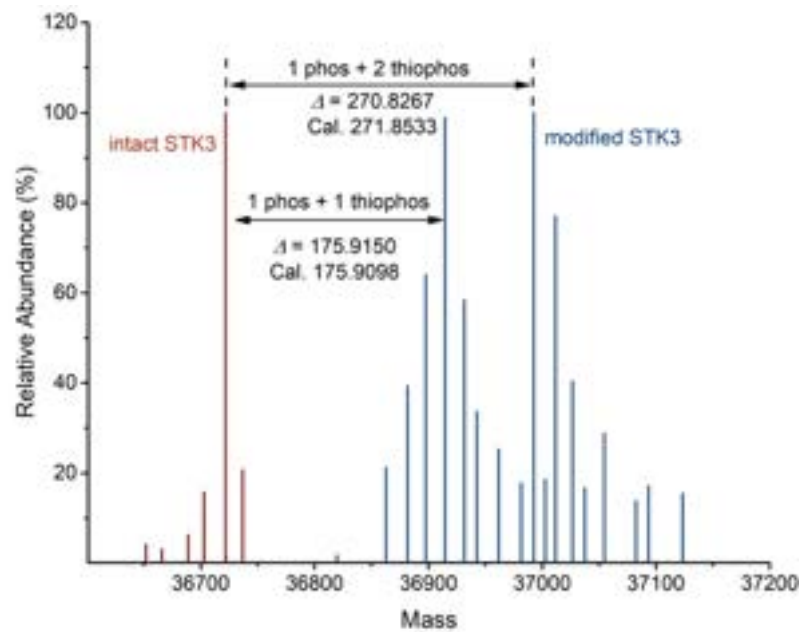


Figure S31. Comparison of deconvoluted mass of intact STK3 and (thio)phosphorylated STK3.

11.3 Liquid Chromatography Tandem Mass Spectrometry

Sample Preparation and Proteolytic Digestion

Approximately 500 µg of STK3 was resuspended in 50 mM ammonium bicarbonate, pH 8.0 with 5M urea and incubated at 37 °C for 1h. The sample was then subjected to reduction and alkylation via incubation with 10 mM TCEP at 55 °C for 15 minutes followed by addition of iodoacetamide to a final concentration of 15mM and incubation at room temperature, protected from light for 20 minutes.

The resulting alkylated protein solution was digested with Glu-C (Promega V165A) and rAsp-N (Promega VA1160) at a 1:20 (w/w) and 1:50 (w/w) protein:protease ratio, respectively. The digestion reaction was allowed to proceed at 37 °C with constant shaking at 750 rpm for 2h and was quenched by addition of formic acid to a final concentration of 1%. The resulting peptide solution was desalted using C18 pipette tips (ThermoFisher 87782) per manufacturer recommendations and dried in a speed vac.

The desalted and dried peptide sample was subjected to phosphopeptide enrichment using Fe-NTA spin columns (ThermoFisher A32992) per manufacturer recommendations. In addition to the final eluate, the initial flow through of the sample was collected for downstream analysis.

LC-MS/MS data acquisition

Liquid Chromatography tandem mass spectrometry experiments were performed on a Thermo Scientific Orbitrap Ascend equipped with a Thermo Scientific Vanquish Neo chromatography system. Peptide mixtures were injected onto a Waters NanoEase m/z BEH C18 column (130 Å pore size, 1.7 µm particle size, 75 µm I.D. × 200mm). Peptides were eluted over a gradient of 3-36% acetonitrile over 74 minutes, followed by a gradient of 36-65% acetonitrile over 12 minutes at a constant flow rate of 350nL/min. Precursor scans were acquired from m/z 375-1800 at a resolving power of 30000. Ions were selected for MS2 based on intensity > 8.0x10³ and charge state between 2-7 with an isolation width of 1.6 m/z. Selected ions were fragmented using HCD activation with an NCE of 26 and detected in the orbitrap with a resolving power of 30000. As many MS2 scans as possible were collected within a 3s cycle time.

LC-MS/MS data analysis

Data from the phosphopeptide enrichment flow through and eluate were analyzed using Thermo Proteome Discoverer 3.1. Data was searched using Sequest HT against the sequence of the recombinant protein construct with Asp N and Glu C semi-specific digestion, allowing for the following dynamic modifications: phospho (S, T, Y); thiophospho (S, T, Y); oxidation (M); carbamidomethyl(C). Precursor mass tolerance was set to 10 ppm and fragment mass tolerance to 0.02 Da. PSMs were validated with the Fixed Value PSM validator node with a maximum Delta Cn of 0.05. Results were filtered for PSMs of high and medium confidence. Spectra for PSMs including phospho- or thiophospho- modifications were manually assessed for ambiguous modification localization.

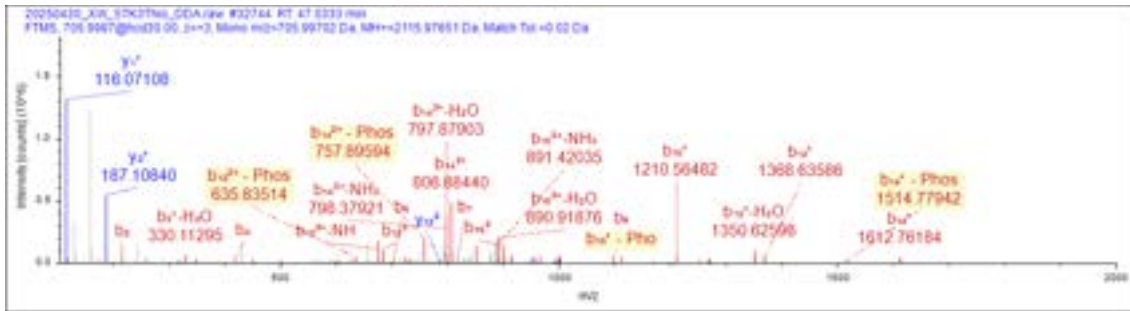


Figure S32. MS/MS fragmentation that suggested T182 phosphorylation.

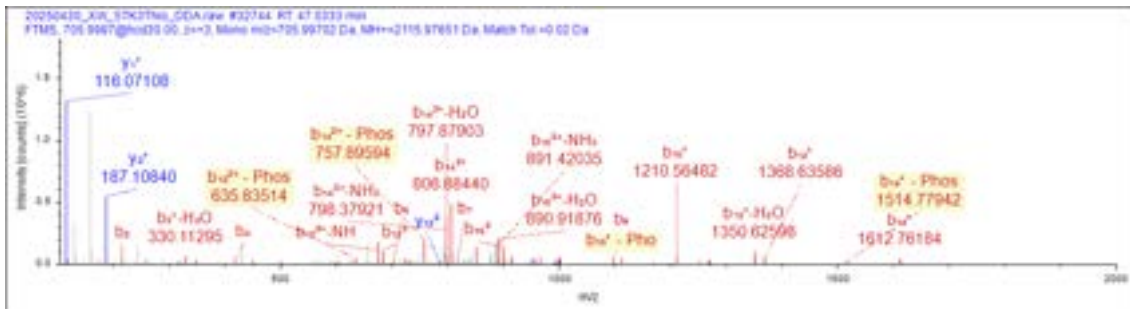


Figure S33. MS/MS fragmentation that suggested T188 phosphorylation.

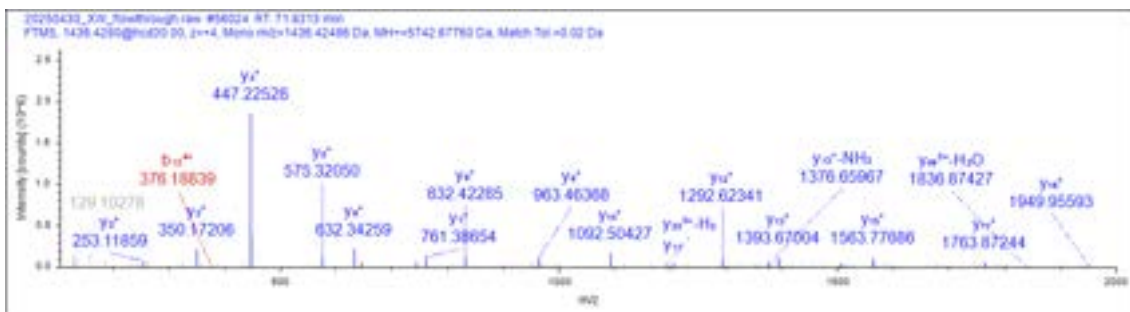


Figure S34. MS/MS fragmentation that suggested T192 thiophosphorylation.

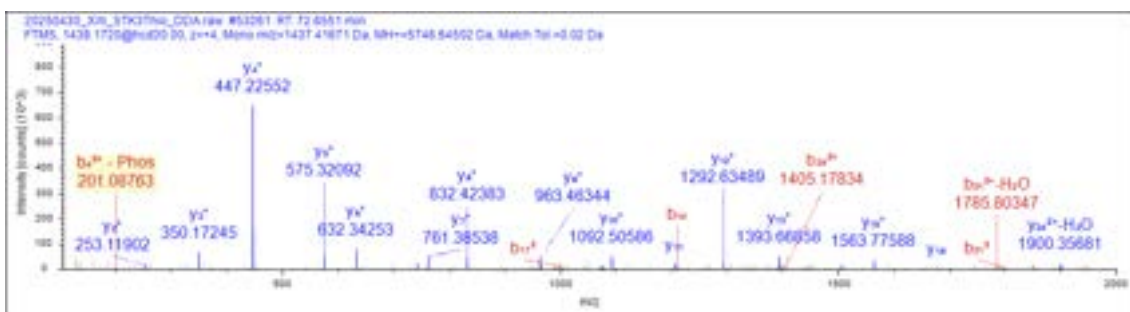


Figure S35. MS/MS fragmentation that suggested T182 phosphorylation and S214 thiophosphorylation.

12. Enzymatic Thiophosphorylation of Oligonucleotide and Characterization

General protocol for oligonucleotides (thio)-phosphorylation

The protocol for thiophosphorylation of oligonucleotides was modified from the protocol in the commercial T4 PNK kit (NEB #M0201).

The following ssDNA oligos were used as substrates for mass spectroscopic analysis (Orbitrap).

ssDNA oligos	Sequence	Molecular Weight
5'-'A'-Oligos	AACGATCAGTGGACGTAGCTA CGATC	8004.2
5'-'T'-Oligos	TAGCTCGAGCACCACCACCAC	6305.1
5'-'G'-Oligos	GCCATTGTCGATACTCATCGTGTGT	7639.9
5'-'C'-Oligos	CGCCGTGCCCCGCTACGCGACATTTGCGGTAAG	9802.4

1. The reaction was set up in a microcentrifuge tube on ice following the tables below for (thio)phosphorylation.

Phosphorylation

Component	Conc.	V(μL)	n (nmol)	equiv
ssDNA	100 μM	30	3	1.0
ATP	10 mM	50	500	167
T4 PNK				100 unit
Reaction Buffer (10X)		50		
Nuclease-free Water		Up to 500		

Thiophosphorylation

Component	Conc.	V(μL)	n (nmol)	equiv
ssDNA	100 μM	30	3	1.0
ADP	10 mM	5	50	16.7
Li _x CrPS	10 mM	50	500	167
Hb-CK	50 mg/mL	10	11.2	3.7
T4 PNK				100 unit
Reaction buffer (10X)		50		
Nuclease-free water		Up to 500		

2. The reactions were incubated at 37°C for 2 h.

3. The reaction was stopped by adding 10 μL of Protease K (NEB #P8111), and incubate at 37°C for 1.5 h.

4. After the digestion, 2.5 volume of EtOH was added to the reaction mixture, and the mixture was stored at -20°C for 1h.

5. The precipitated DNA was recovered by centrifugation at 15,000 rpm for 5 min at 4°C. The pellets were resuspended in 50 μL nuclease-free water and purified by Monarch Spin RNA clean-up kit (NEB #T2050), which is used for the mass spectroscopic analysis (Orbitrap).

Protocol for thiophosphorylated oligonucleotides orthogonal labeling with fluorophore S76

1. Following general procedure of oligonucleotides thiophosphorylation described above, the following ssDNA oligos was used as substrates for fluorescent detection.

ssDNA oligos	Sequence	Molecular Weight
Fluoro-seq	GGATCCTTGGAAGTATAGATTTTCAC	8000.3

2. To the 10 μ L of purified thiophosphorylated-oligos solution was added 5 μ L 50 mM Tris-HCl (50 mM, pH 6.5) and 1 mg of fluorescent dye **S76** (>4000 equiv) in 10 μ L MeCN.

3. The reaction mixture was incubated at room temperature for 2 h in the dark.

4. After the reaction was complete, oligos were extracted by phenol-chloroform, and precipitated with EtOH. The labeled oligos can be stored stably as dry powder or dissolved in nuclease-free water.

5. The labeled oligos were separated on 12% Urea-PAGE and characterized with in-gel fluorescent detection ($\lambda_{\text{ex}} = 472$ nm, $\lambda_{\text{em}} = 595$ nm).

6. As a comparison, the same gel was immersed in a solution of ethidium bromide (EtBr) in the dark for 10 min and characterized with fluorescent detection ($\lambda_{\text{ex}} = 302$ nm, $\lambda_{\text{em}} = 595$ nm).



Figure S36. In-gel fluorescence of a representative thiophosphorylated ss-DNA (left: phosphorylation, right: thiophosphorylation).

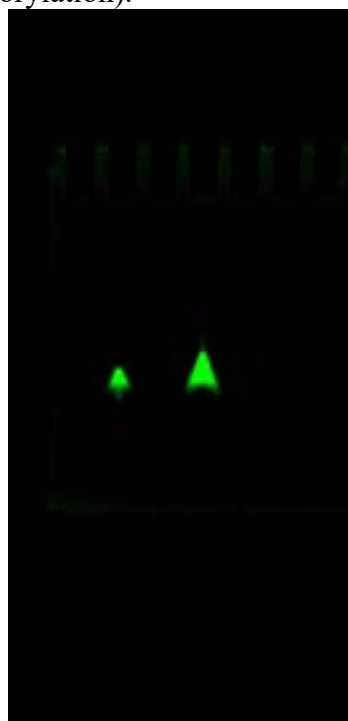


Figure S37. EtBr fluorescence of a representative thiophosphorylated ss-DNA (left: phosphorylation, right: thiophosphorylation).

Intact Oligonucleotide Mass Analysis

Liquid Chromatography tandem mass spectrometry experiments were performed on a Thermo Scientific Orbitrap Ascend equipped with a Thermo Scientific Vanquish Neo chromatography system. Mobile Phase composition was A: 20 mM ammonium acetate B: 100% acetonitrile. Oligonucleotide samples were injected onto a Thermo Scientific PepMap Neo C18 column (100 Å pore size, 2 µm particle size, 75 µm I.D.×150 mm). Oligonucleotides were eluted over a gradient of 1-30% acetonitrile over 12 minutes at a constant flow rate of 350 nL/min. Spectra were acquired in negative polarity from m/z 350-2000 at a resolving power of 120000.

Spectral Deconvolution

Data was processed in Thermo Scientific BioPharma Finder 5.1. Scans were merged using the sliding windows algorithm with a 25% offset, 30 ppm merge tolerance and a maximum retention time gap of 1 minute. Spectra were deconvoluted with the Xtract algorithm with the following parameters: S/N threshold 3; relative abundance threshold 0; charge range 5 to 50; nucleotide isotope table.

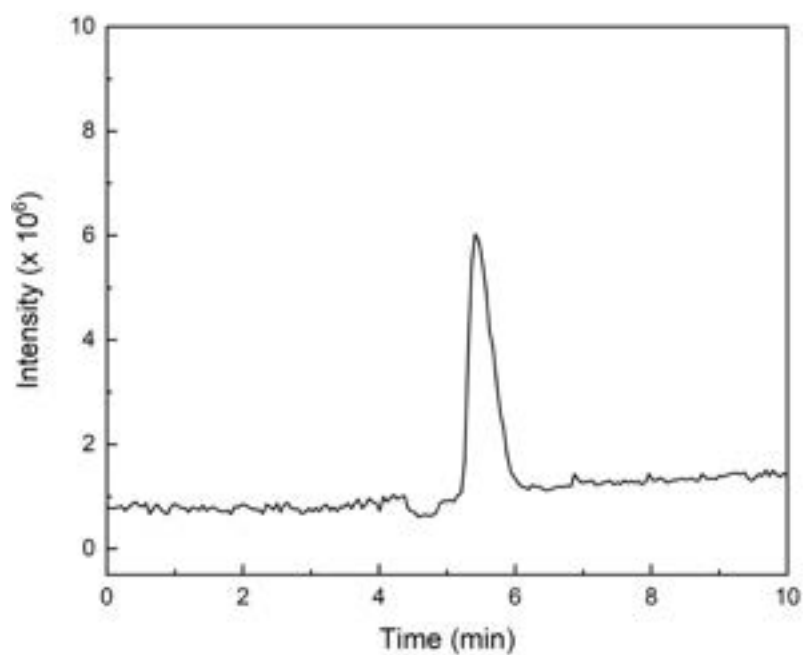


Figure S38. Chromatogram of modified ss DNA (5'-A)

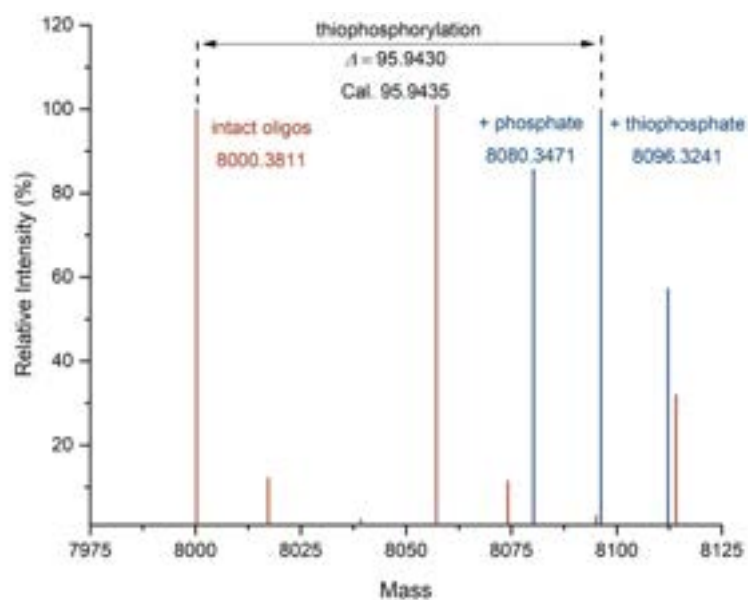


Figure S39. Comparison of deconvoluted mass between intact and modified ss DNA (5'-A)

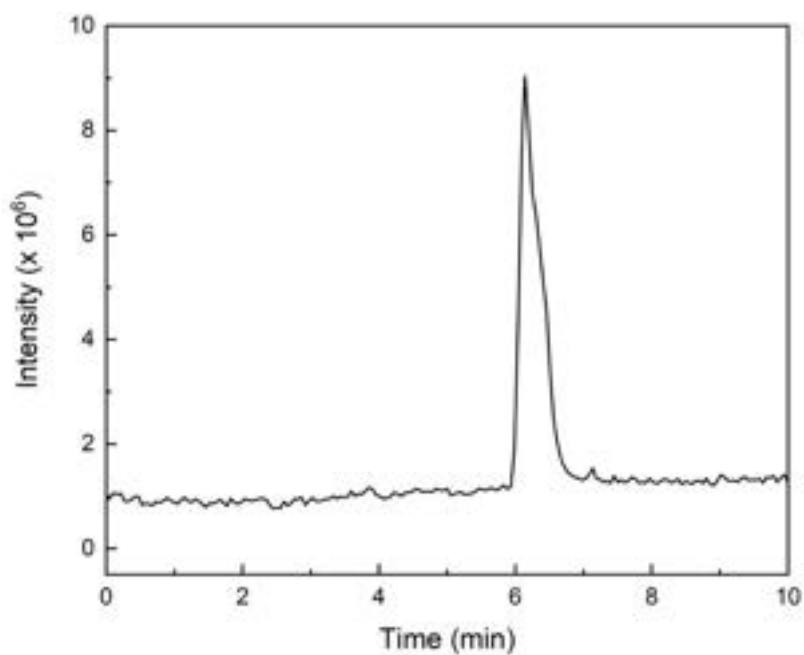


Figure S40. Chromatogram of modified ss DNA (5'-T)

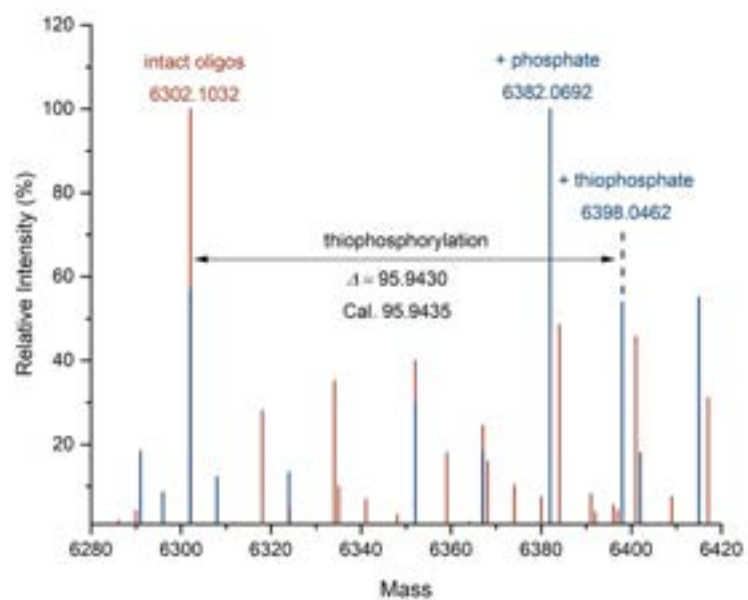


Figure S41. Comparison of deconvoluted mass between intact and modified ss DNA (5'-T)

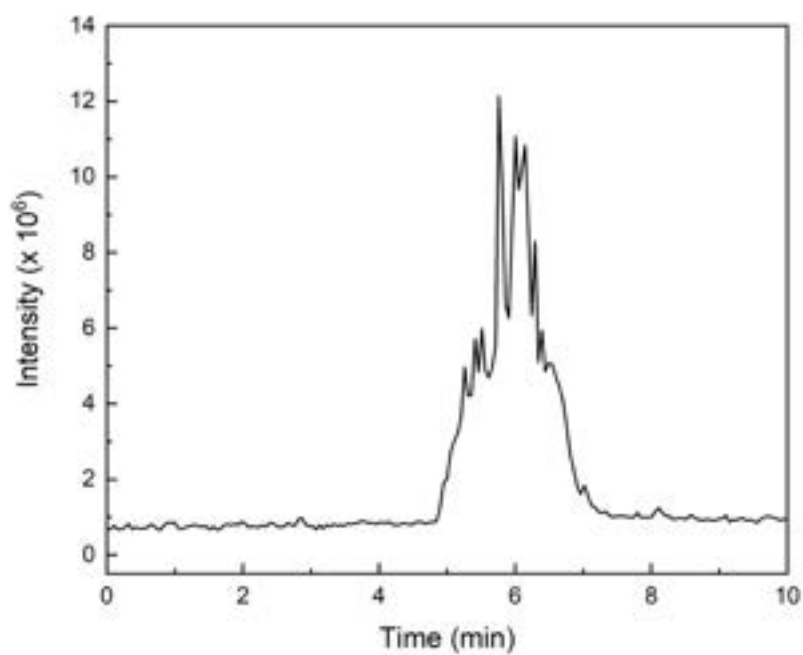


Figure S42. Chromatogram of modified ss DNA (5'-G)

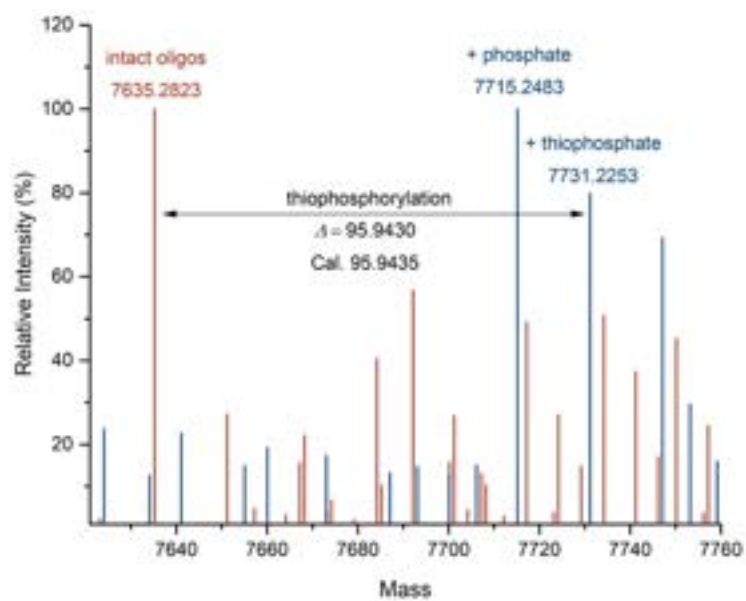


Figure S43. Comparison of deconvoluted mass between intact and modified ss DNA (5'-G)

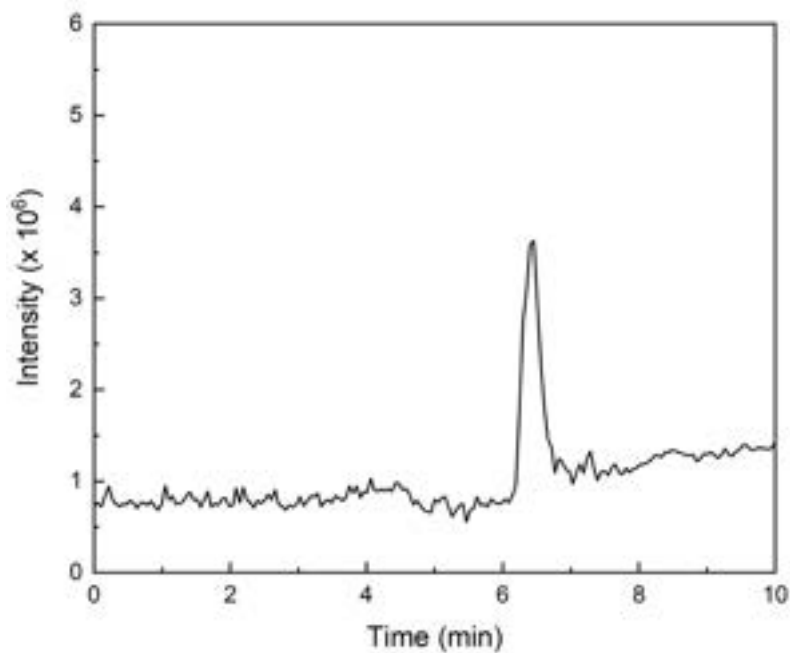


Figure S44. Chromatogram of modified ss DNA (5'-C)

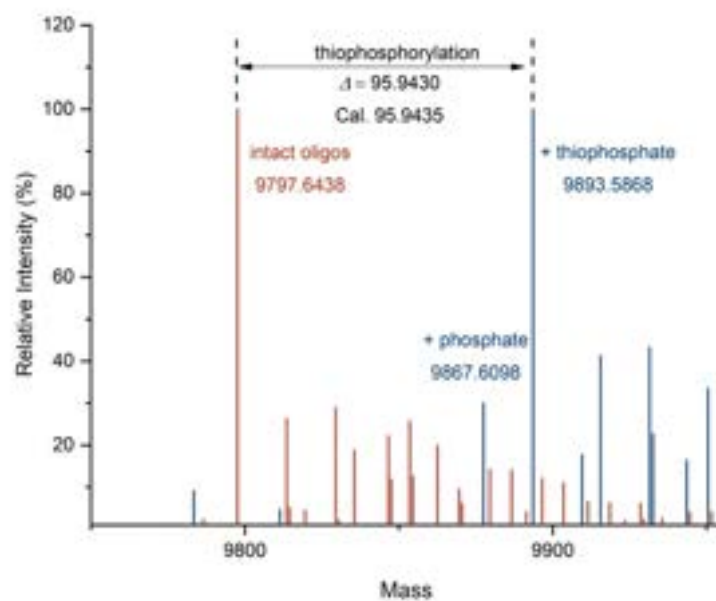


Figure S45. Comparison of deconvoluted mass between intact and modified ss DNA (5'-C)

Thiophosphorylation of 3'-OH modified oligonucleotides and MALDI experiment

Following general thiophosphorylation protocol described above, a 25-bp 3'-6-FAM-modified ssDNA was used to minimize potential hydrolysis from 3' terminus.

ssDNA oligos	Sequence	Molecular Weight			
5-'C'-Oligos	CGATTGAGGGCGTAGAATCAGAAAGT-FAM	8364.2			

Component	Mw	Conc.	V(μ L)	n (nmol)	equiv
ssDNA	8364.2	100 μ M	30	3	1.0
ADP		10 mM	5	50	16.7
Li _x CrPS		10 mM	50	500	167
Hb-CK	44.81kDa	50 mg/mL	10	11.2	3.7
T4 PNK					100 unit
Reaction buffer (10X)			50		
Nuclease-free water			Up to 500		

After cleaning up with Monarch Spin RNA clean-up kit, the sample was lyophilized and used for MALDI experiment.

The samples were run on Bruker AutoFlex Speed LRF MALDI in linear positive mode using 3-Hydroxypicolinic acid (3-HPA) as matrix (saturated in 10 mg/mL ammonium citrate in 50% ACN). The sample was dissolved in 50 μ L water and mixed with the matrix at the volume ratio of 1:10.

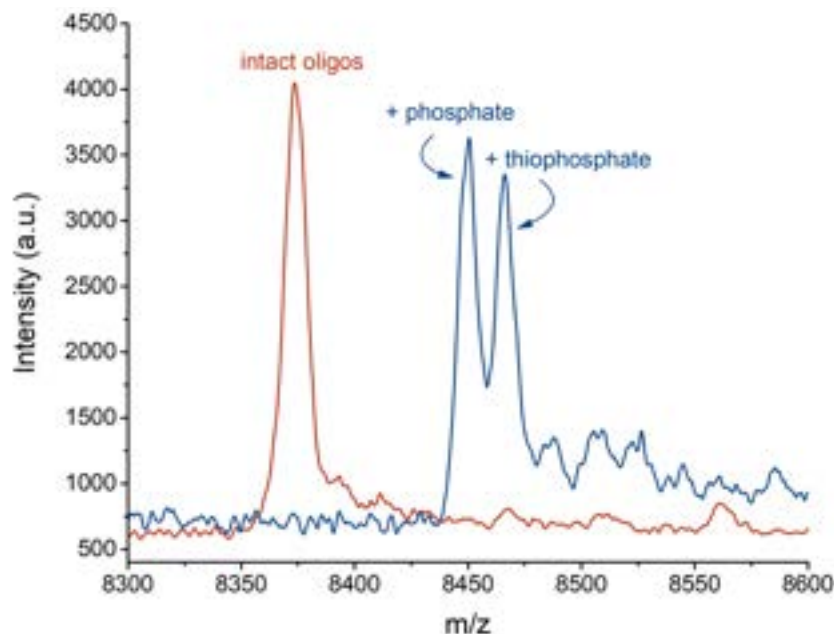


Figure S46. MALDI spectra of intact and modified 3'-6-FAM-ss DNA (5'-C)

13. X-ray Crystallographic Data

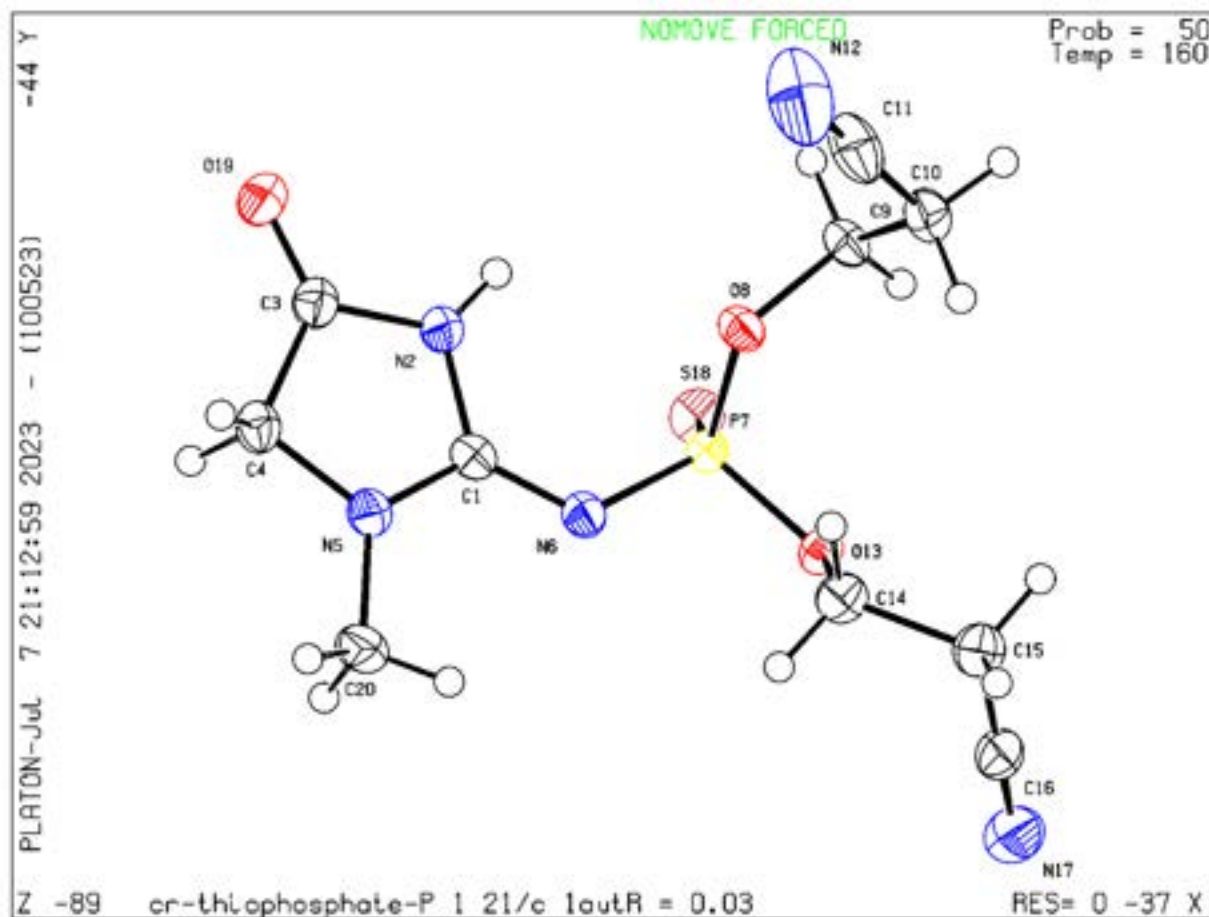


Table S17. Crystal structure data and refinement for the **S1-Int 1**

CCDC deposition number	2467026
Empirical formula	C ₁₀ H ₁₄ N ₅ O ₃ PS
Formula weight	315.29
Temperature/K	162(30)
Crystal system	monoclinic
Space group	P2 ₁ /c
a/Å	6.99180(10)
b/Å	23.8229(2)
c/Å	8.59390(10)
α/°	90
β/°	92.9620(10)
γ/°	90
Volume/Å ³	1429.53(3)
Z	4
ρ _{calc} /cm ³	1.465
μ/mm ⁻¹	3.228

F(000)	656.0
Crystal size/mm ³	0.28 × 0.205 × 0.121
Radiation	Cu K α (λ = 1.54184)
2 Θ range for data collection/°	7.422 to 145.682
Index ranges	-8 ≤ h ≤ 8, -24 ≤ k ≤ 29, -10 ≤ l ≤ 10
Reflections collected	8395
Independent reflections	2745 [R _{int} = 0.0166, R _{sigma} = 0.0147]
Data/restraints/parameters	2745/0/183
Goodness-of-fit on F ²	1.099
Final R indexes [I ≥ 2 σ (I)]	R ₁ = 0.0295, wR ₂ = 0.0864
Final R indexes [all data]	R ₁ = 0.0301, wR ₂ = 0.0869
Largest diff. peak/hole / e Å ⁻³	0.36/-0.36

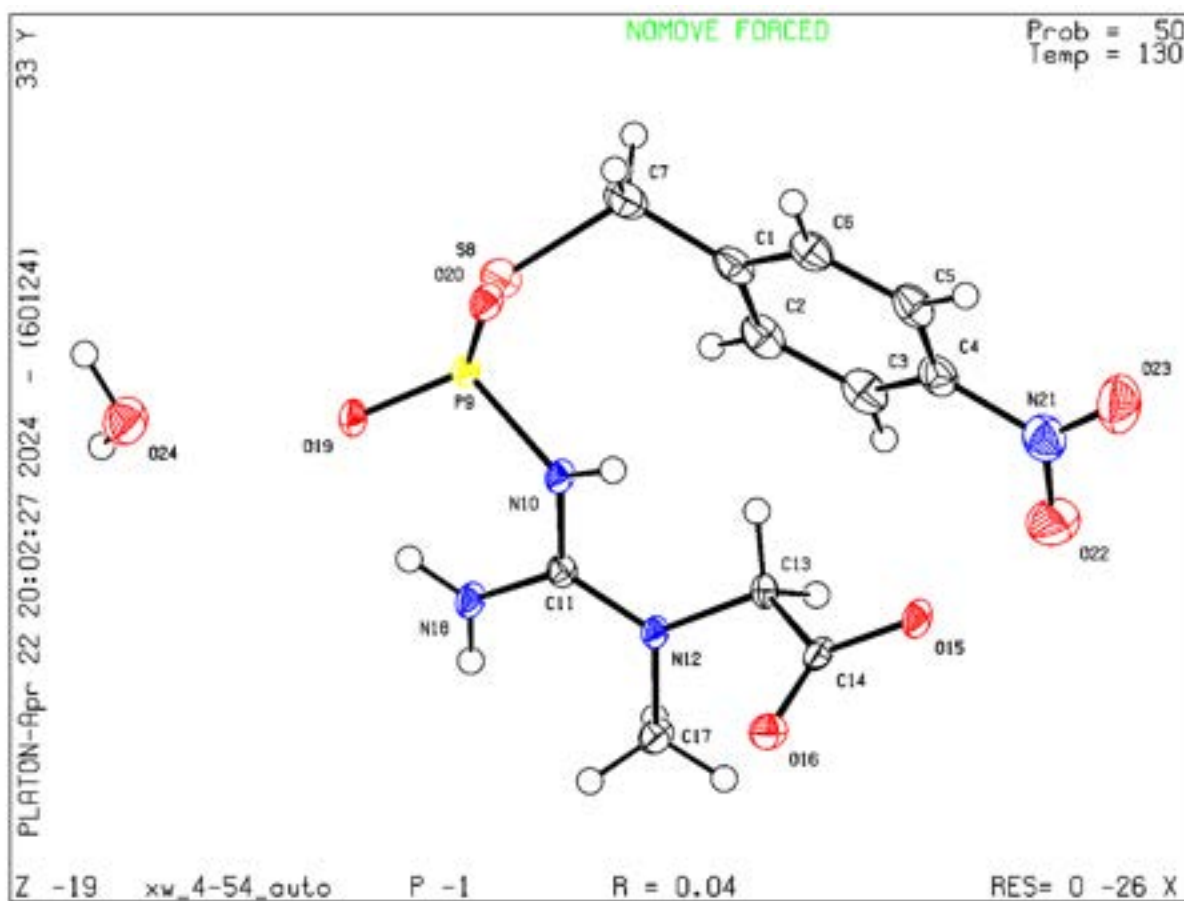


Table S18. Crystal structure data and refinement for the **S1-Int 2**

CCDC deposition number	2467027
Empirical formula	C ₁₁ H ₁₆ N ₄ O ₇ PS
Formula weight	379.31
Temperature/K	125(30)
Crystal system	triclinic
Space group	P-1

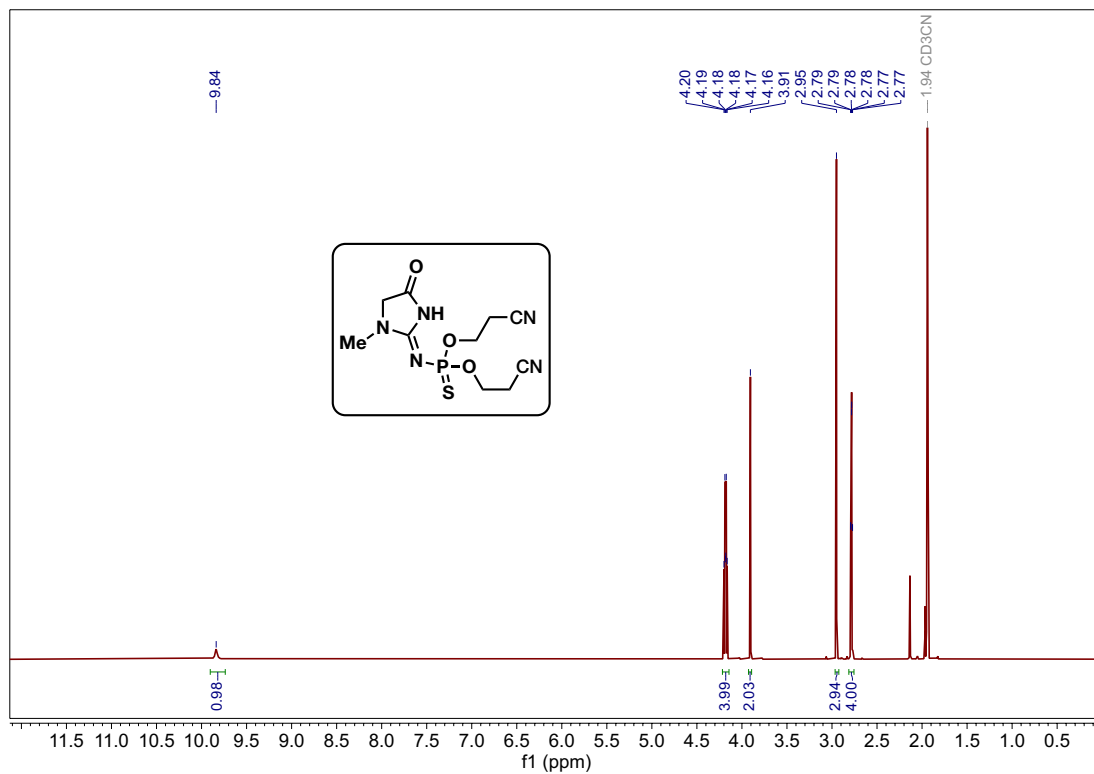
a/Å	7.46930(10)
b/Å	8.7809(2)
c/Å	13.4559(2)
$\alpha/^\circ$	106.146(2)
$\beta/^\circ$	97.291(2)
$\gamma/^\circ$	95.8810(10)
Volume/Å ³	832.03(3)
Z	2
$\rho_{\text{calc}}/\text{cm}^3$	1.514
μ/mm^{-1}	3.045
F(000)	394.0
Crystal size/mm ³	$0.333 \times 0.161 \times 0.113$
Radiation	Cu K α ($\lambda = 1.54184$)
2 Θ range for data collection/ $^\circ$	6.932 to 147.256
Index ranges	$-8 \leq h \leq 9, -10 \leq k \leq 10, -16 \leq l \leq 16$
Reflections collected	8975
Independent reflections	3182 [$R_{\text{int}} = 0.0258, R_{\text{sigma}} = 0.0282$]
Data/restraints/parameters	3182/3/224
Goodness-of-fit on F^2	0.828
Final R indexes [$I \geq 2\sigma(I)$]	$R_1 = 0.0384, wR_2 = 0.1038$
Final R indexes [all data]	$R_1 = 0.0401, wR_2 = 0.1053$
Largest diff. peak/hole / e Å ⁻³	0.78/-0.61

14. References

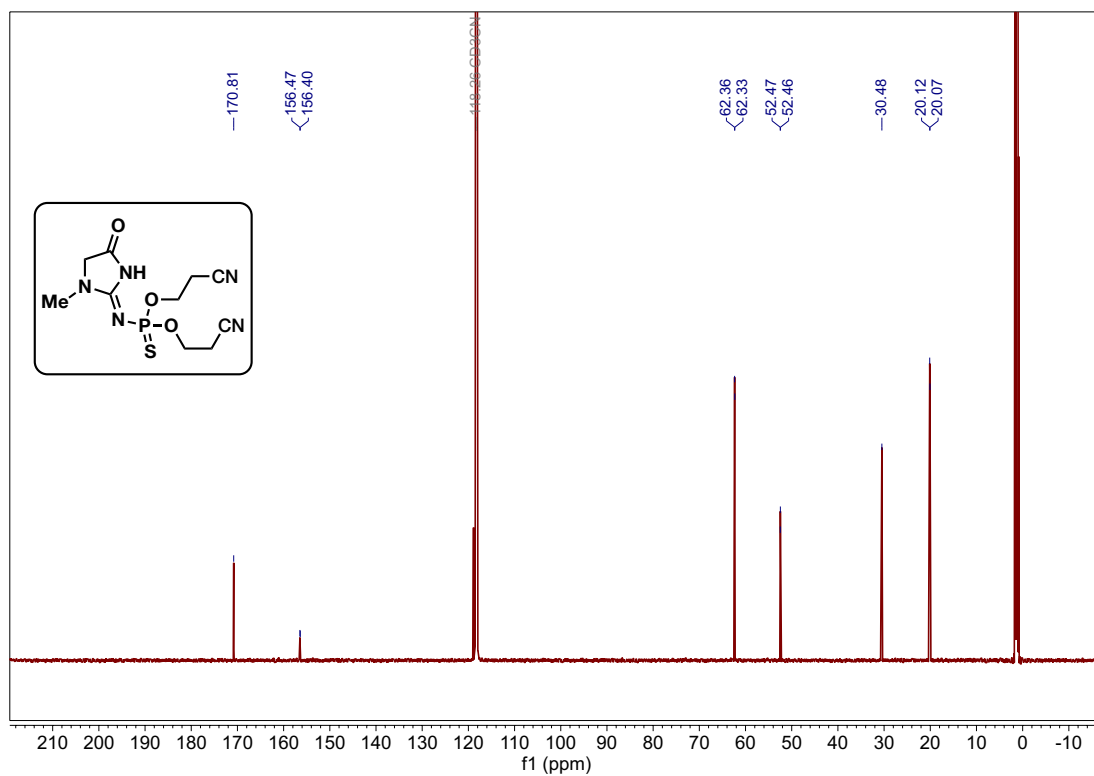
1. Kim, A. *et al.* Selective Phosphorylation of Phenols and Benzenediols by the Kinase PsiK and Variants Thereof. *Angew. Chem. Int. Ed.* **64**, e202503538 (2025).
2. Crans, D. C. & Whitesides, G. M. A convenient synthesis of disodium acetyl phosphate for use in in situ ATP cofactor regeneration. *J. Org. Chem.* **48**, 3130–3132 (1983).
3. Li, J., Wei, H. & Zhou, M.-M. Structure-Guided Design of a Methyl Donor Cofactor That Controls a Viral Histone H3 Lysine 27 Methyltransferase Activity. *J. Med. Chem.* **54**, 7734–7738 (2011).
4. Debarge, S., Balzarini, J. & Maguire, A. R. Design and Synthesis of α -Carboxy Phosphononucleosides. *J. Org. Chem.* **76**, 105–126 (2011).
5. Zhu, X. & and Ian Scott, A. Novel Synthetic Approach to Multibenzoylated Nucleosides. *Synth. Commun.* **38**, 1346–1354 (2008).
6. Zhang, H.-J. *et al.* Stereocontrolled access to thioisosteres of nucleoside di- and triphosphates. *Nat. Chem.* **16**, 249–258 (2024).
7. Suydam, I. T. & Strobel, S. A. Fluorine Substituted Adenosines As Probes of Nucleobase Protonation in Functional RNAs. *J. Am. Chem. Soc.* **130**, 13639–13648 (2008).
8. Meldal, M. Large-scale synthesis of d-mannose 6-phosphate and other hexose 6-phosphates. *Carbohydr. Res.* **235**, 115–127 (1992).
9. Li, L. *et al.* A highly efficient galactokinase from *Bifidobacterium infantis* with broad substrate specificity. *Carbohydr. Res.* **355**, 35–39 (2012).
10. Strohalm, M., Hassman, M., Kořata, B. & Koldíček, M. mMass data miner: an open source alternative for mass spectrometric data analysis. *Rapid Commun. Mass Spectrom.* **22**, 905–908 (2008).

15. NMR Spectra of products

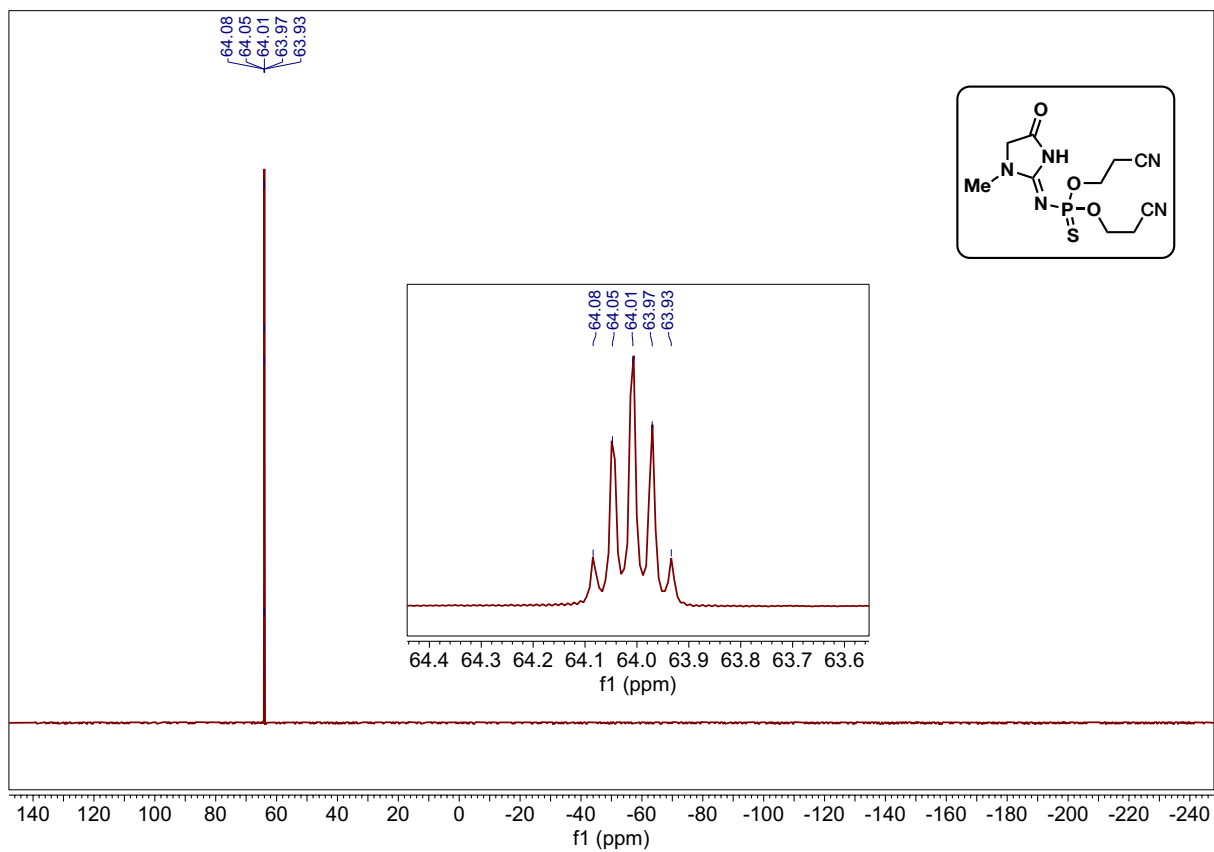
¹H NMR of compound S1-Int 1 (600 MHz, CD₃CN)



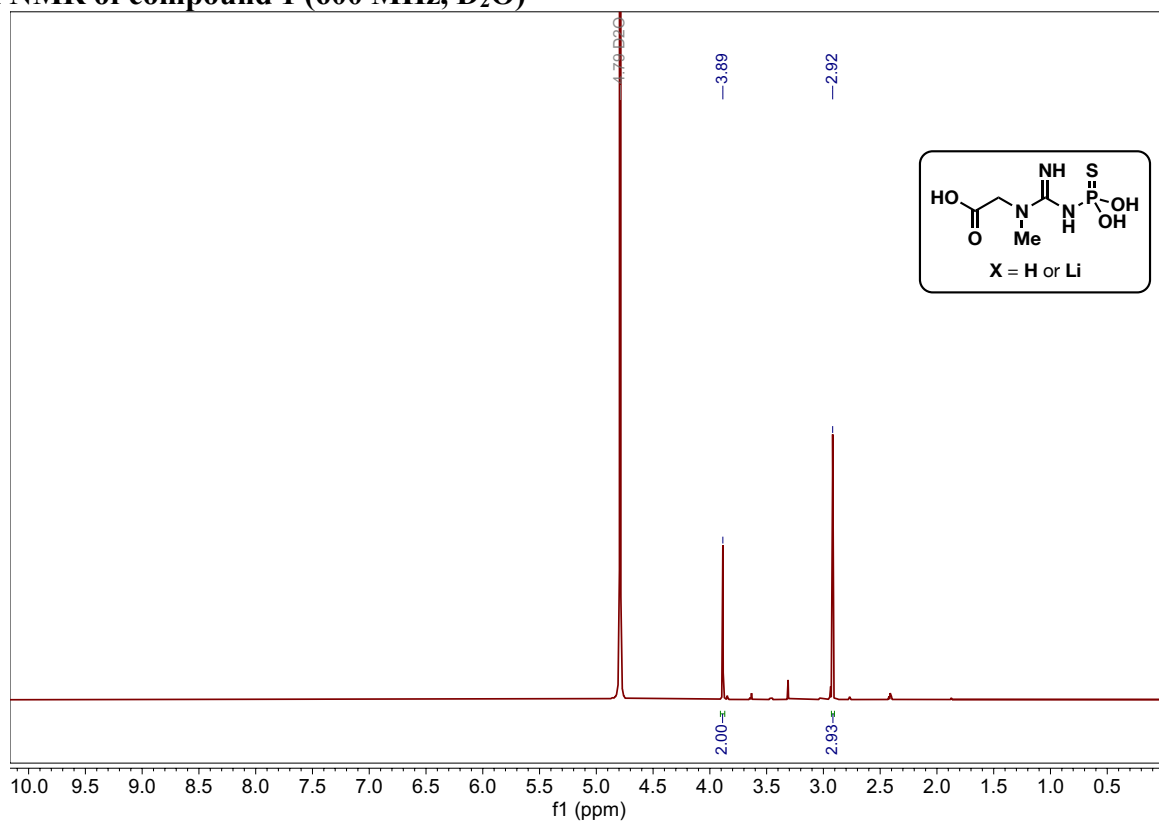
¹³C NMR of compound S1-Int 1 (151 MHz, CD₃CN)



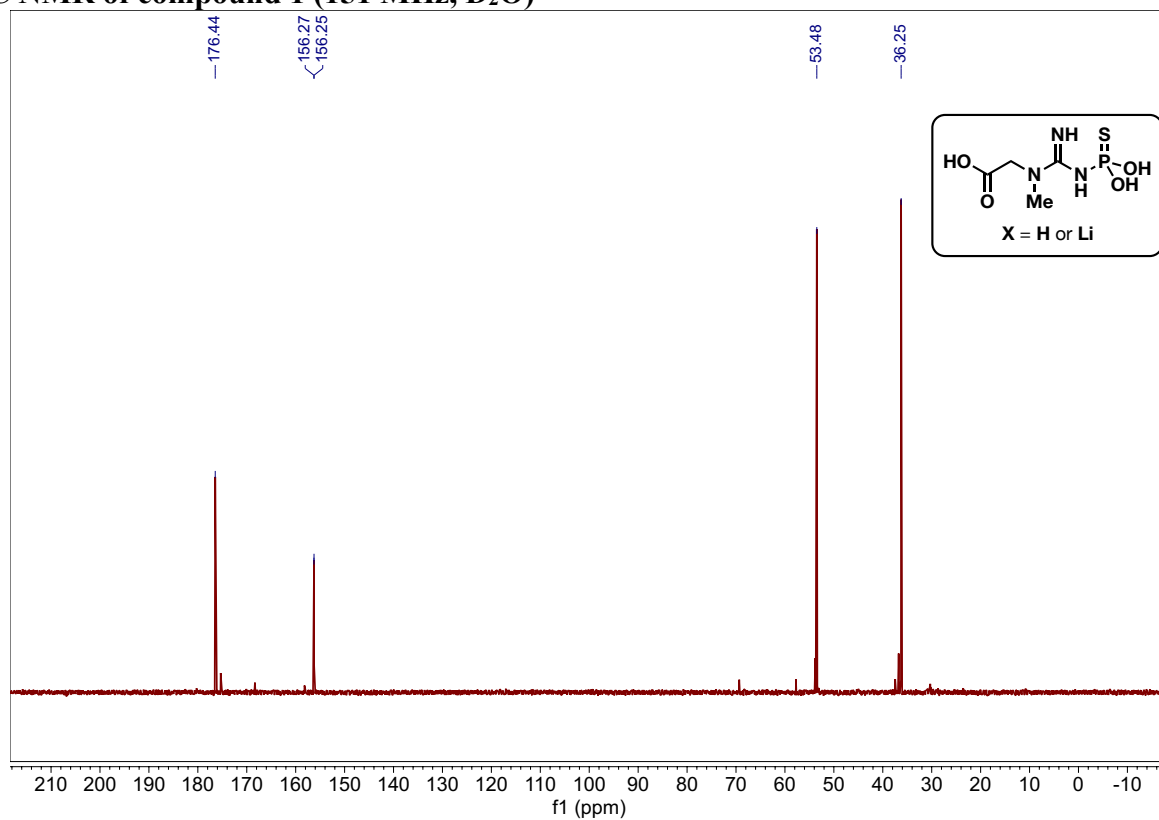
^{31}P NMR of compound S1-Int 1 (243 MHz, CD_3CN)



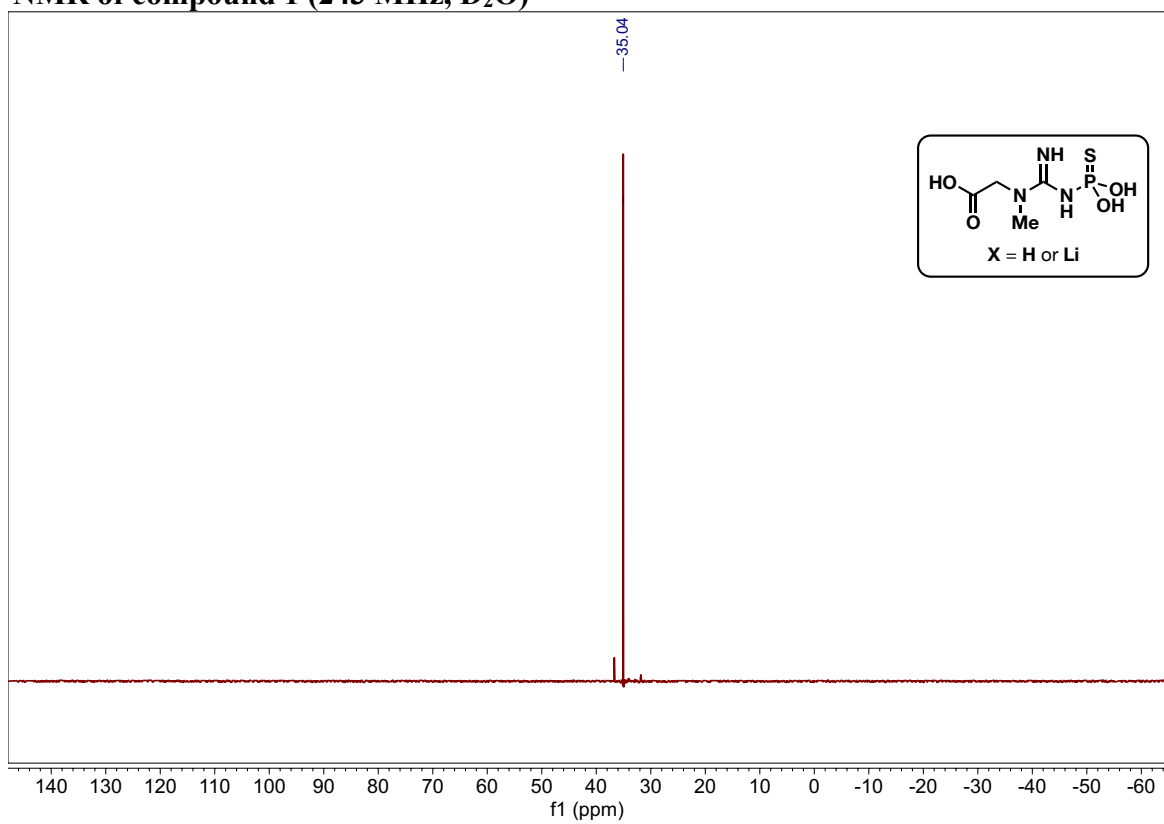
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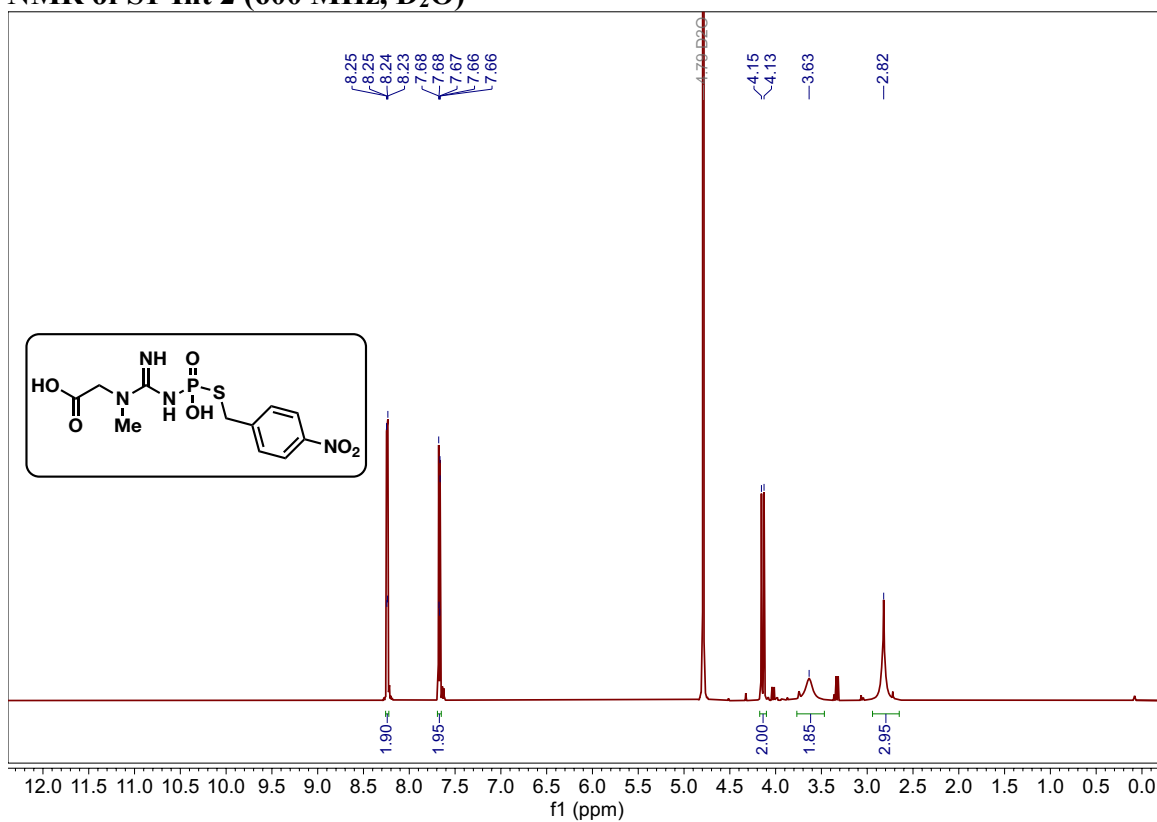
^{13}C NMR of compound 1 (151 MHz, D_2O)



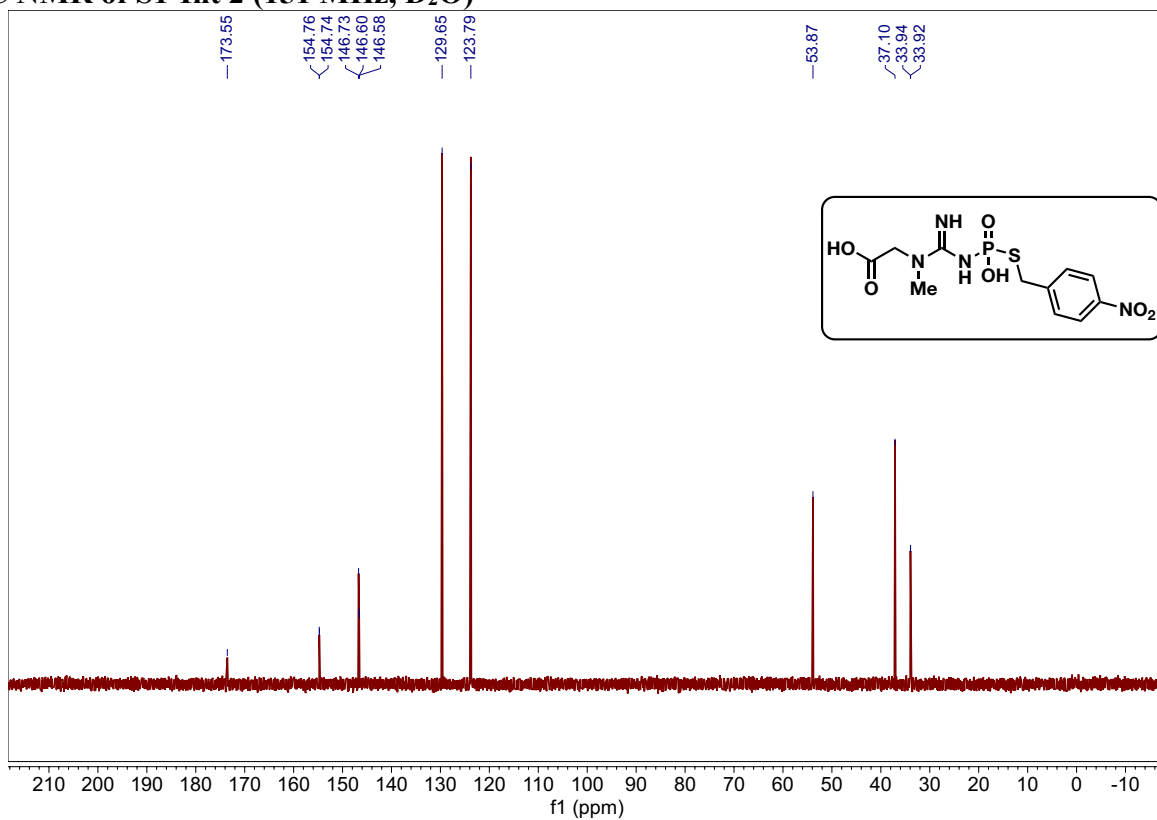
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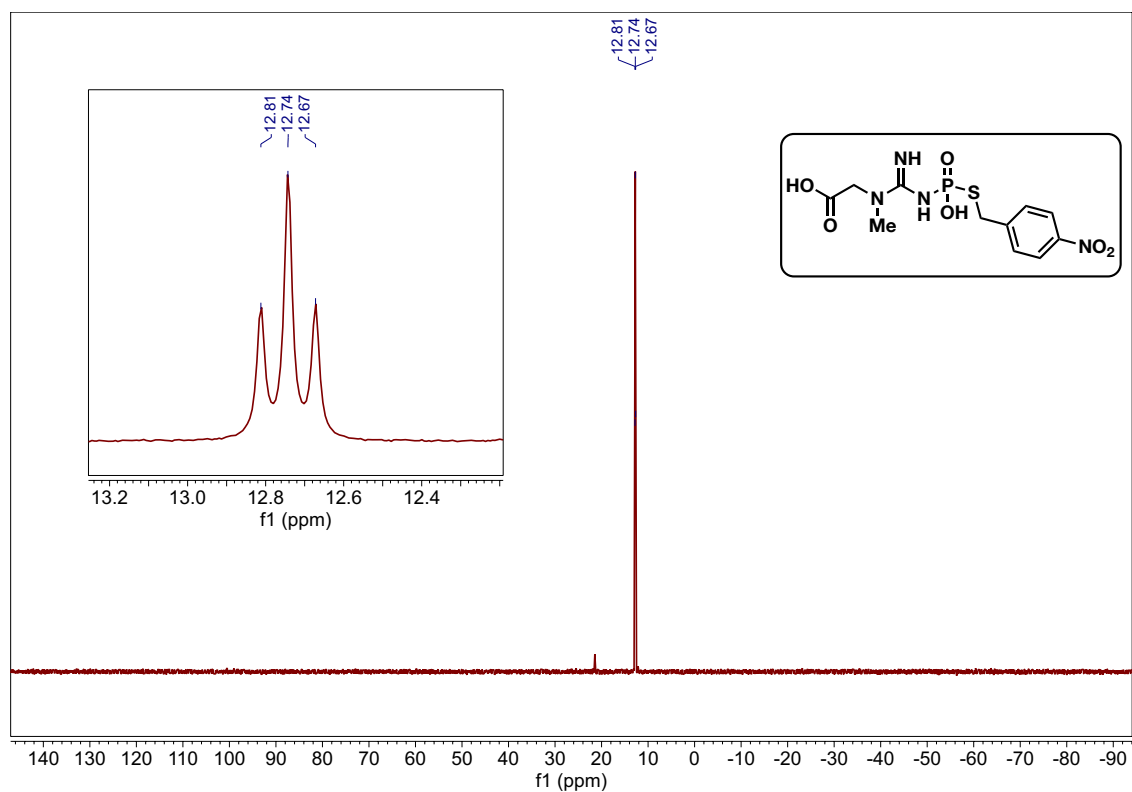
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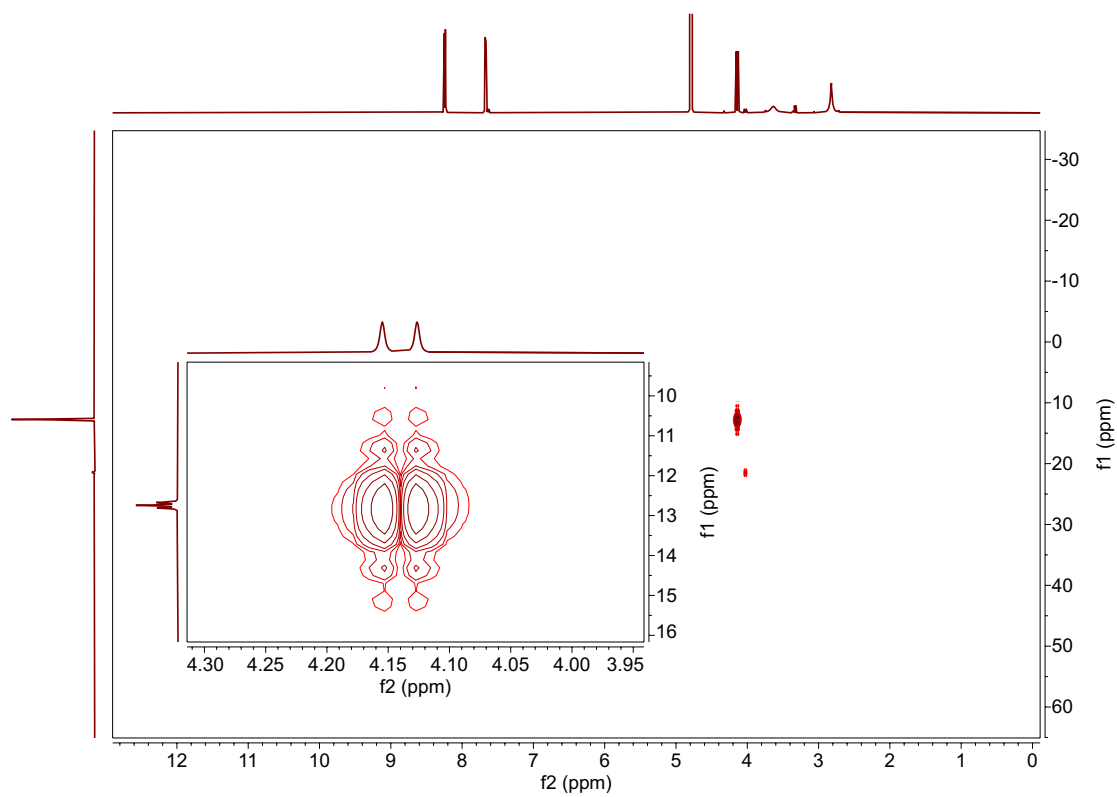
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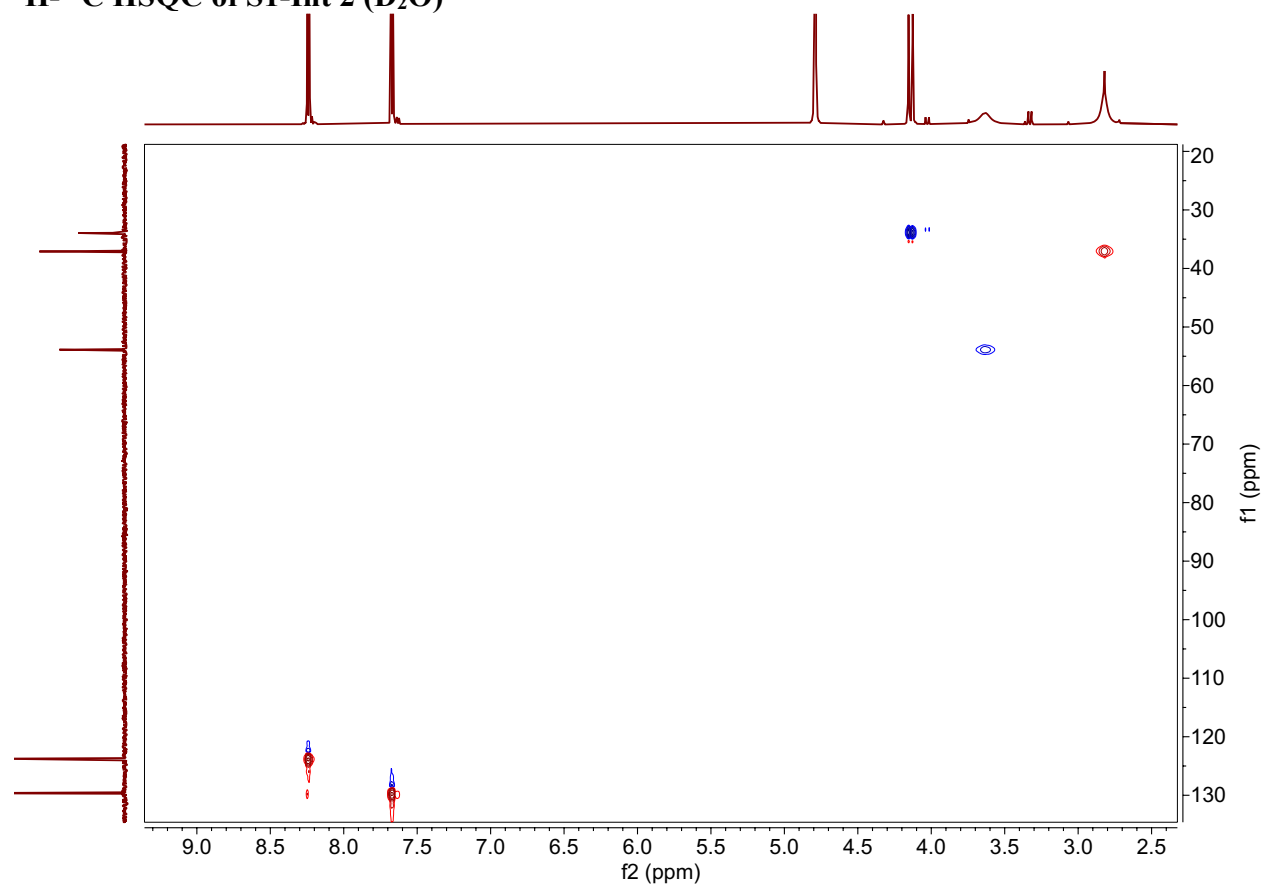
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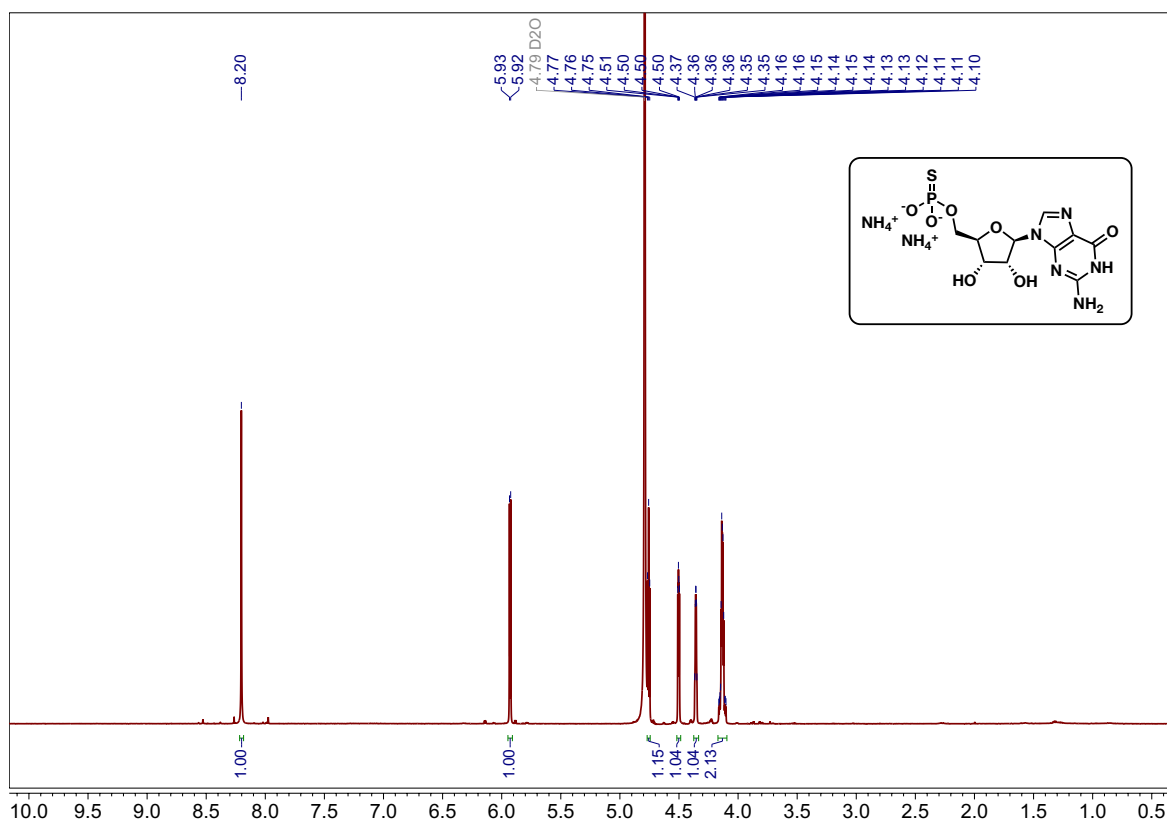
^1H - ^{31}P HMBC of S1-Int 2 (D_2O)



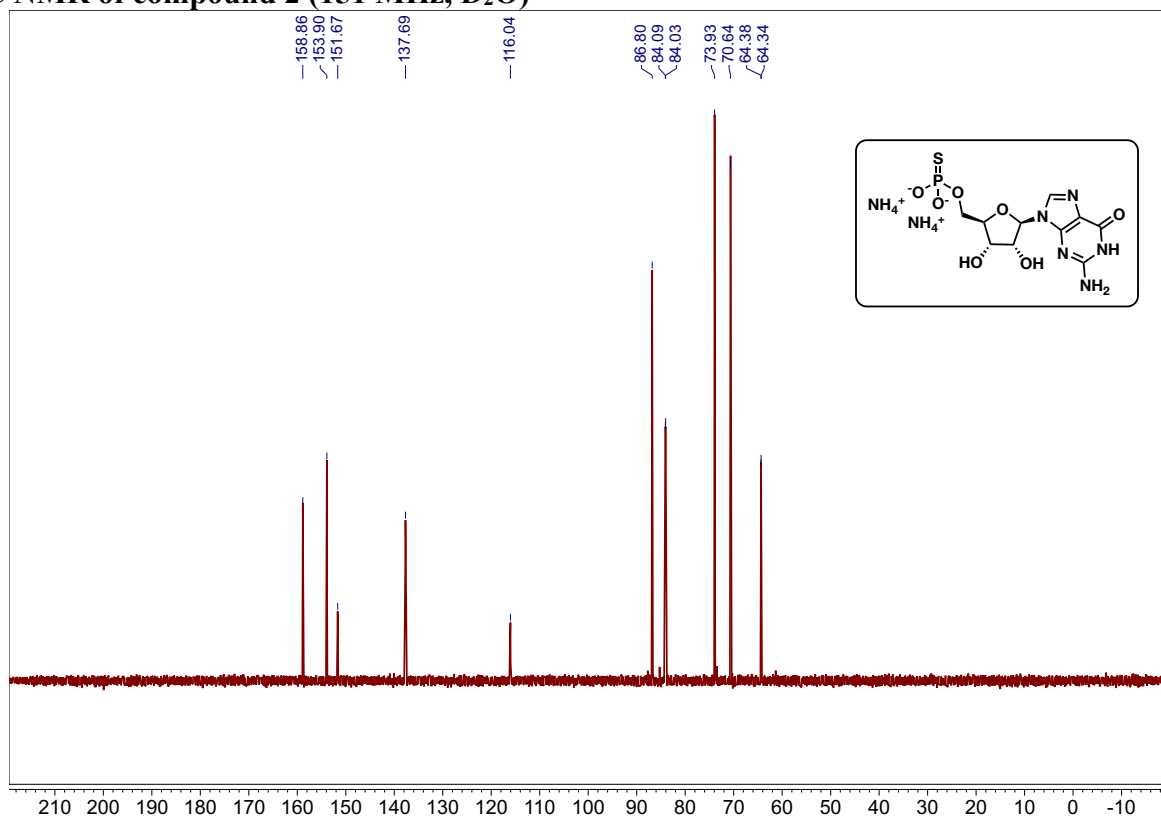
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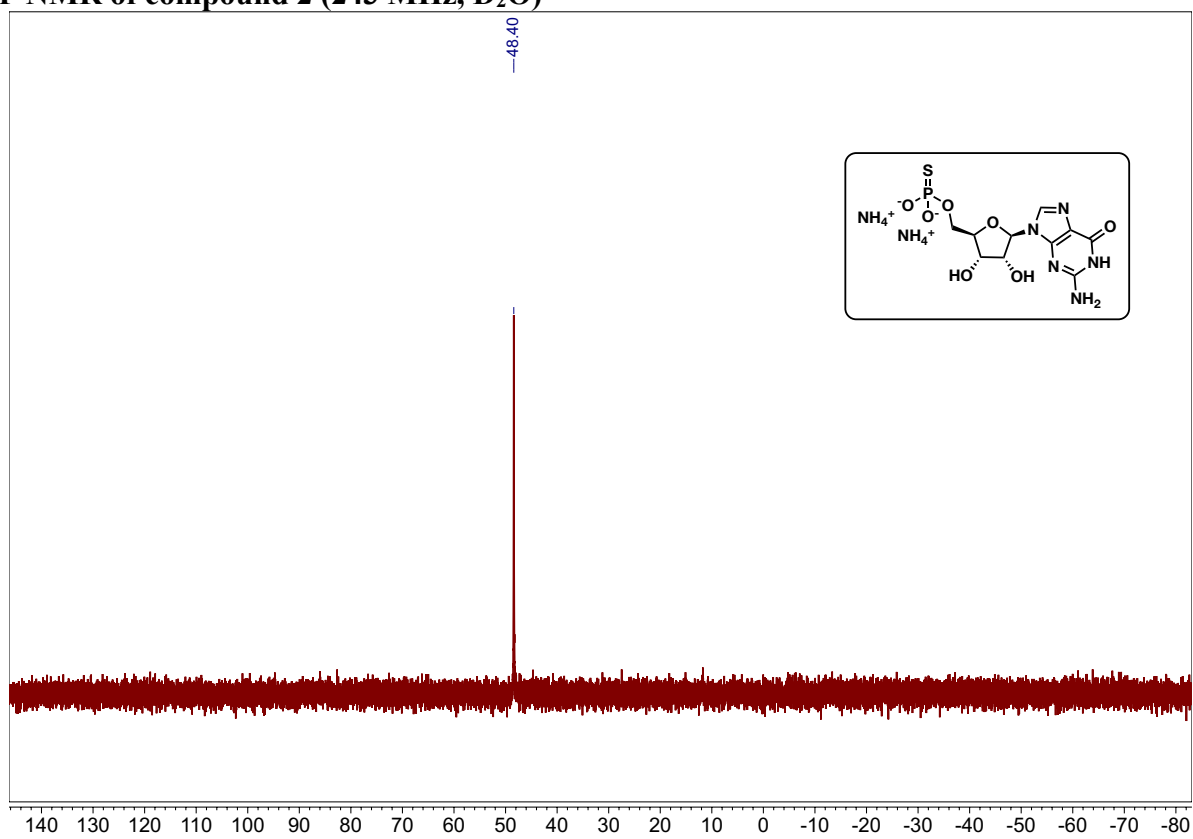
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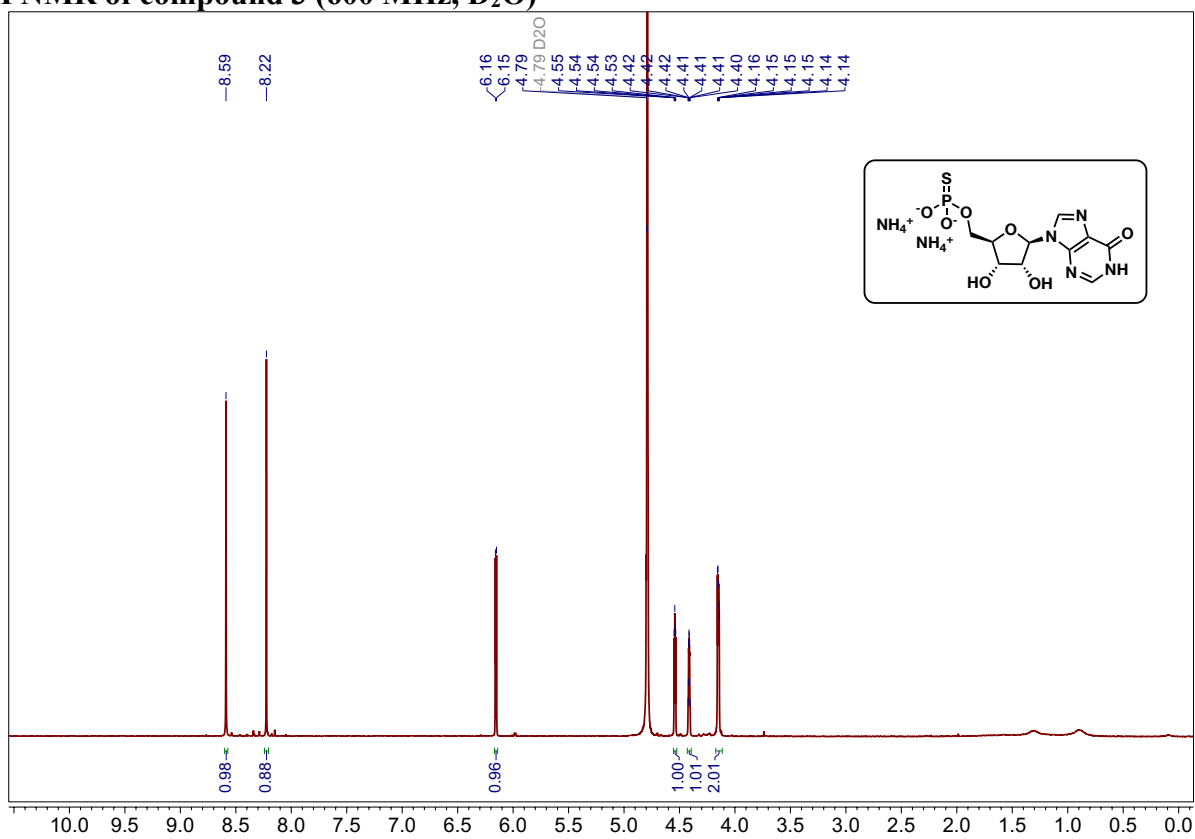
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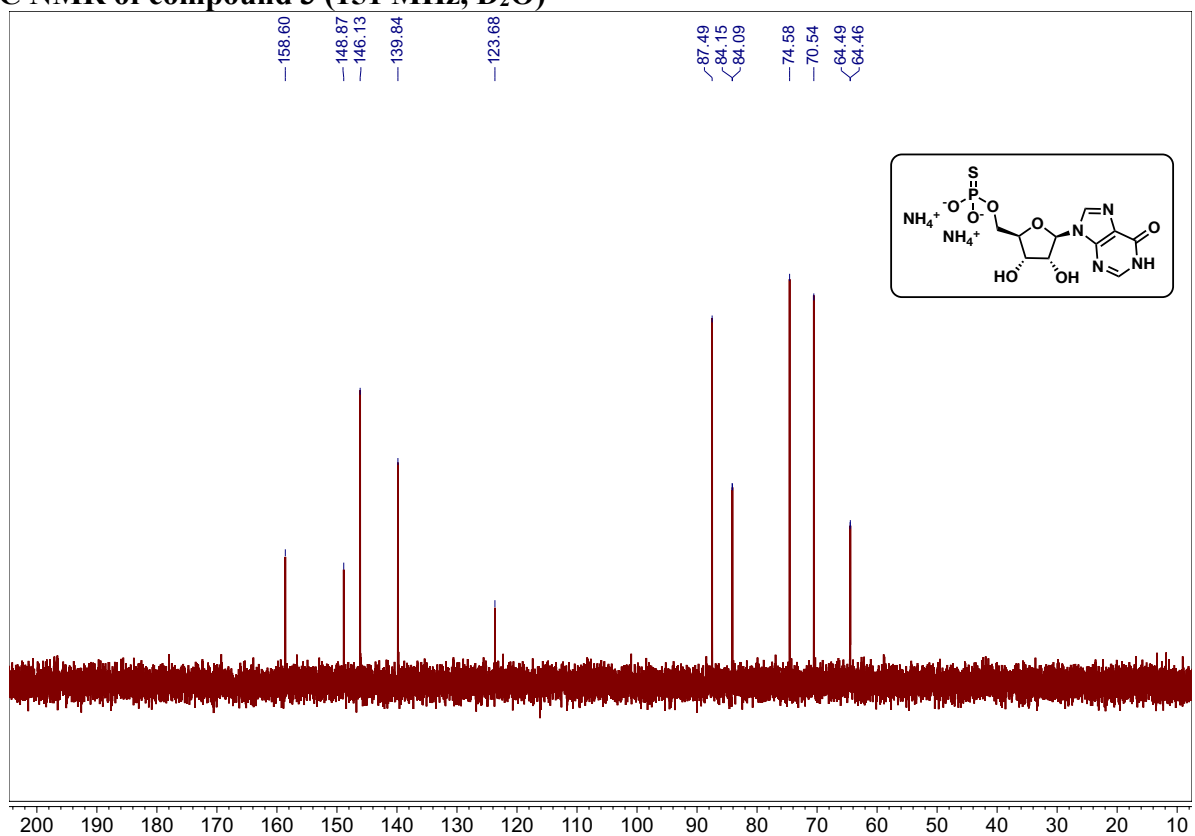
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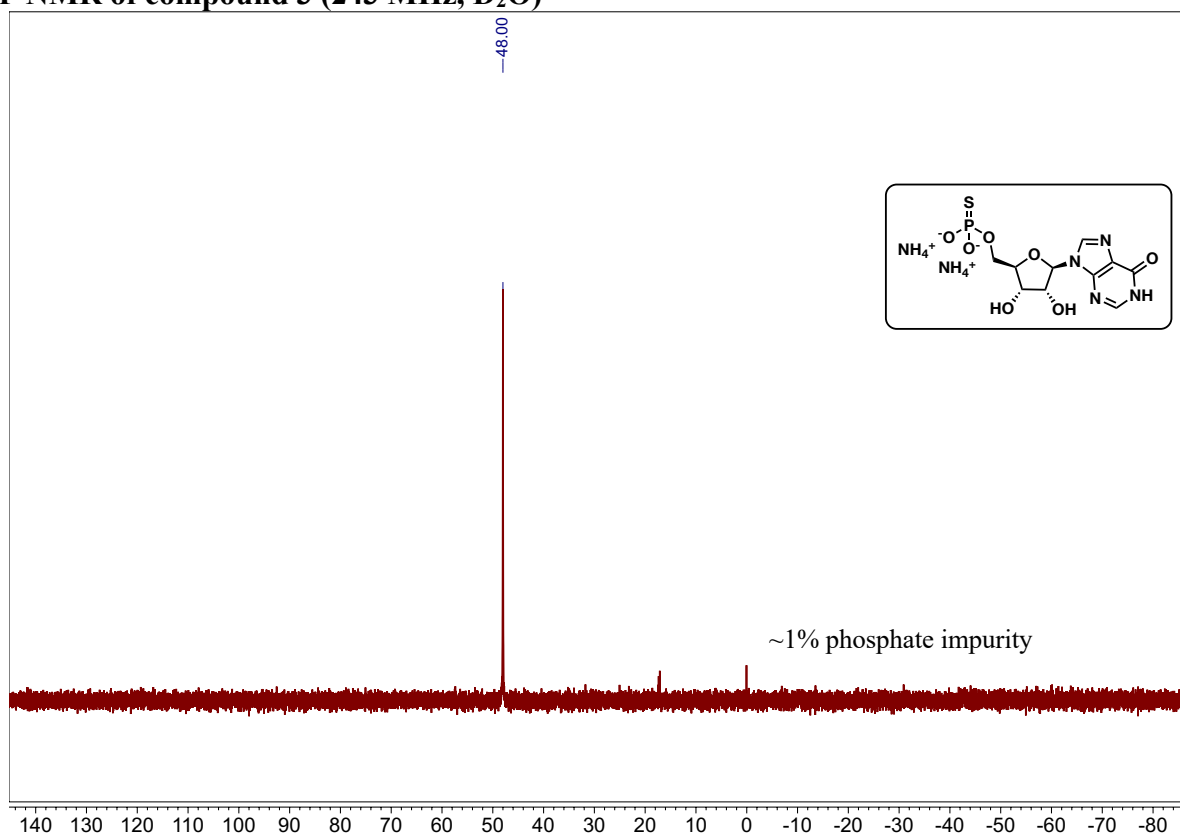
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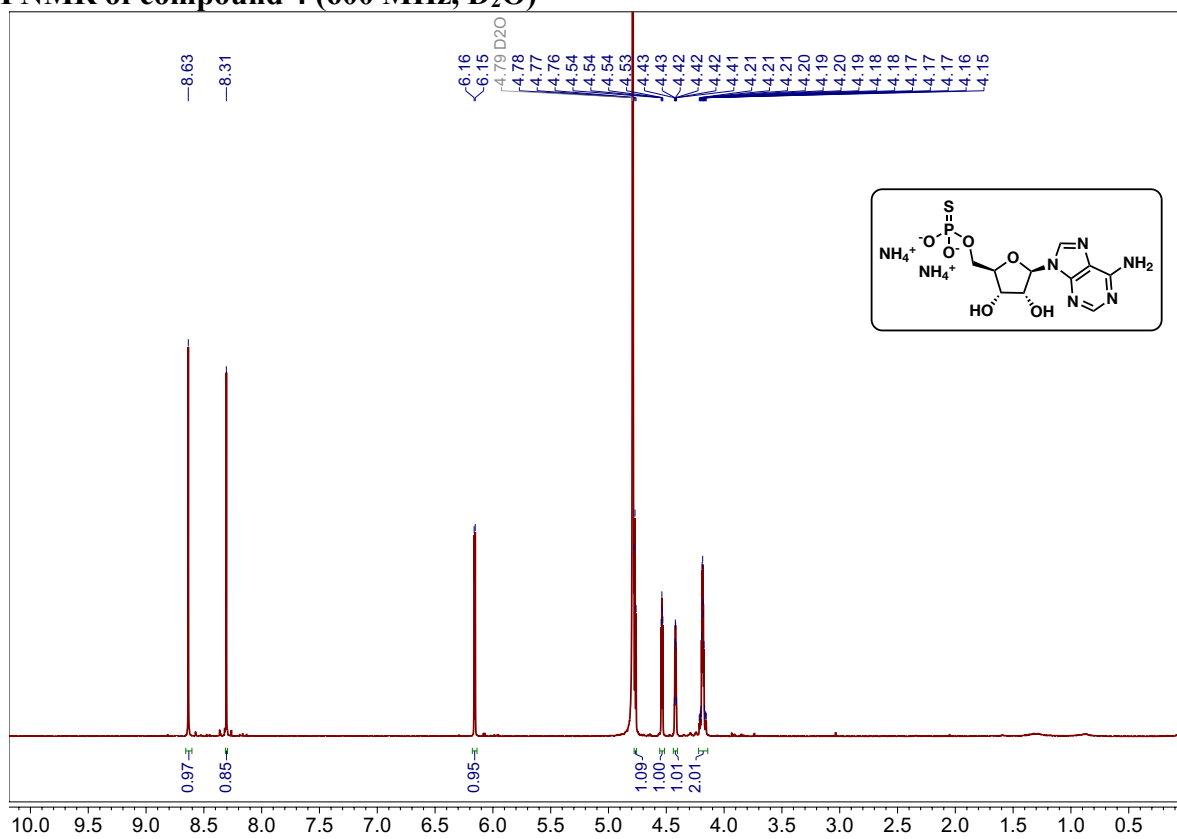
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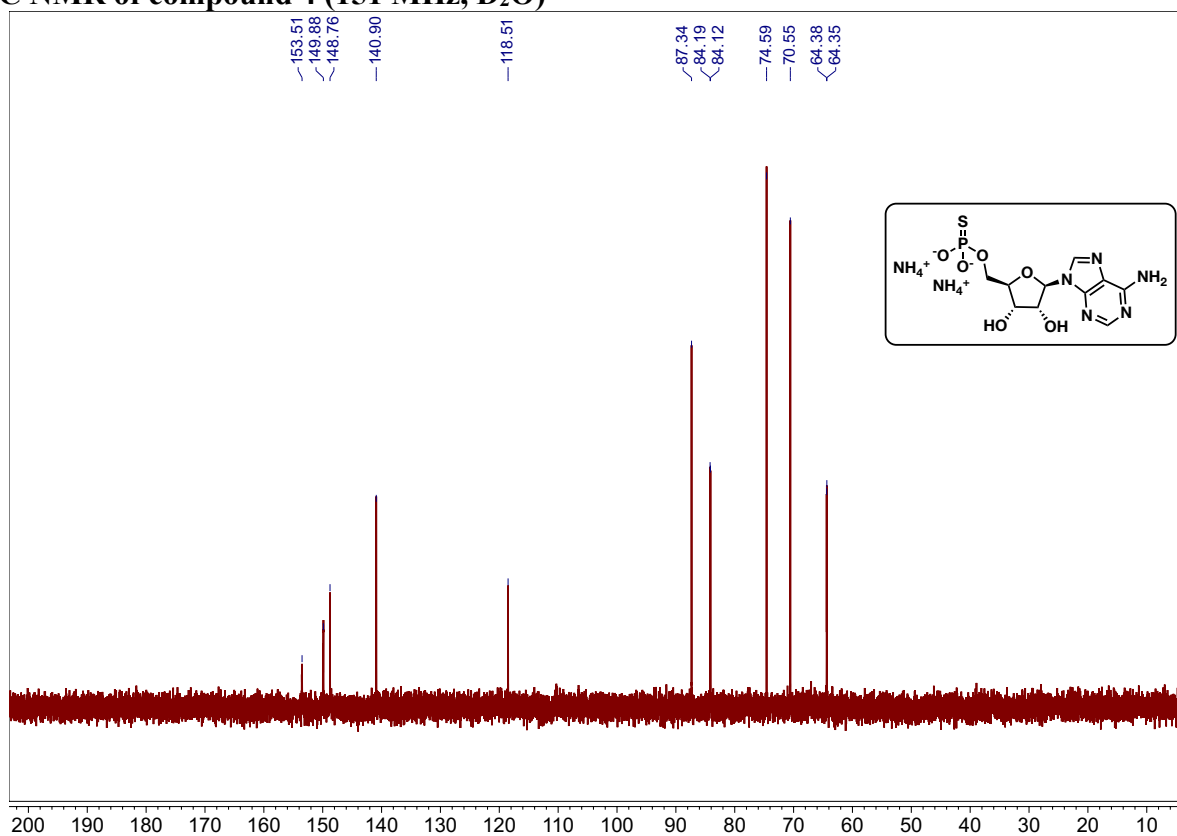
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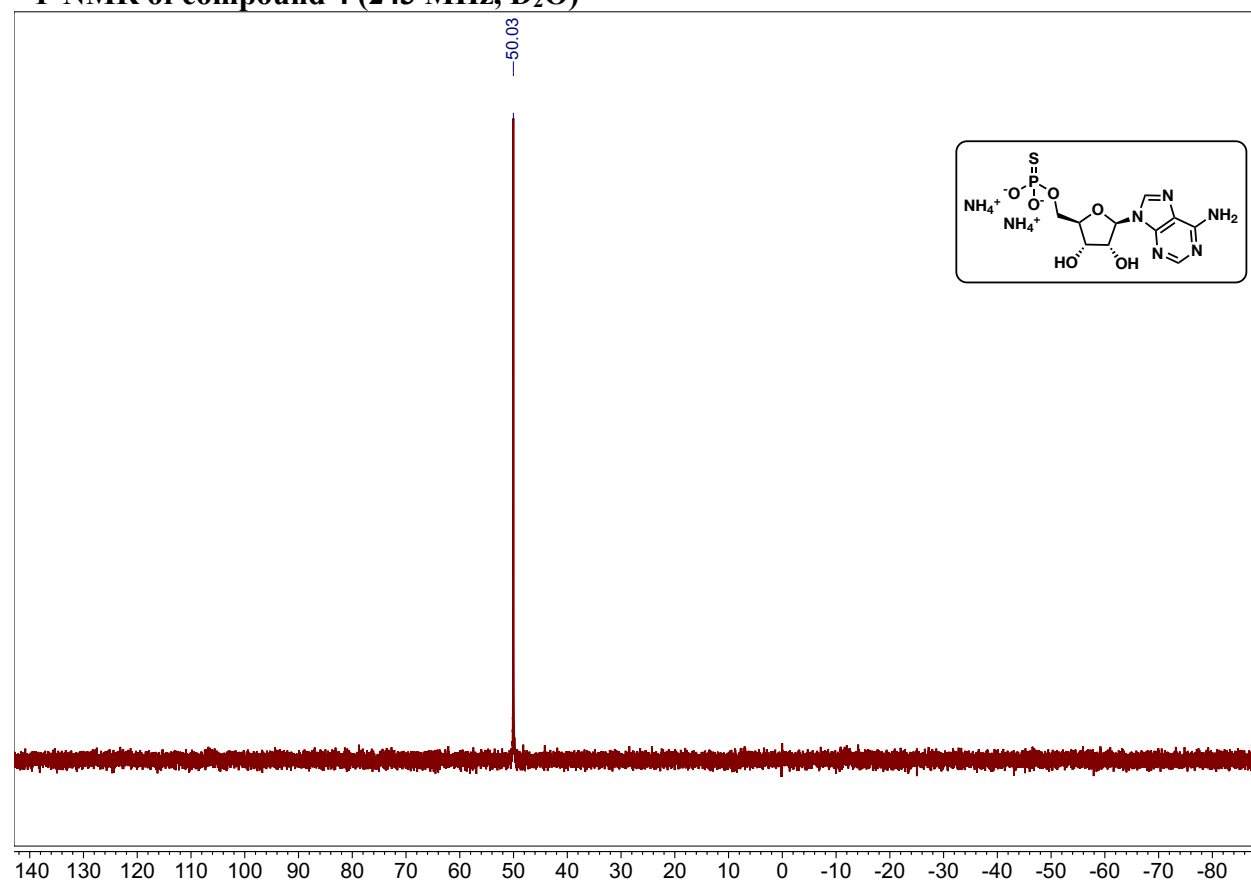
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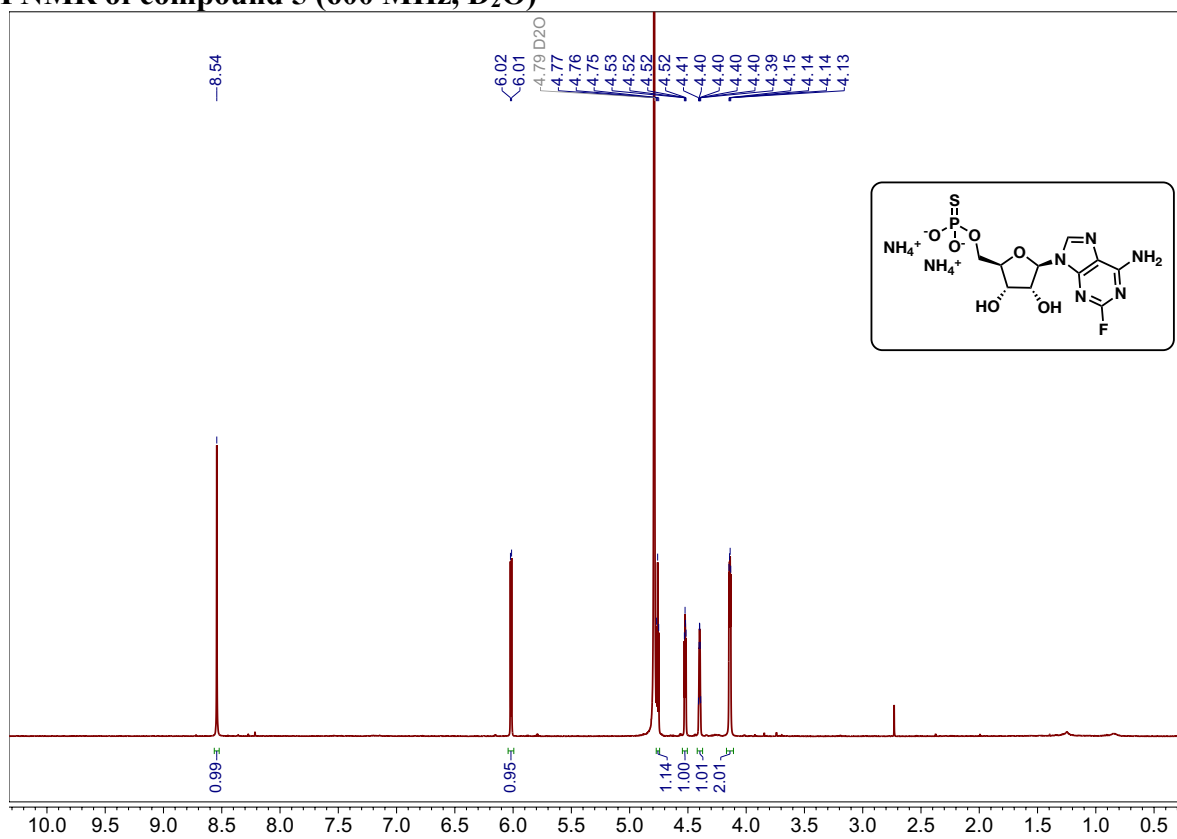
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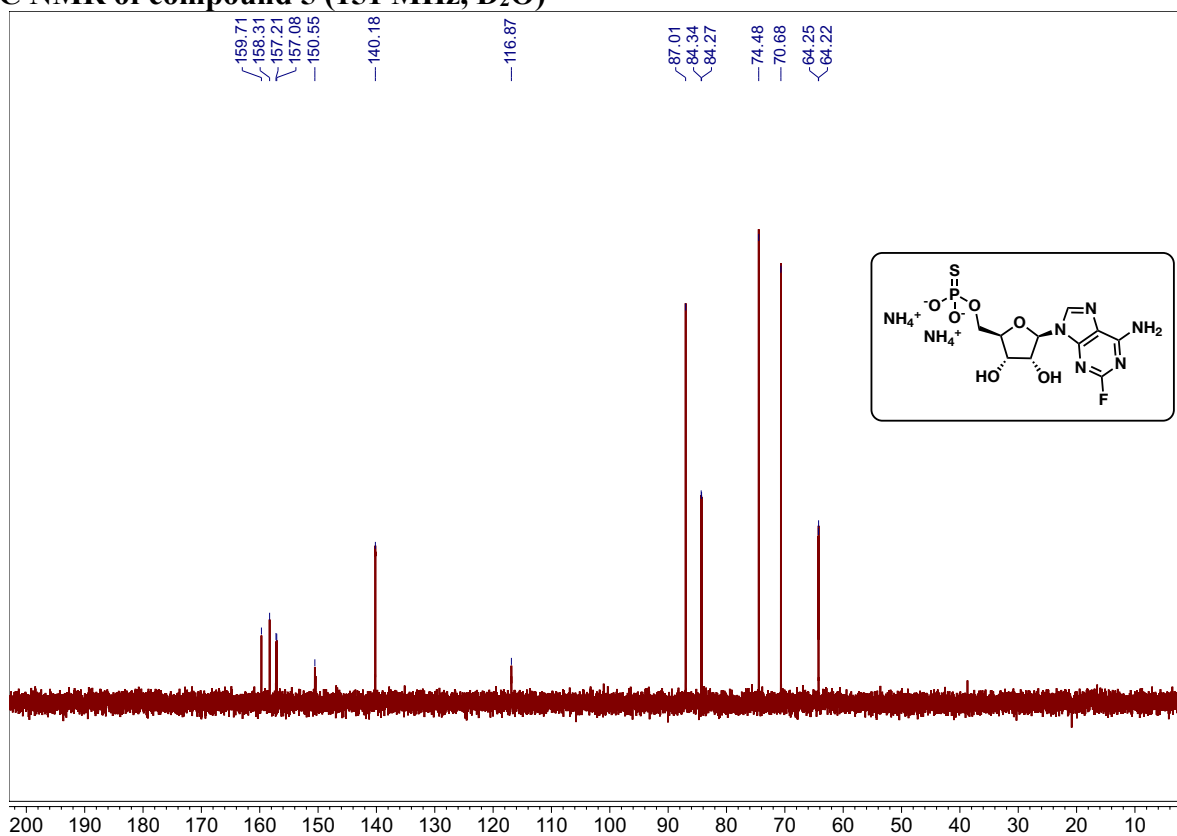
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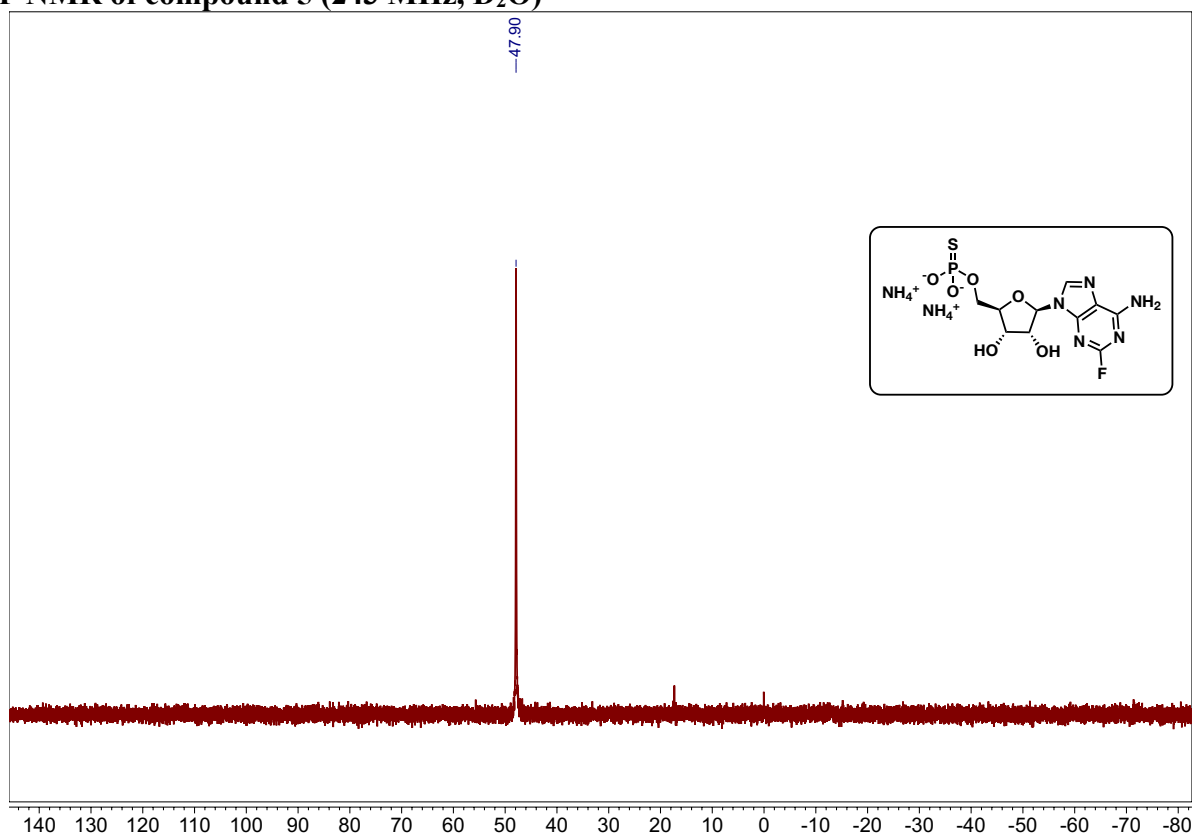
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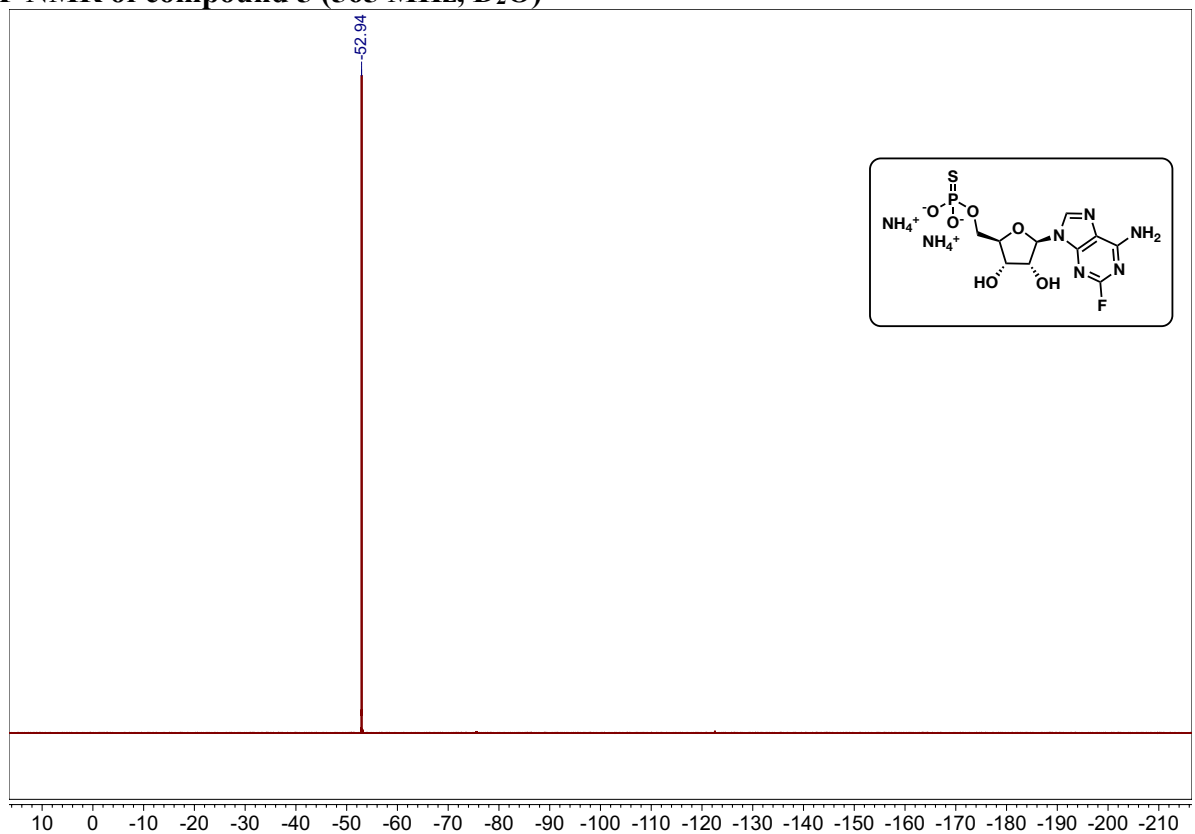
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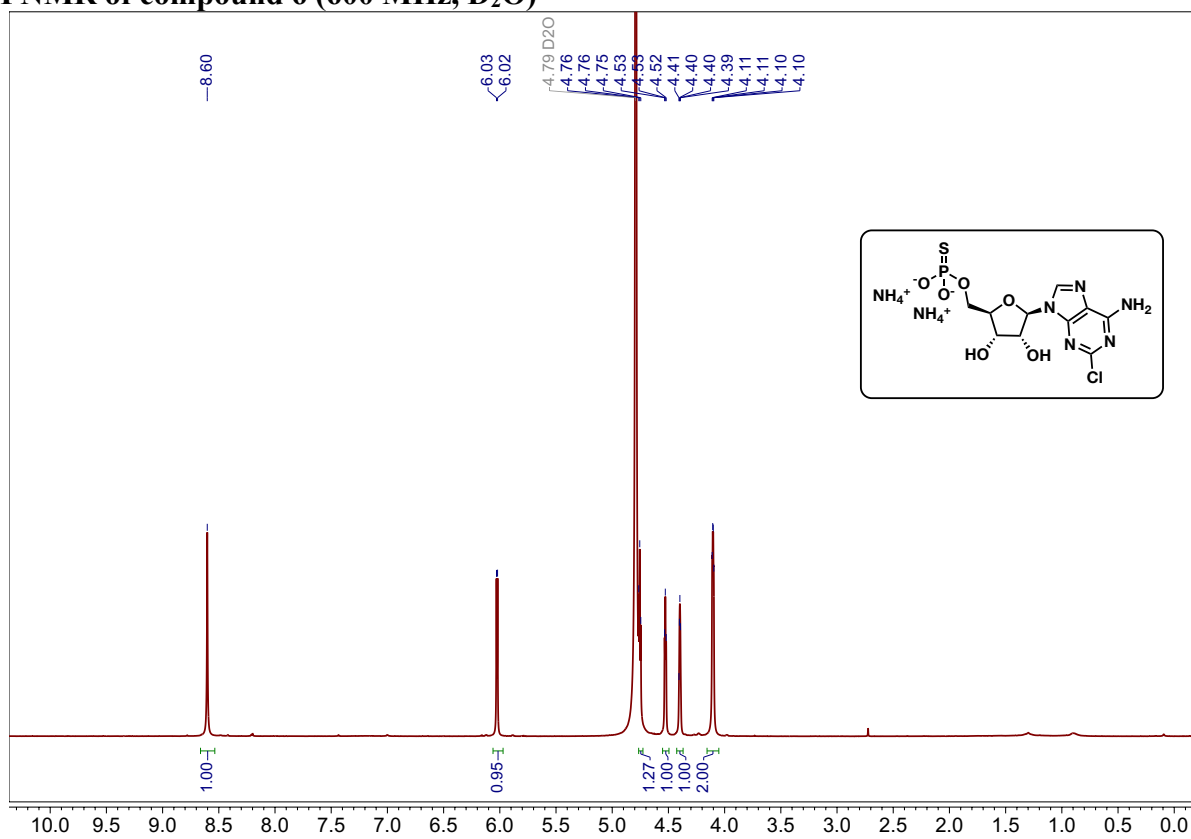
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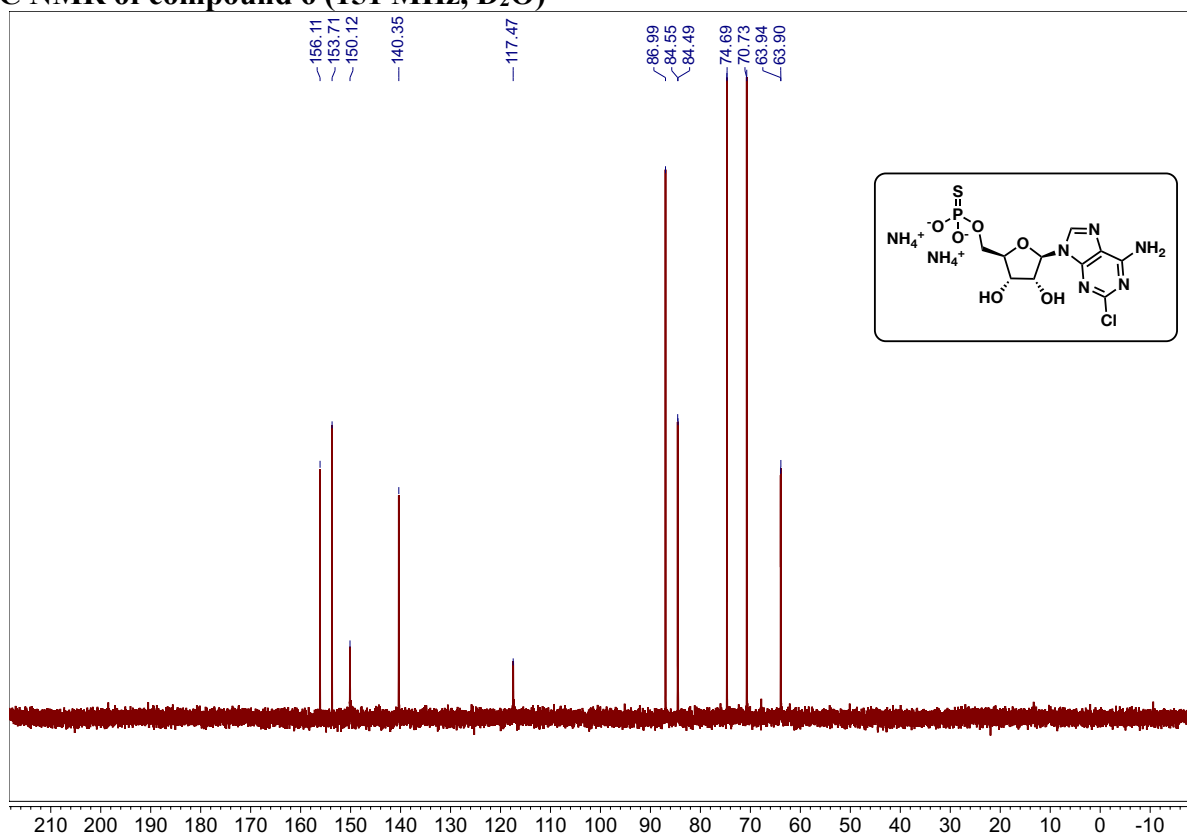
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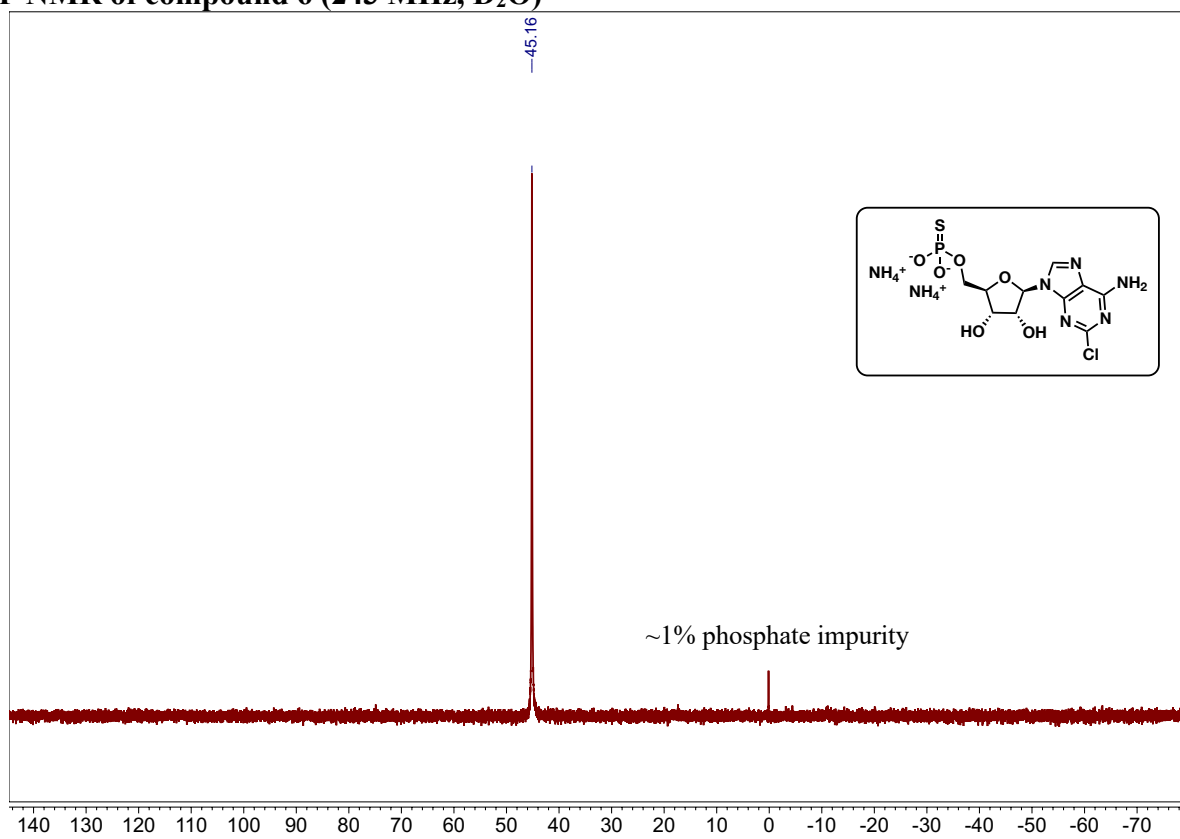
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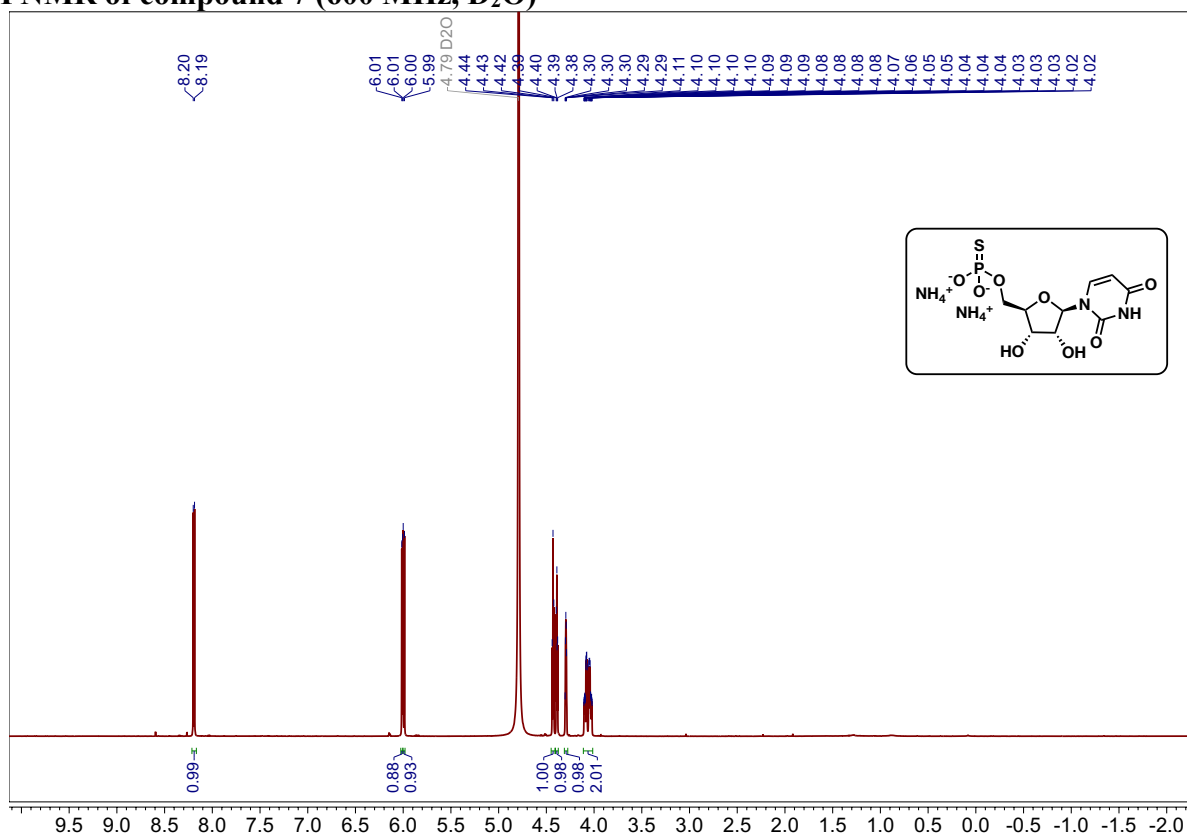
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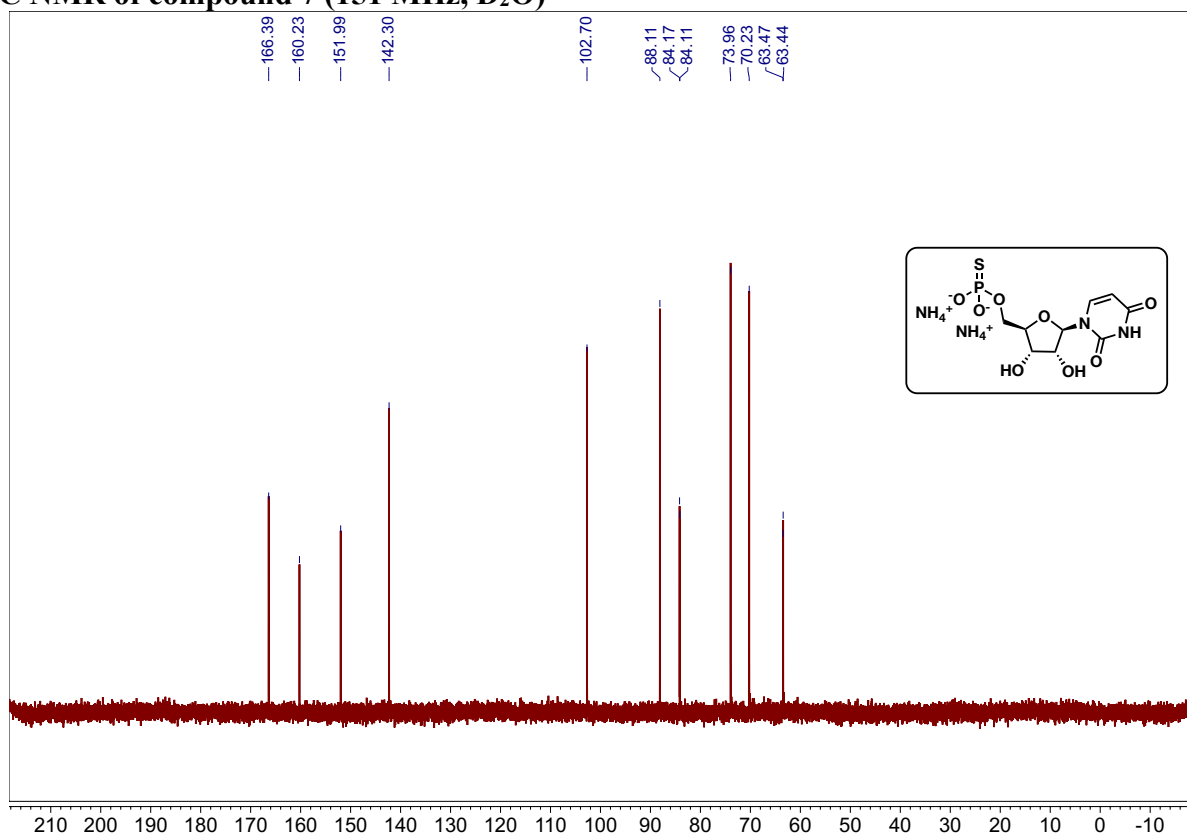
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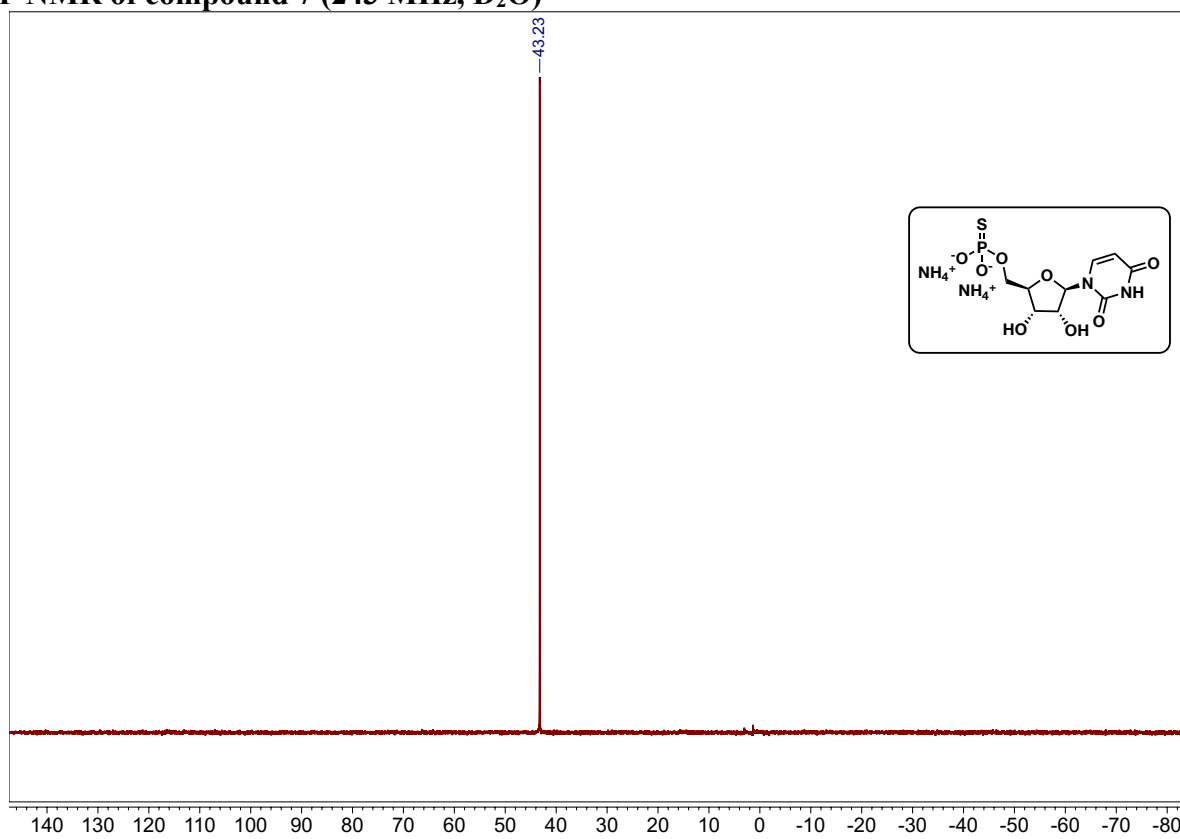
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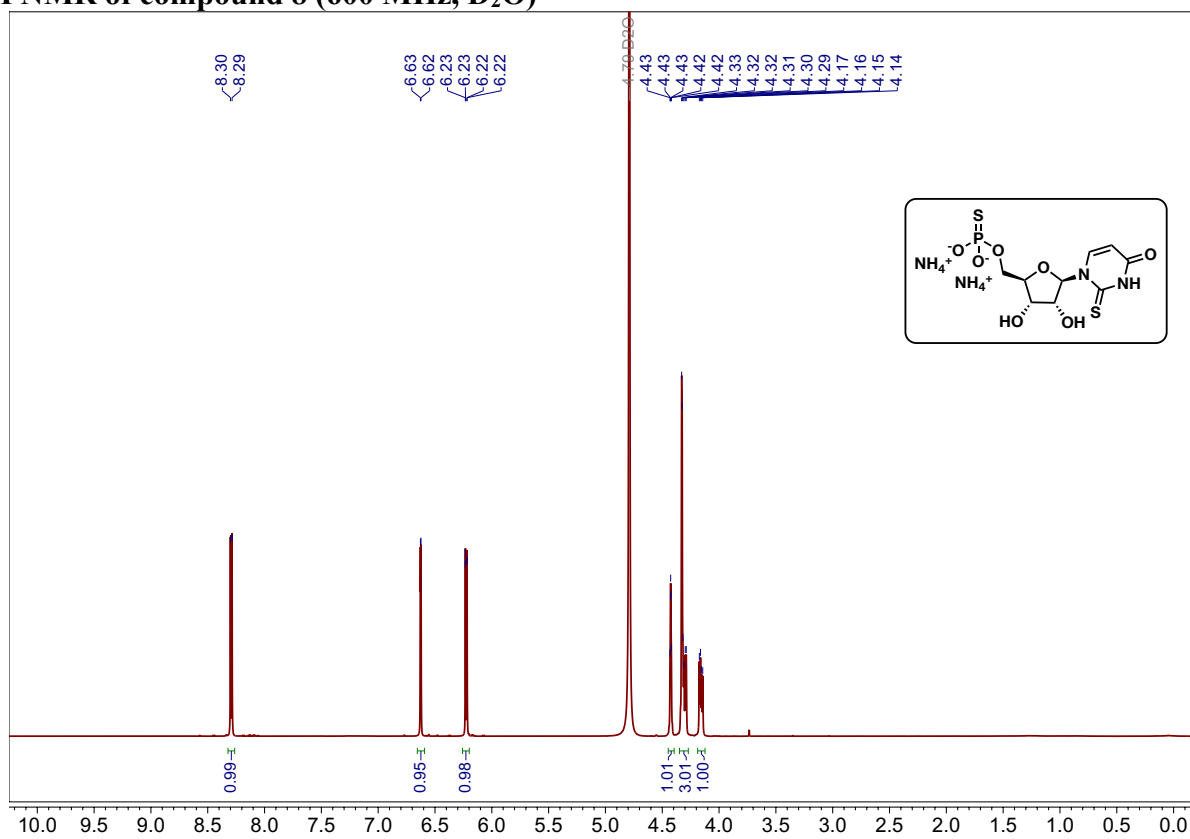
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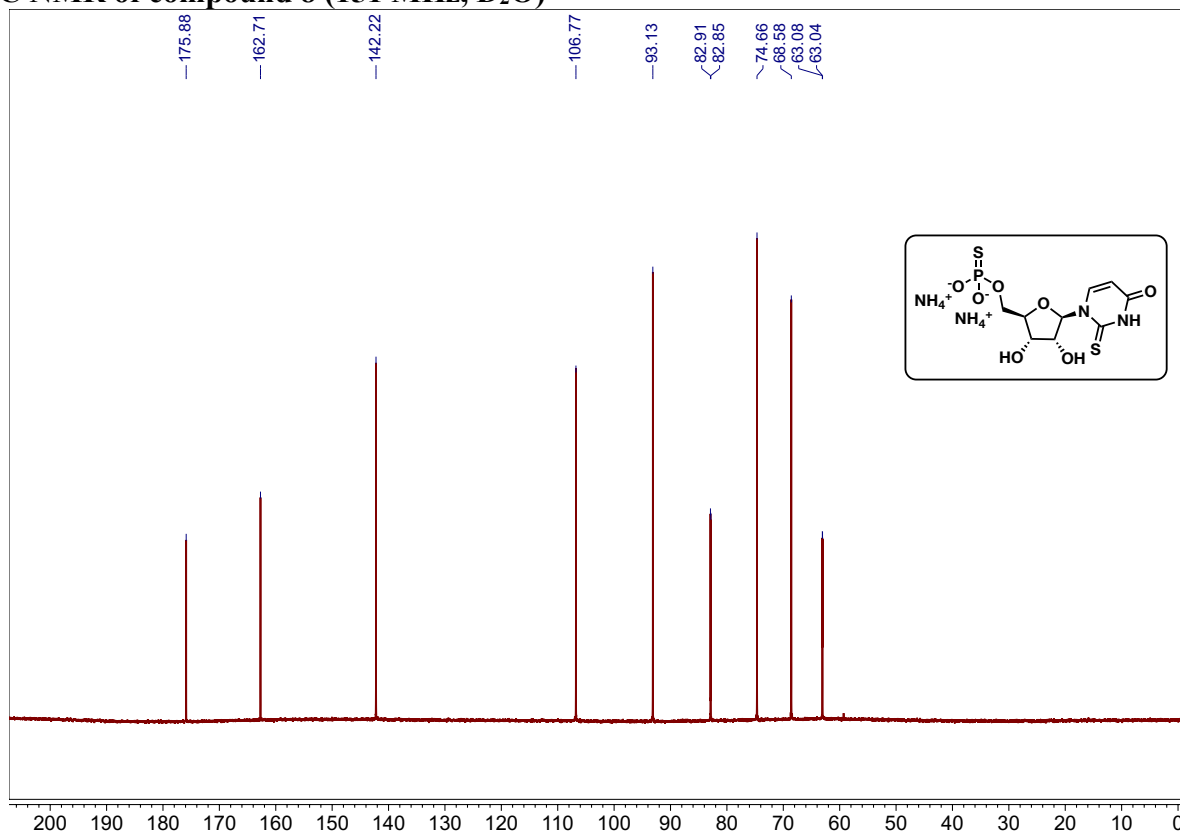
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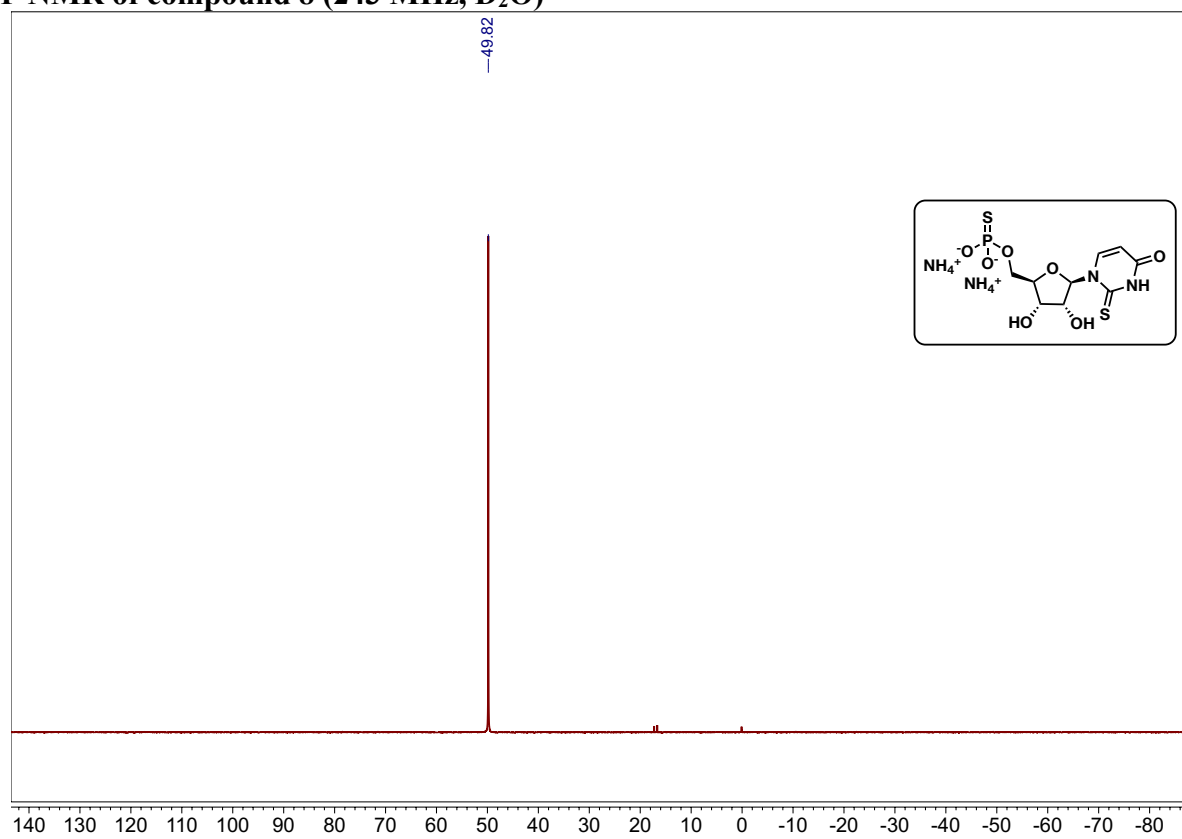
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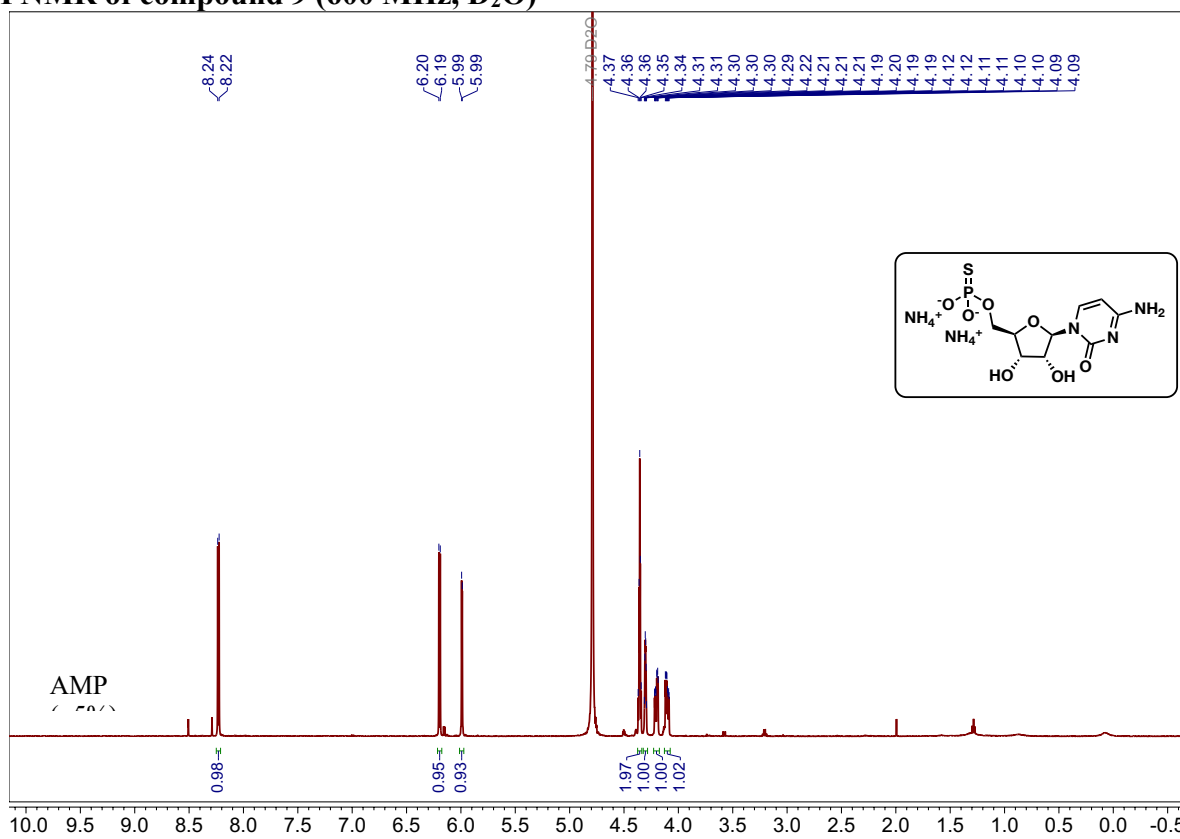
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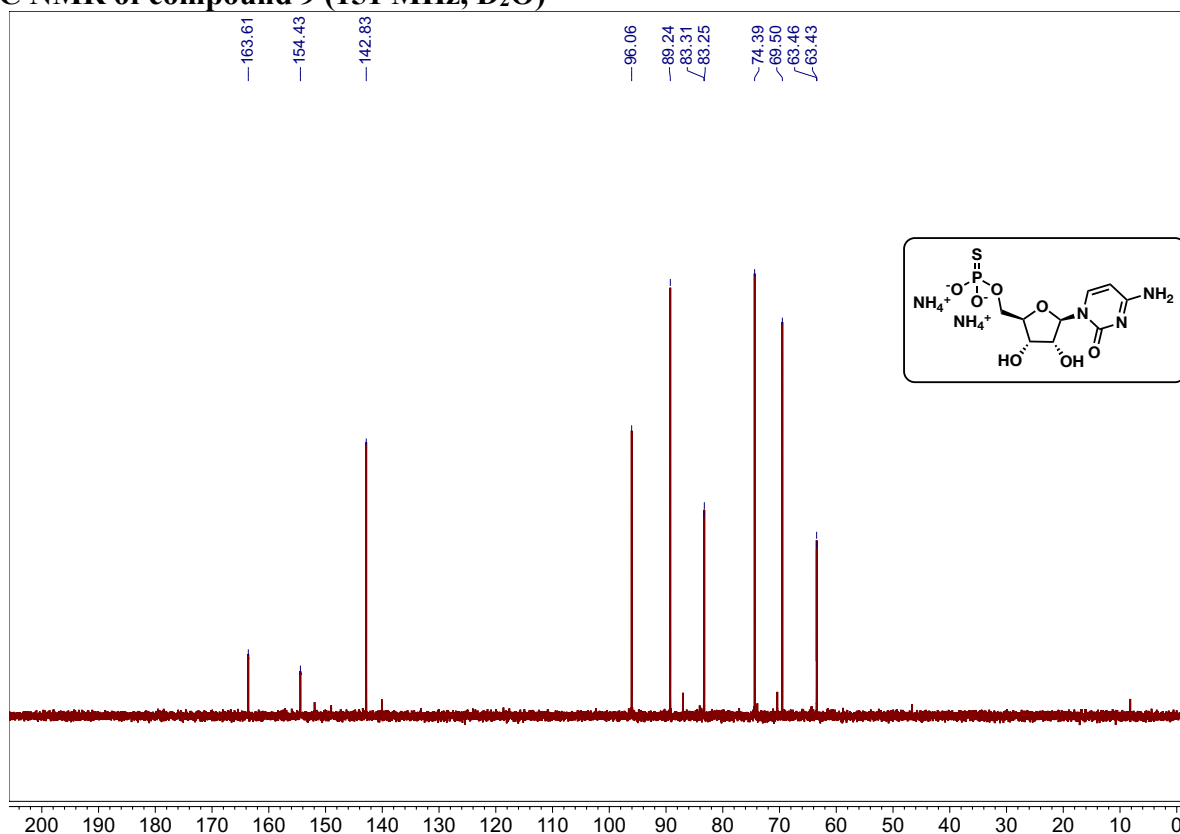
^{31}P NMR of compound 8 (243 MHz, D_2O)



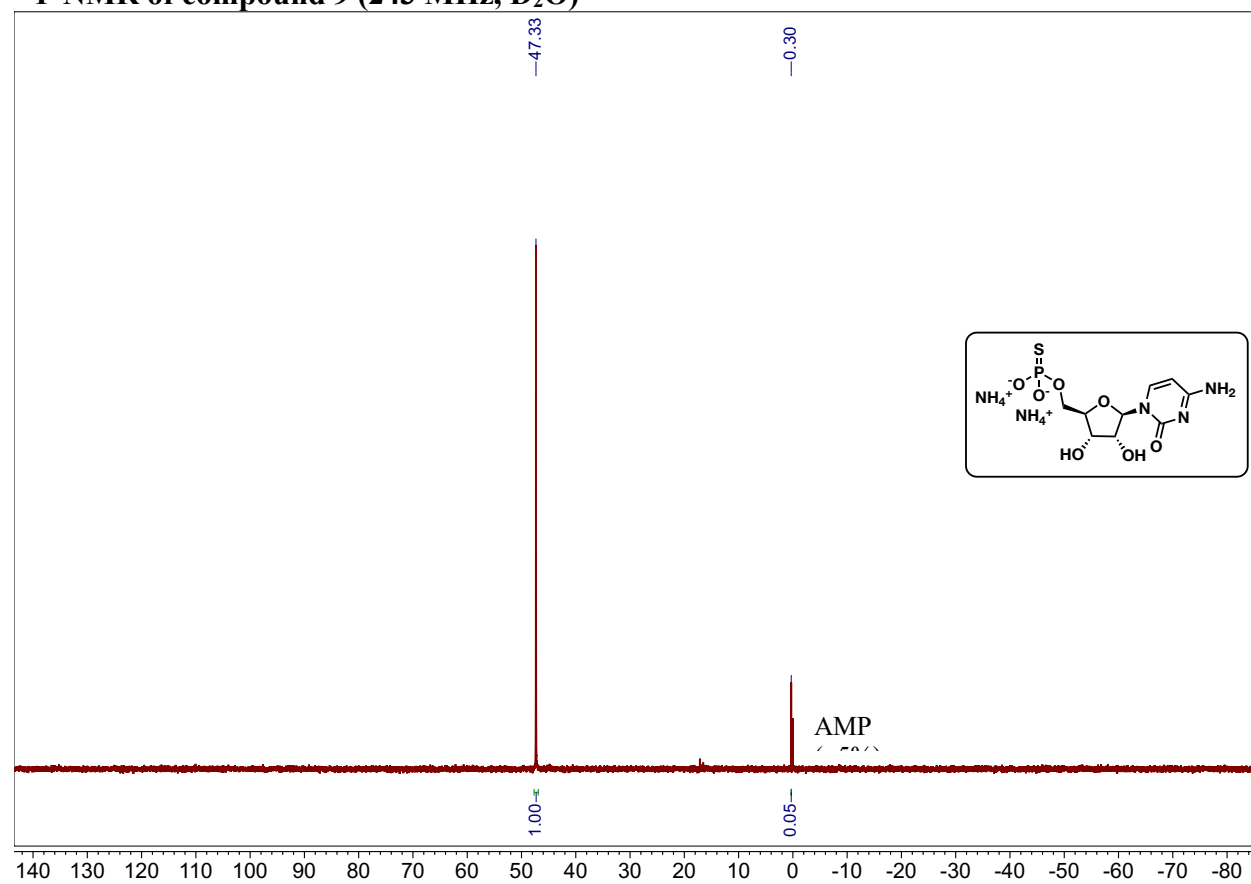
¹H NMR of compound 9 (600 MHz, D₂O)



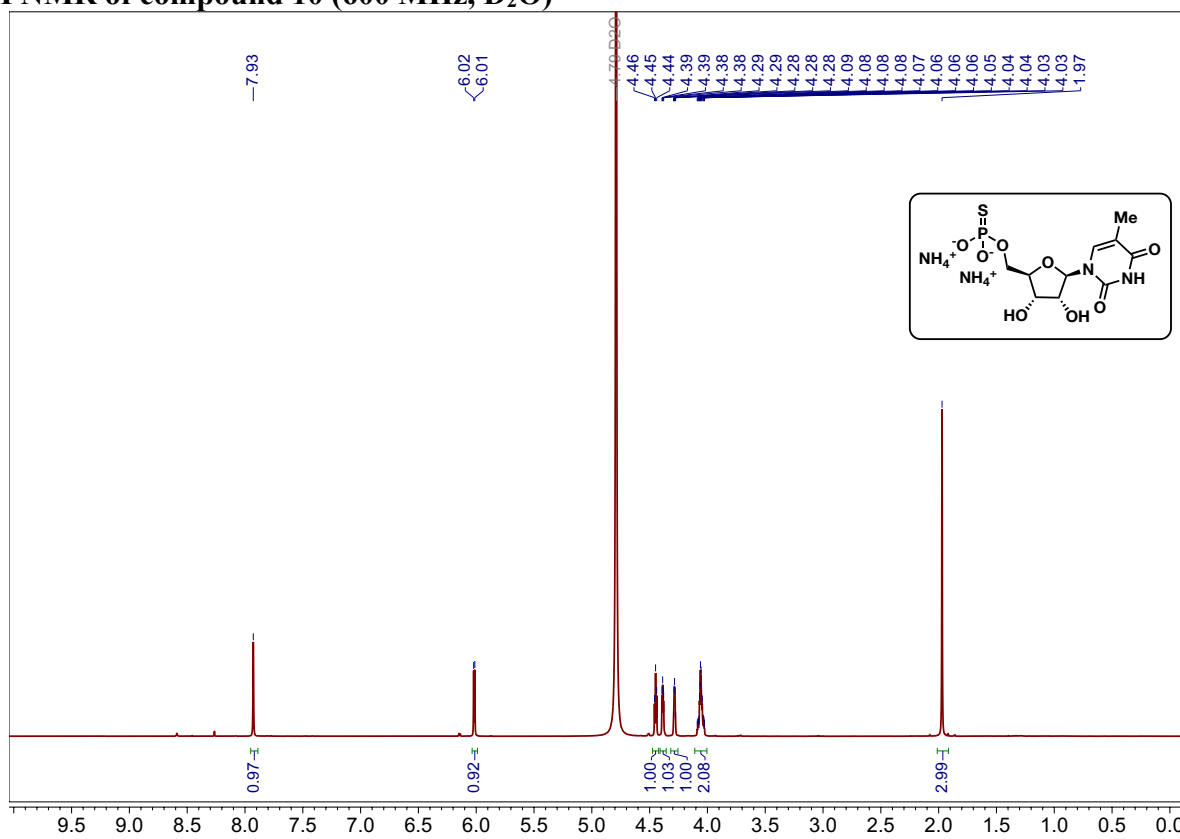
¹³C NMR of compound 9 (151 MHz, D₂O)



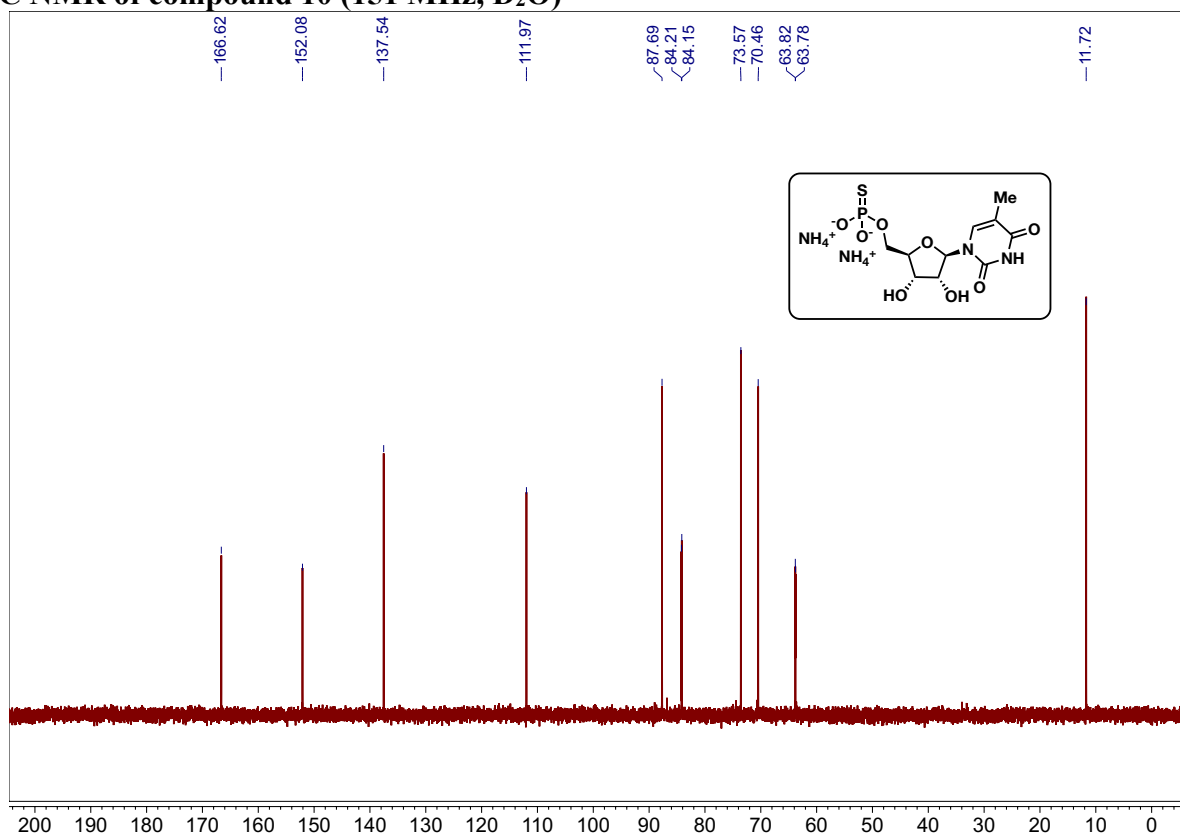
^{31}P NMR of compound 9 (243 MHz, D_2O)



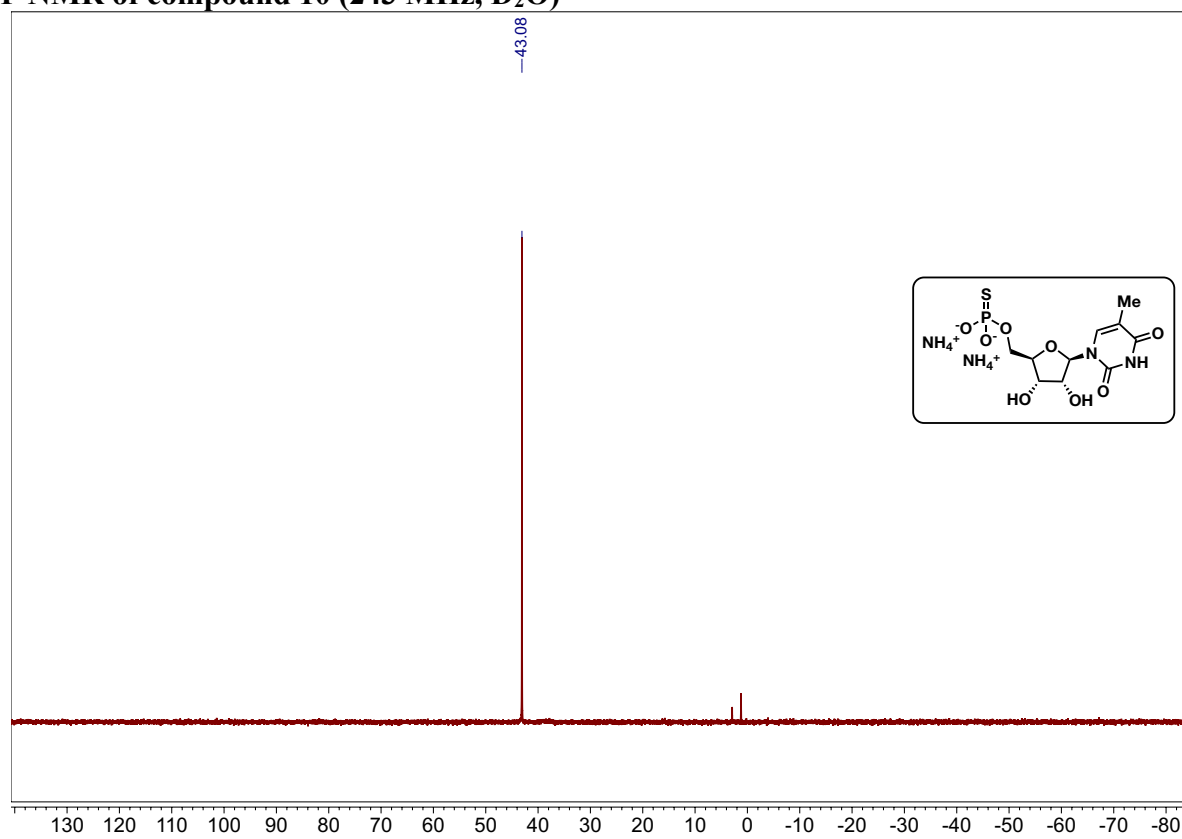
¹H NMR of compound 10 (600 MHz, D₂O)



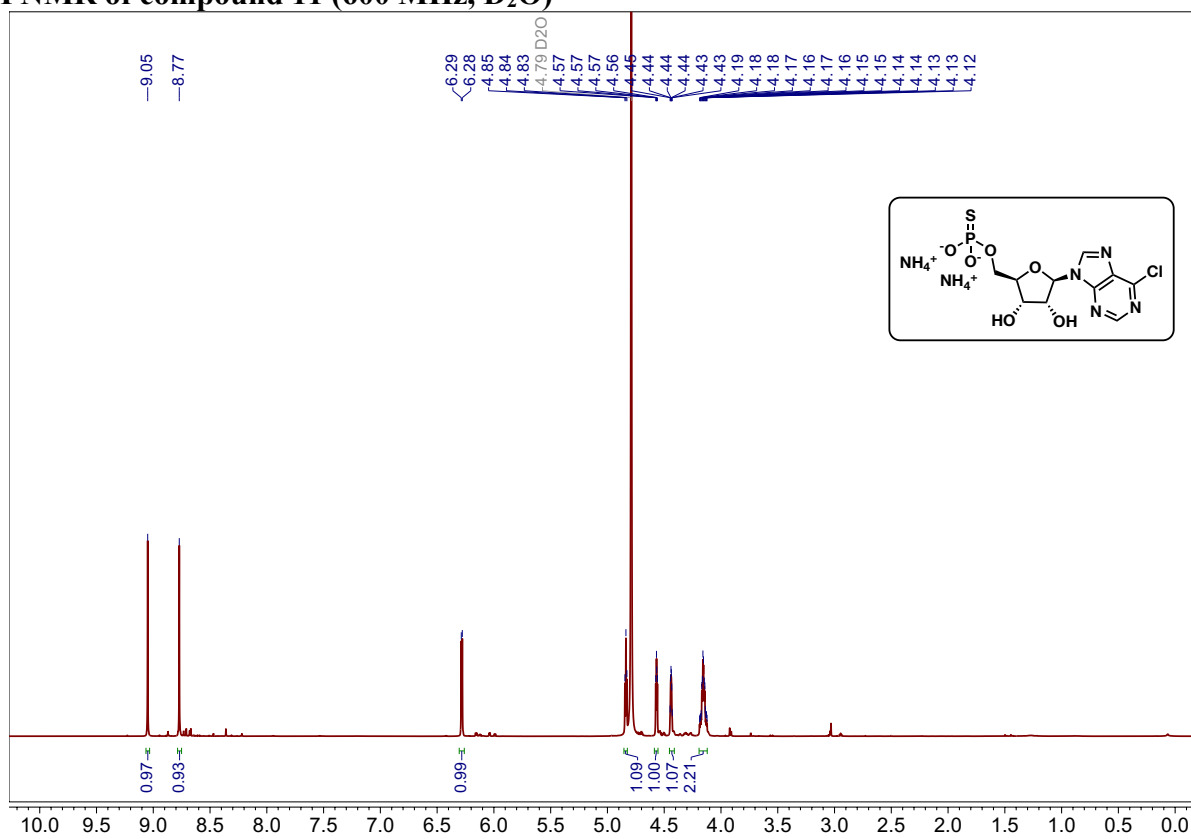
¹³C NMR of compound 10 (151 MHz, D₂O)



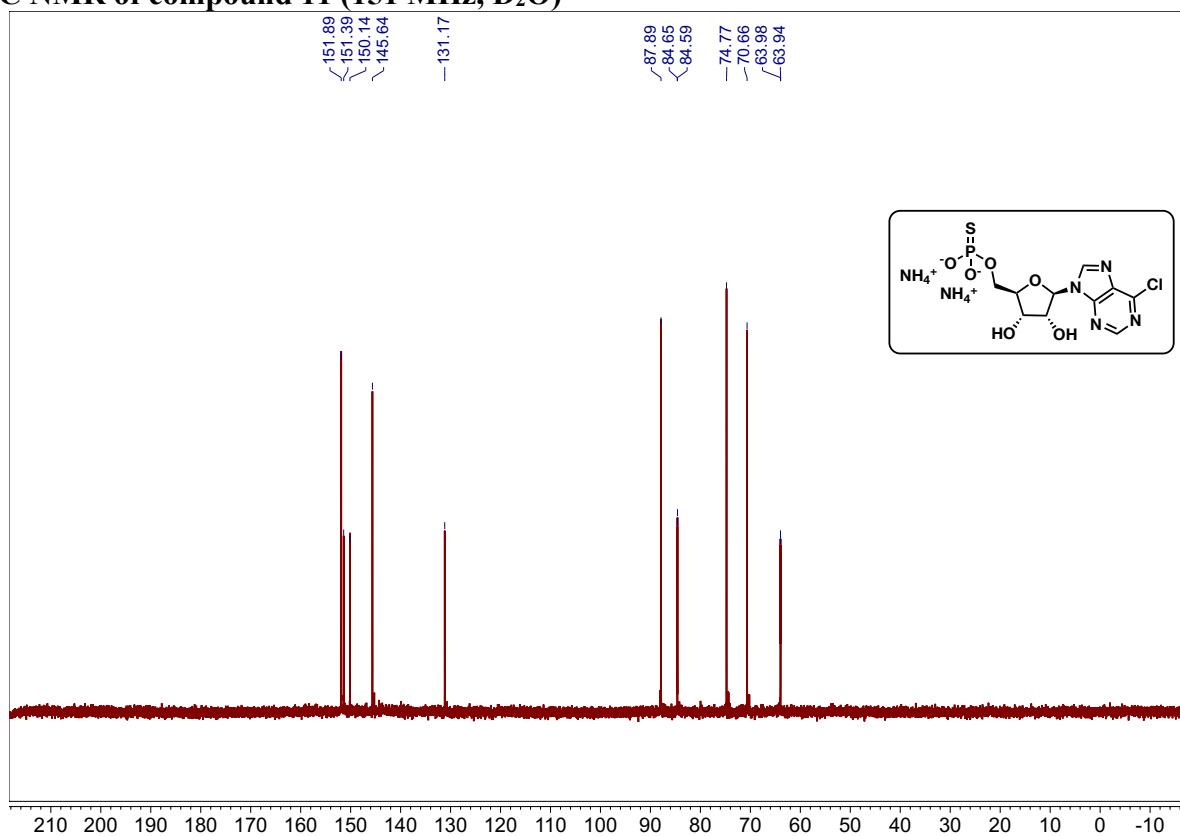
^{31}P NMR of compound 10 (243 MHz, D_2O)



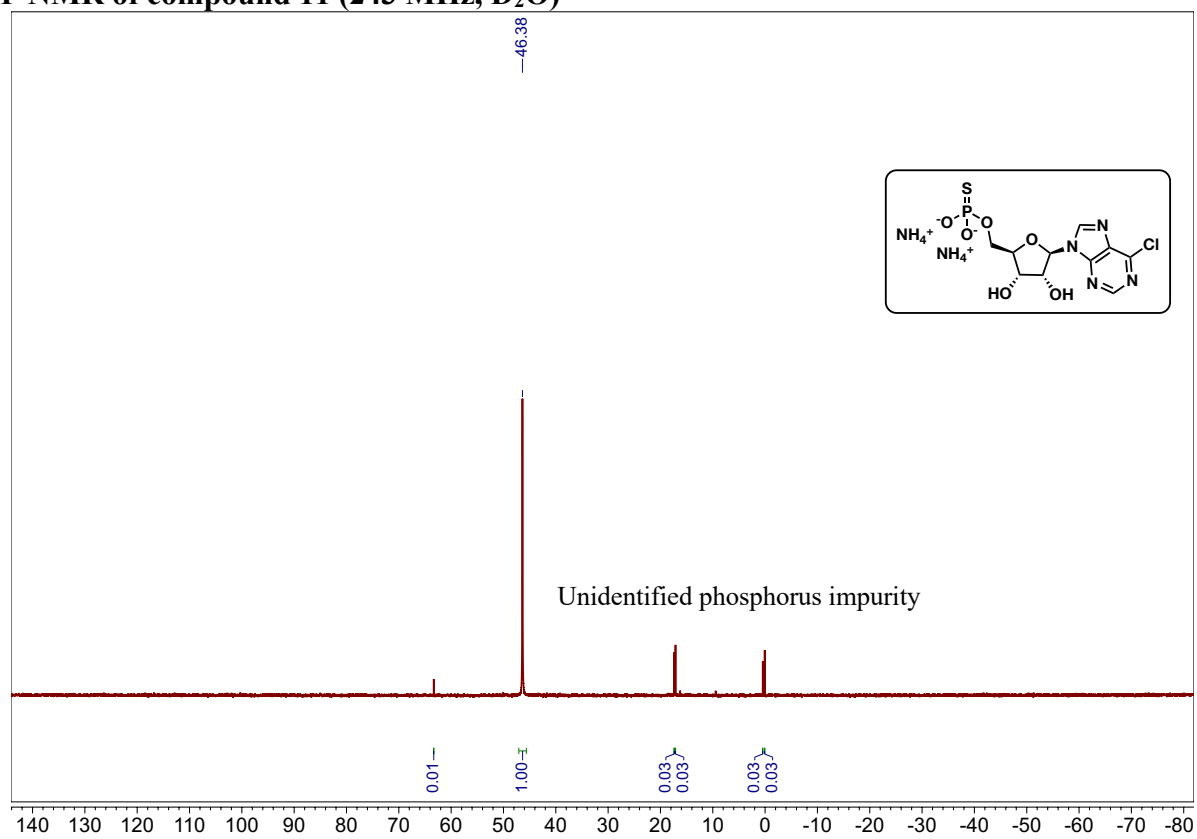
¹H NMR of compound 11 (600 MHz, D₂O)



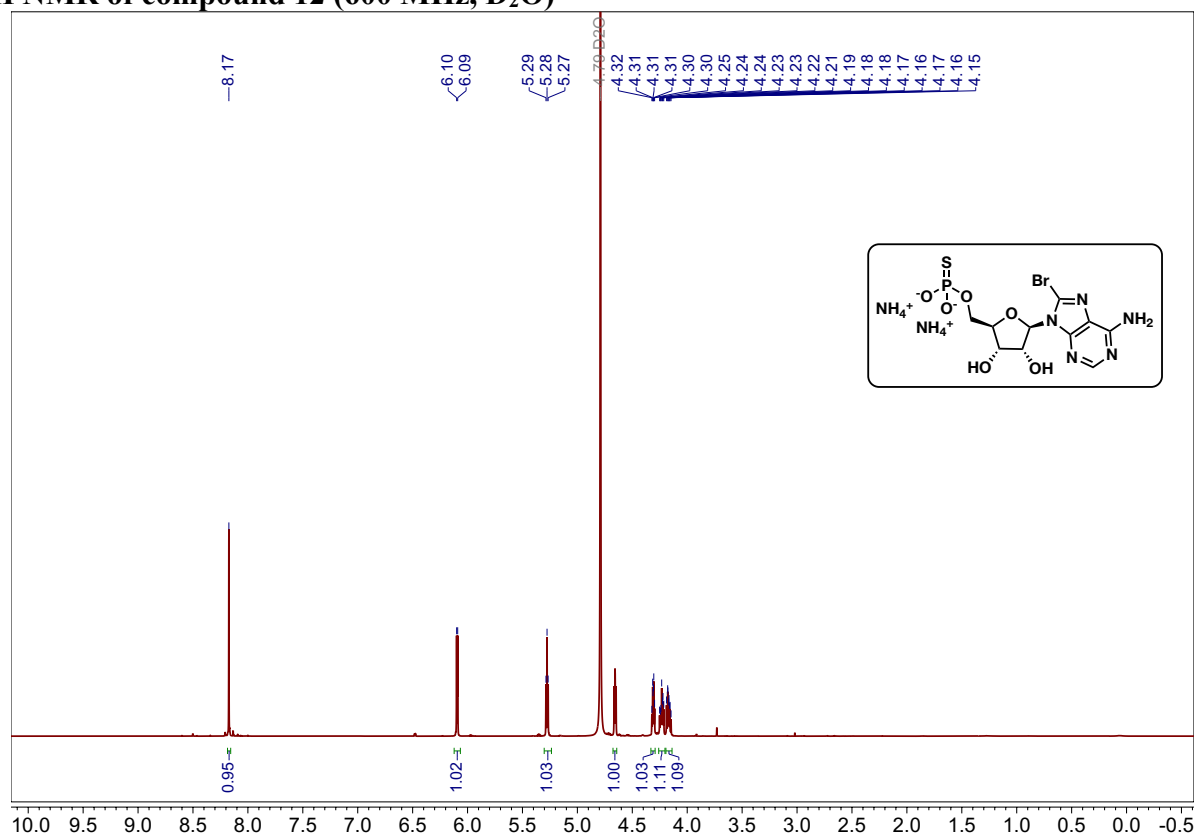
¹³C NMR of compound 11 (151 MHz, D₂O)



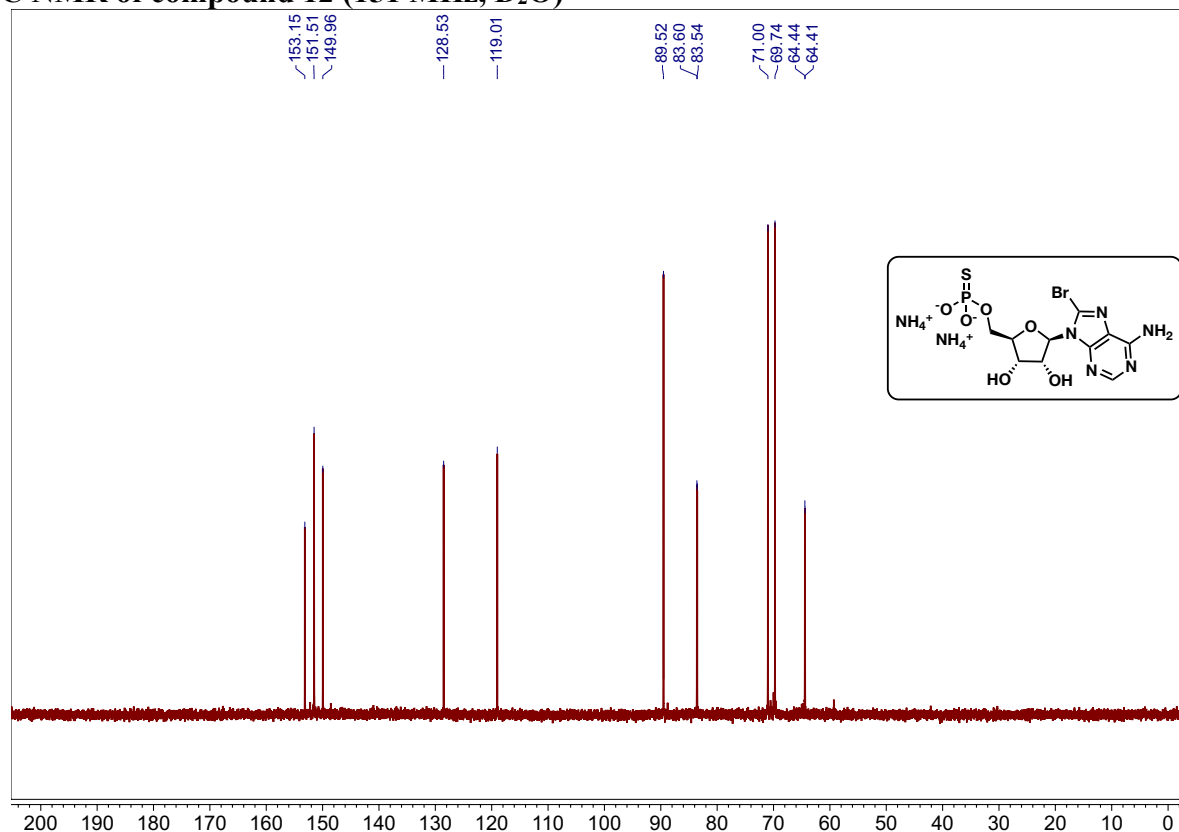
^{31}P NMR of compound 11 (243 MHz, D_2O)



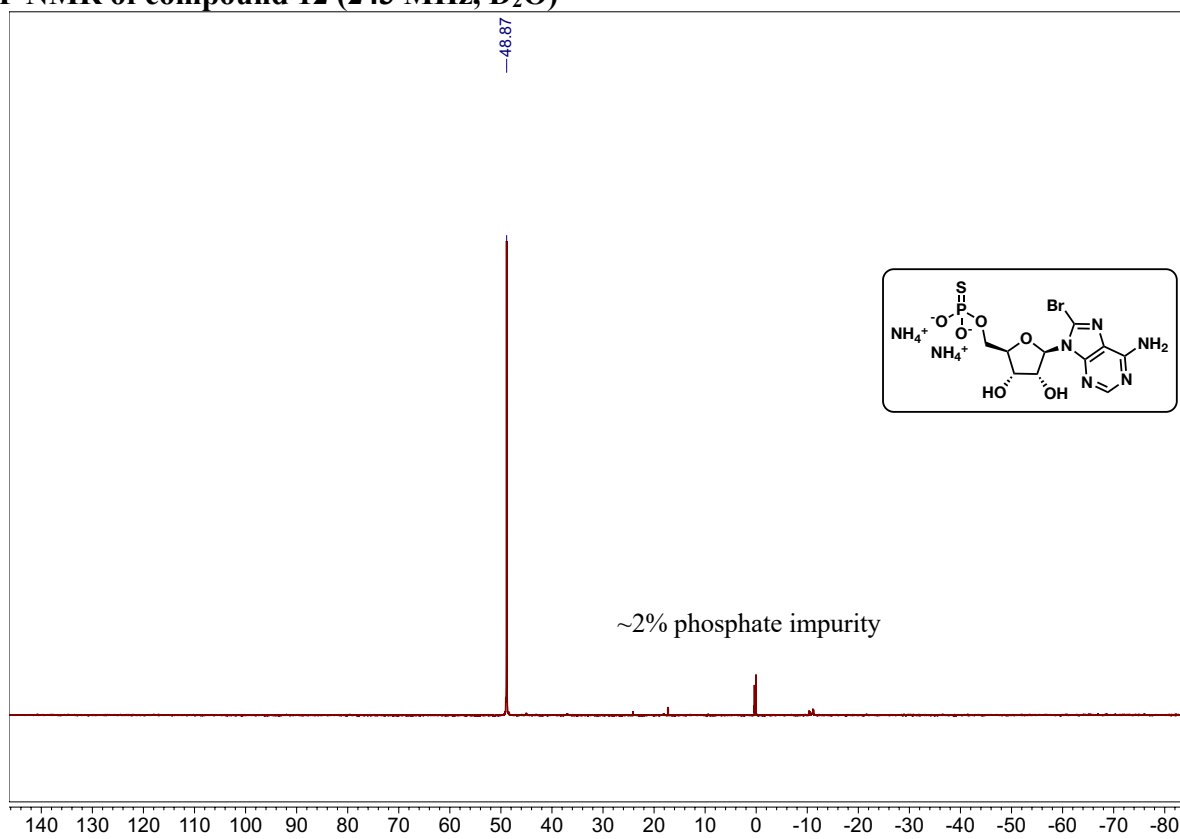
¹H NMR of compound 12 (600 MHz, D₂O)



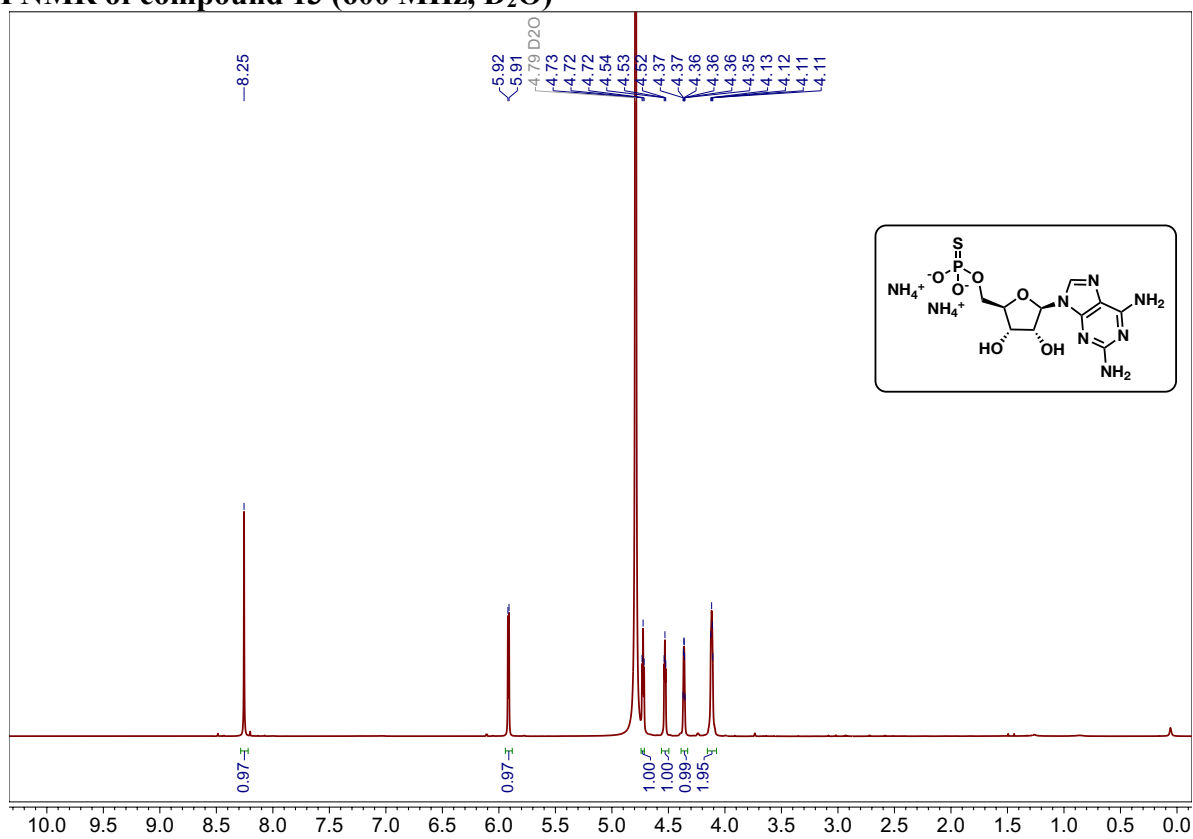
¹³C NMR of compound 12 (151 MHz, D₂O)



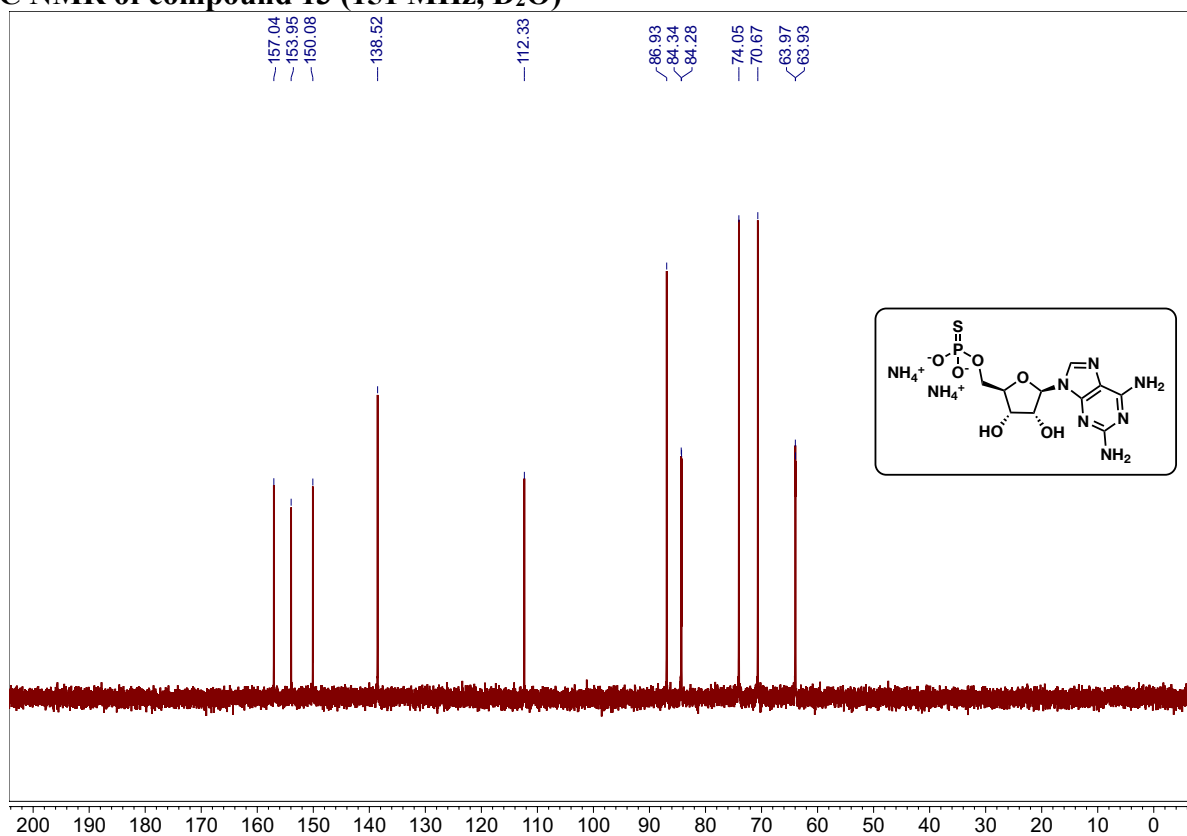
^{31}P NMR of compound 12 (243 MHz, D_2O)



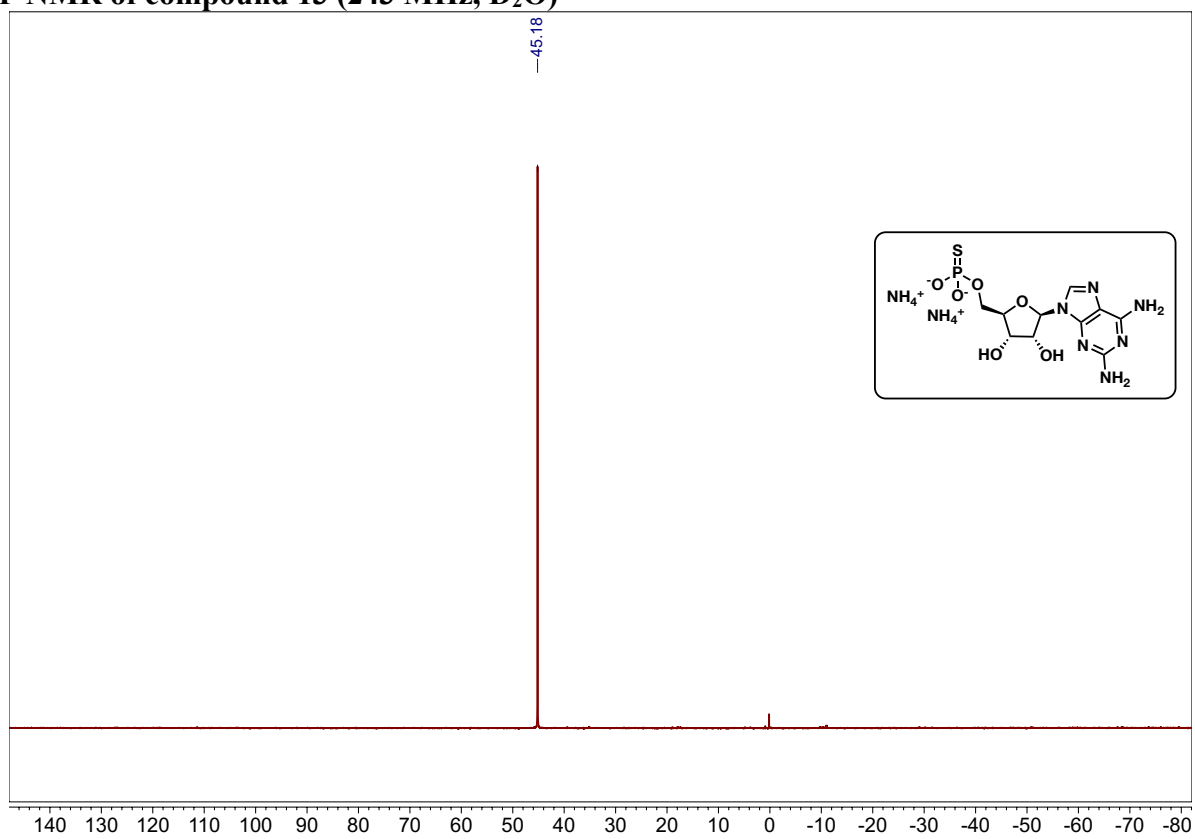
^1H NMR of compound 13 (600 MHz, D_2O)



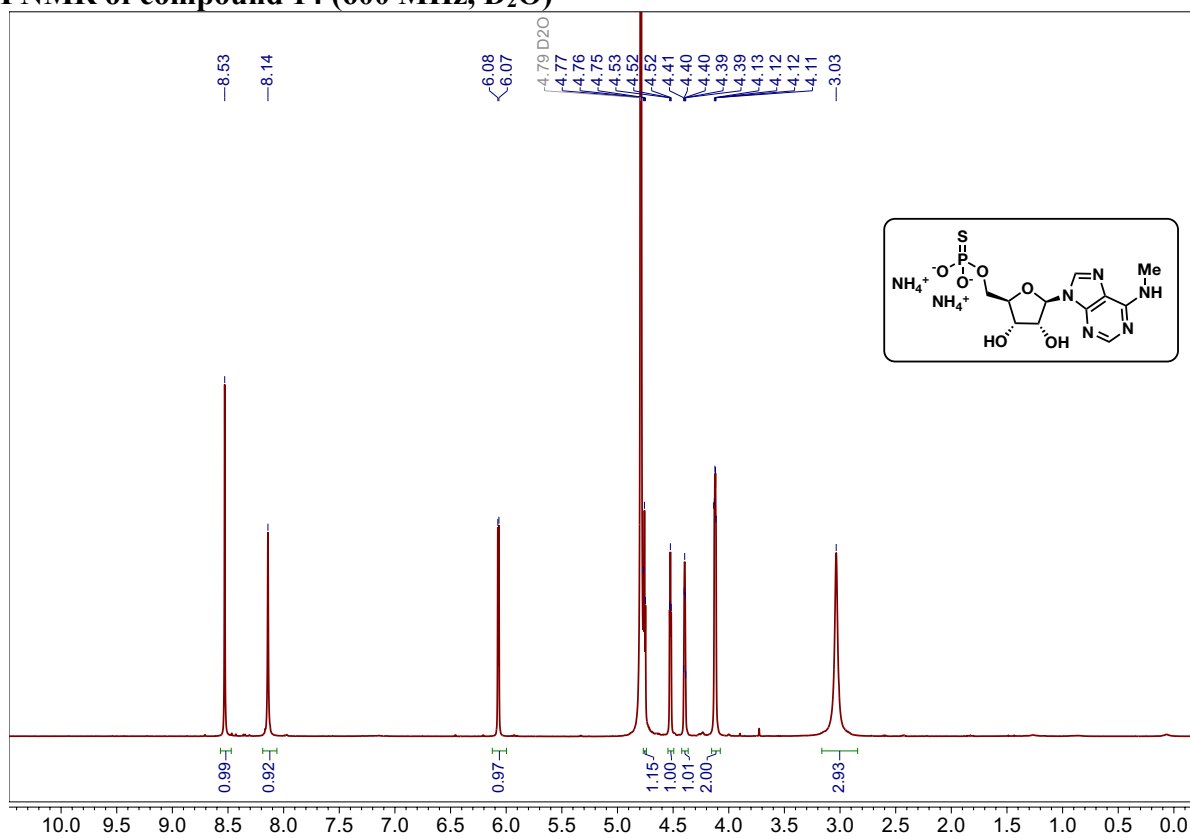
^{13}C NMR of compound 13 (151 MHz, D_2O)



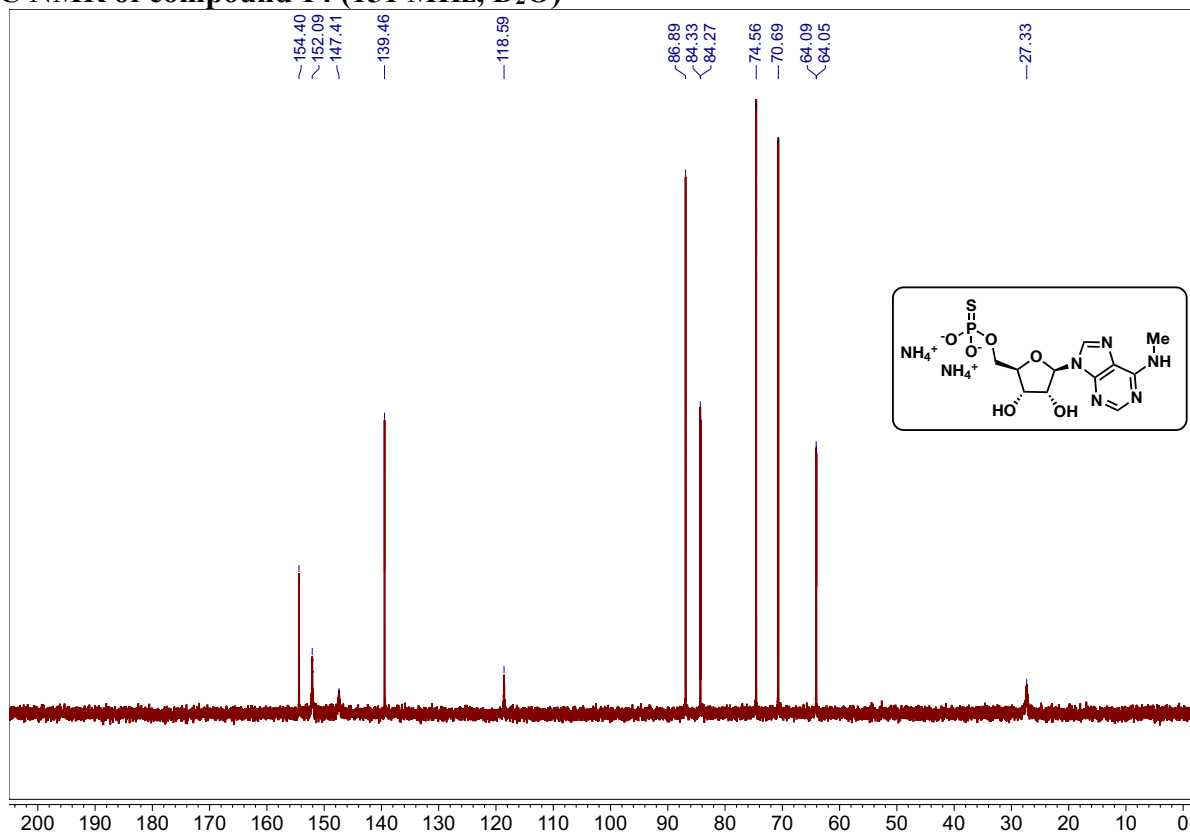
^{31}P NMR of compound 13 (243 MHz, D_2O)



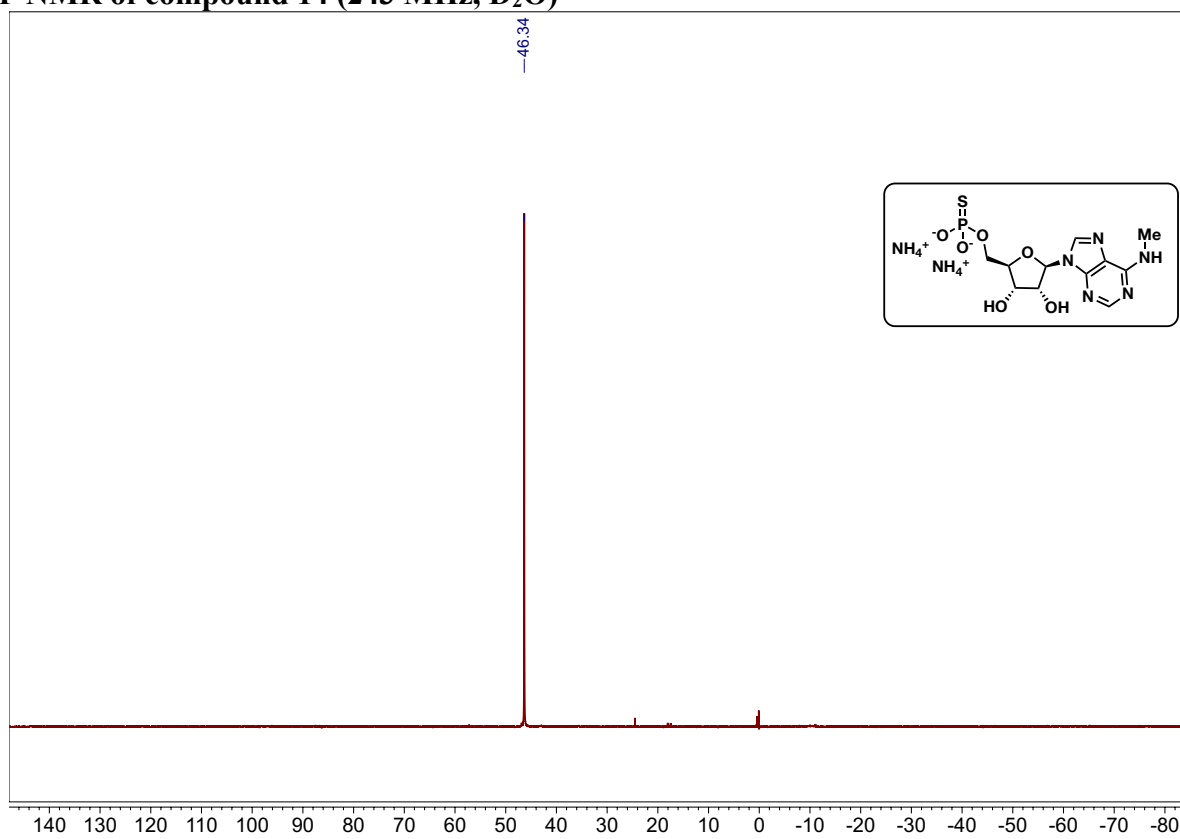
¹H NMR of compound 14 (600 MHz, D₂O)



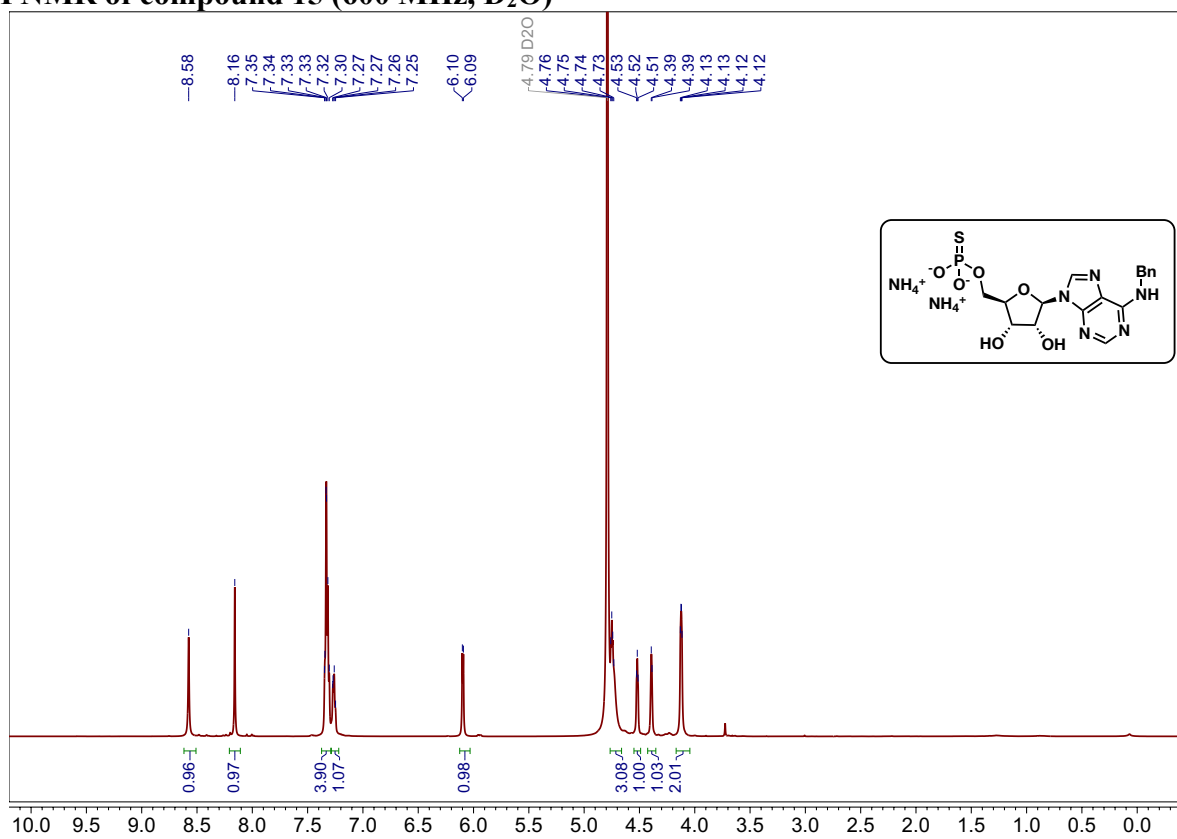
¹³C NMR of compound 14 (151 MHz, D₂O)



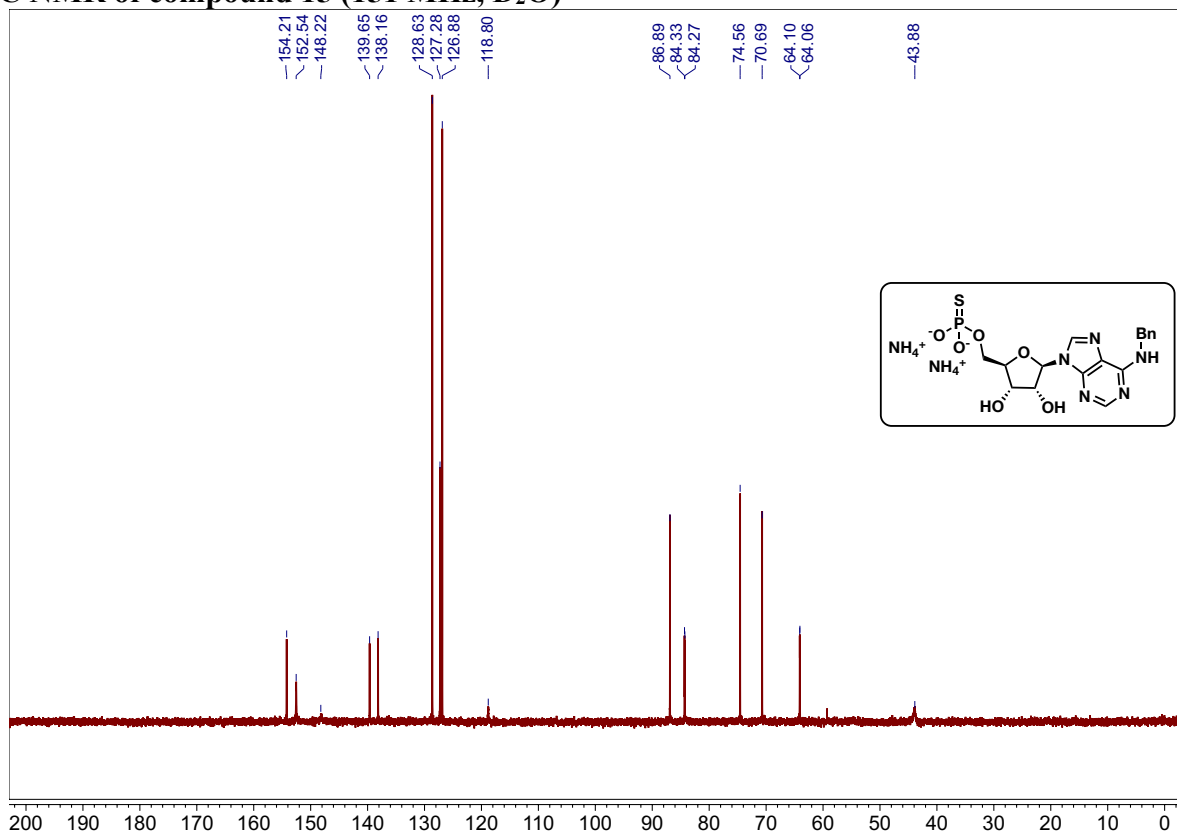
^{31}P NMR of compound 14 (243 MHz, D_2O)



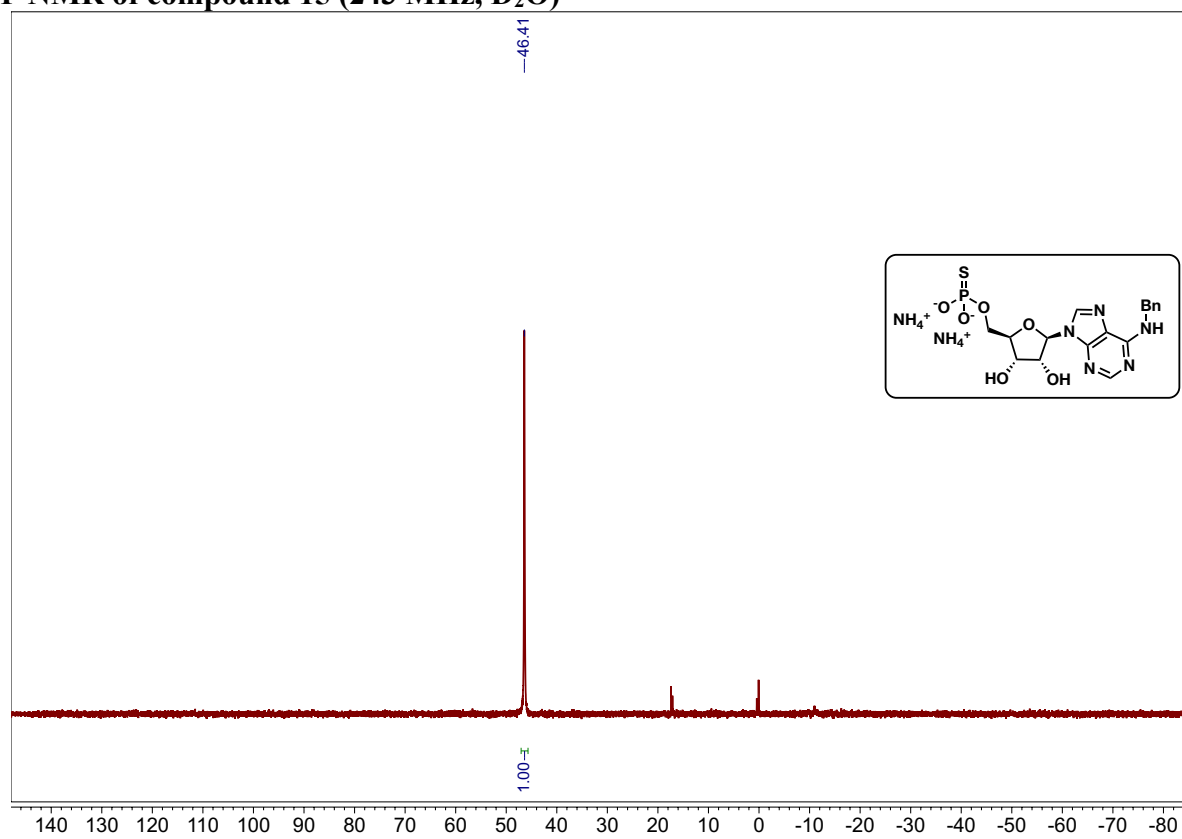
¹H NMR of compound 15 (600 MHz, D₂O)



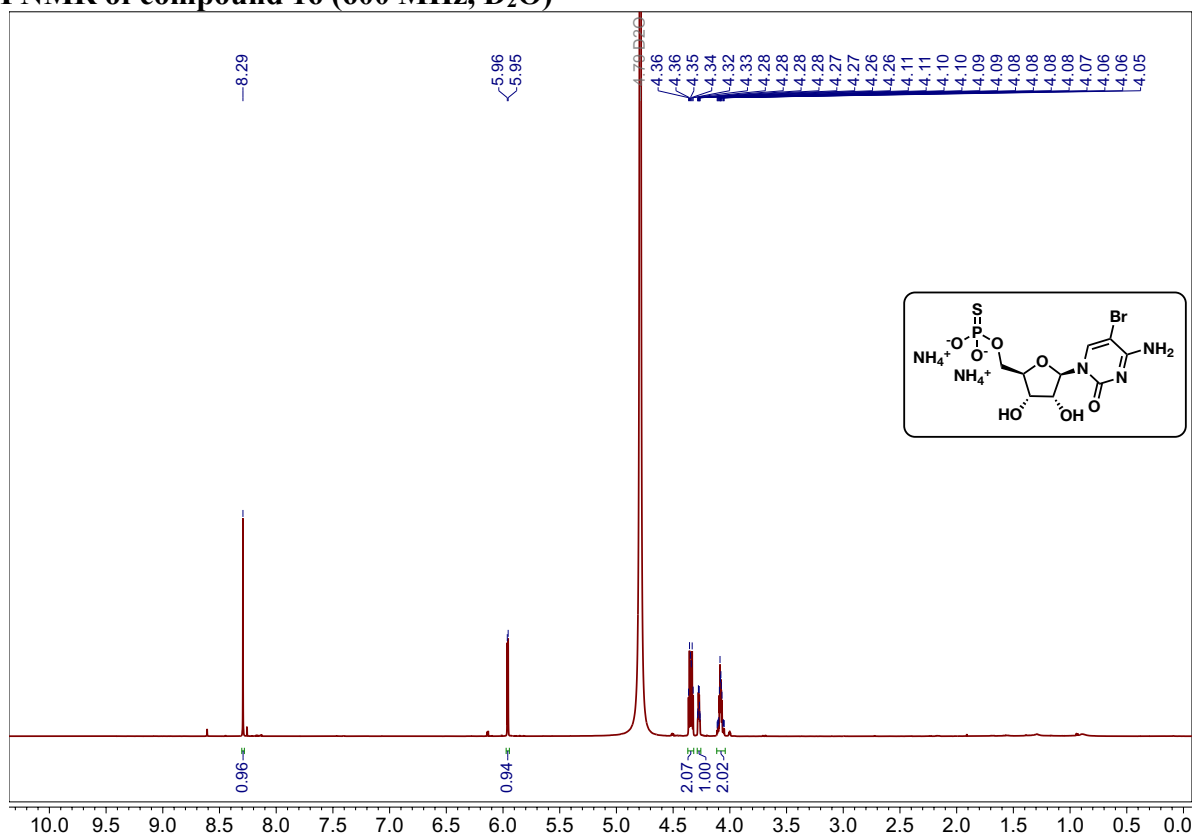
¹³C NMR of compound 15 (151 MHz, D₂O)



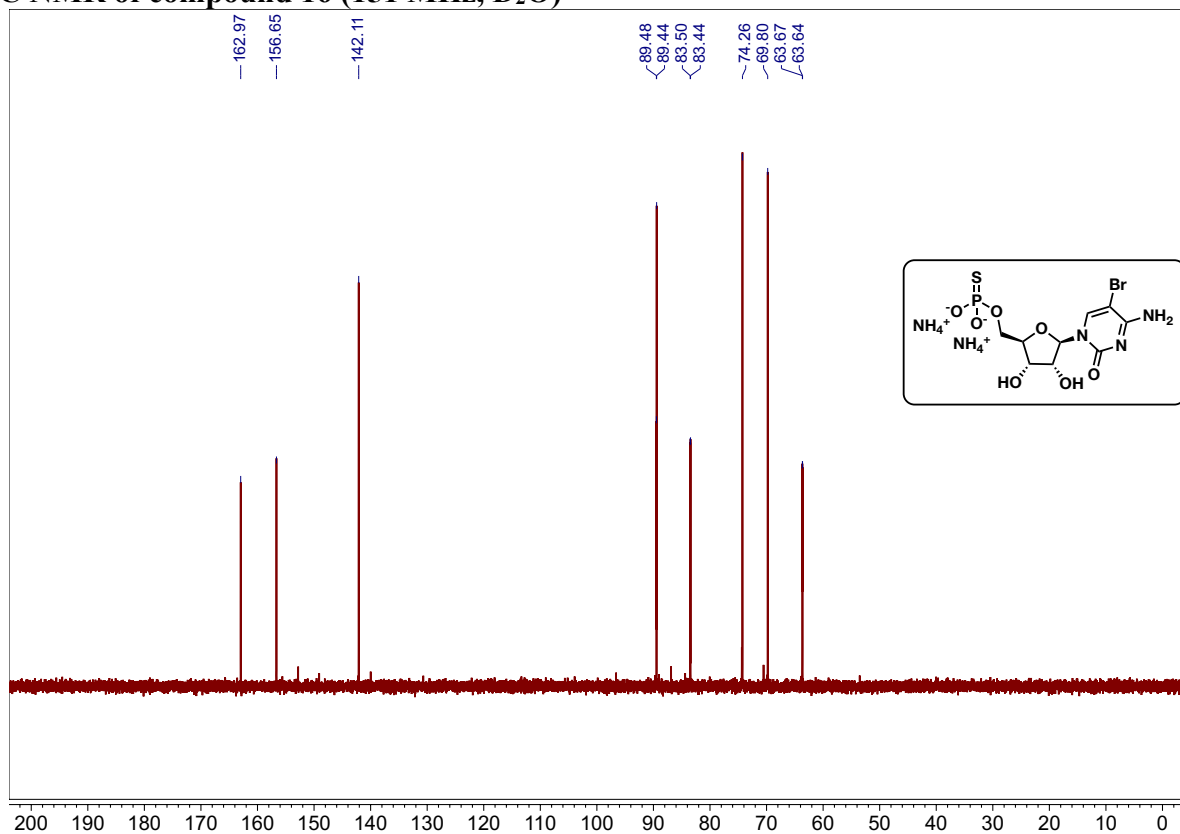
^{31}P NMR of compound 15 (243 MHz, D_2O)



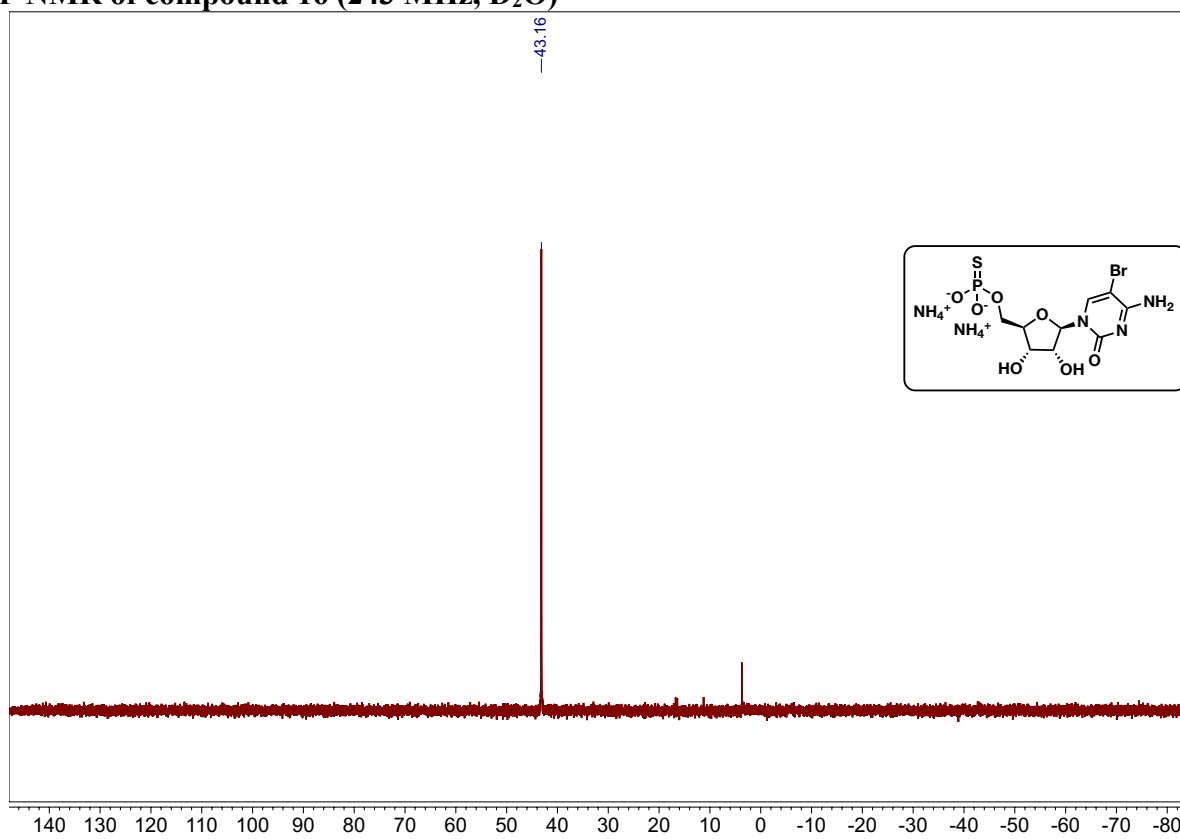
¹H NMR of compound 16 (600 MHz, D₂O)



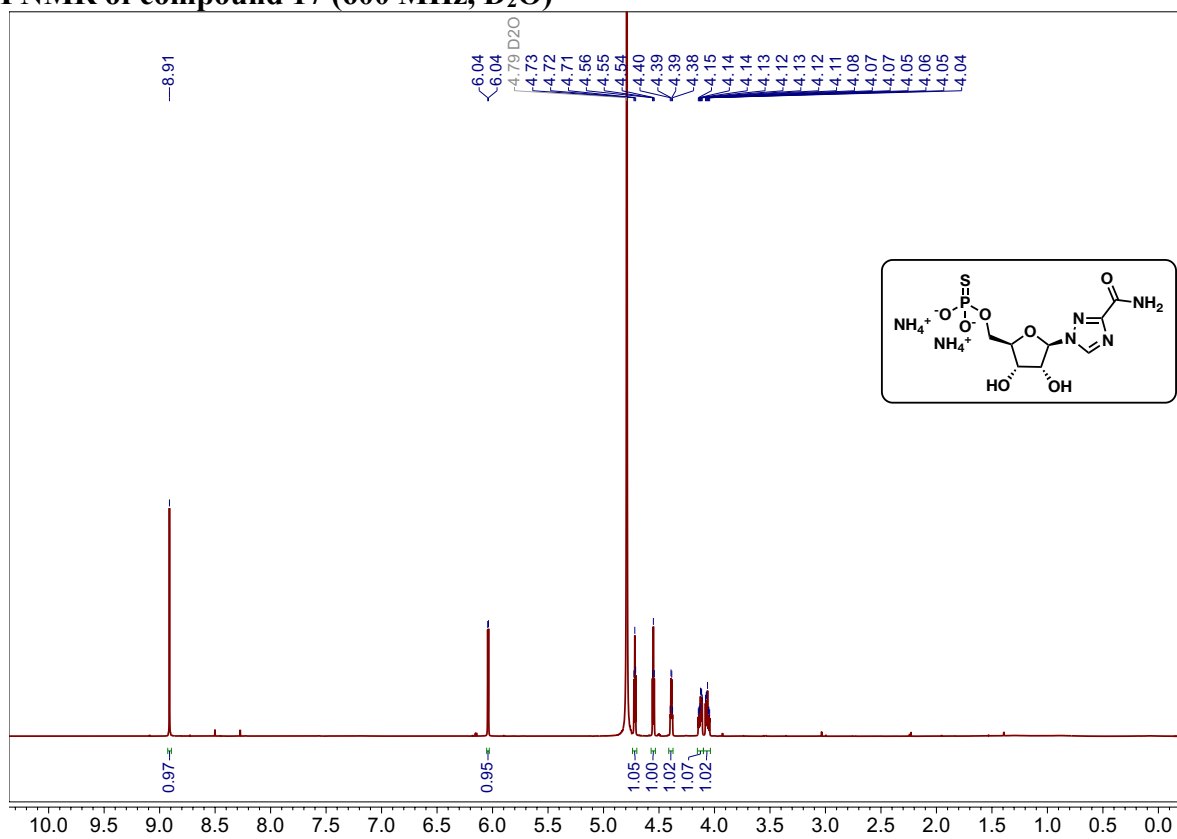
¹³C NMR of compound 16 (151 MHz, D₂O)



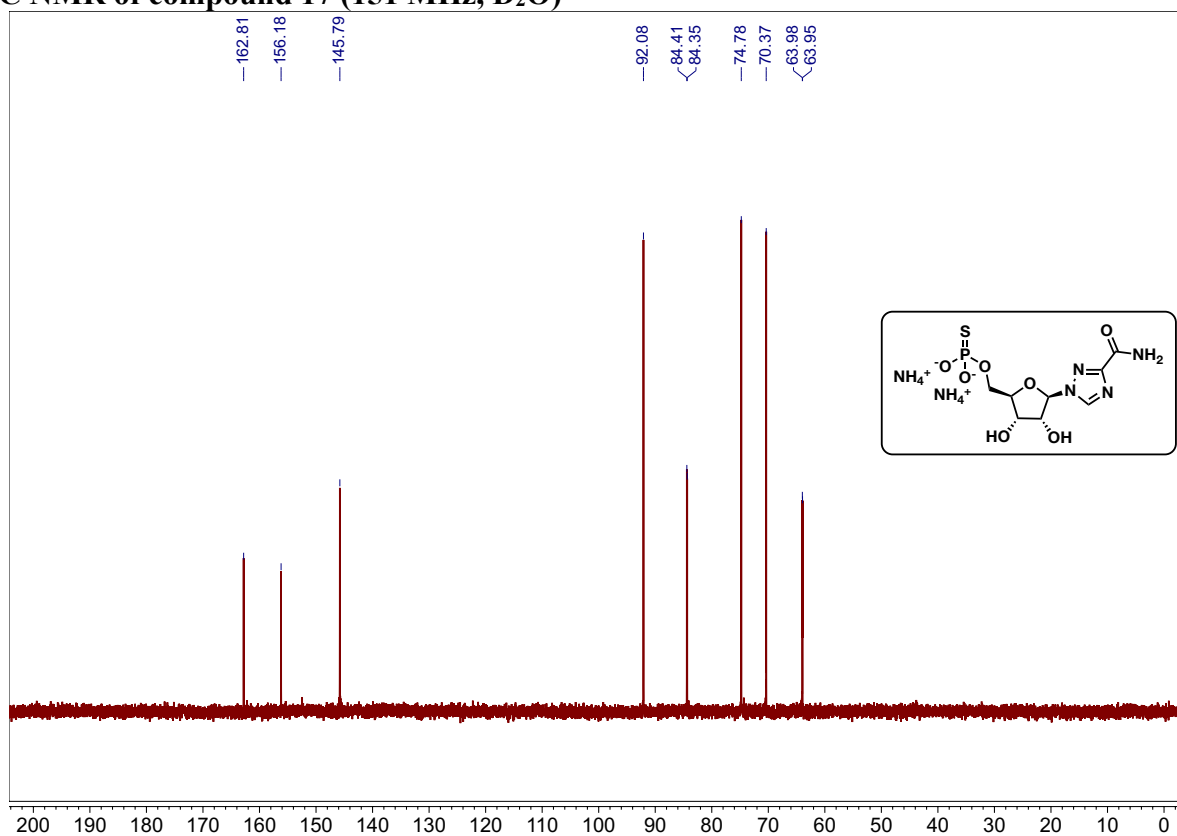
^{31}P NMR of compound 16 (243 MHz, D_2O)



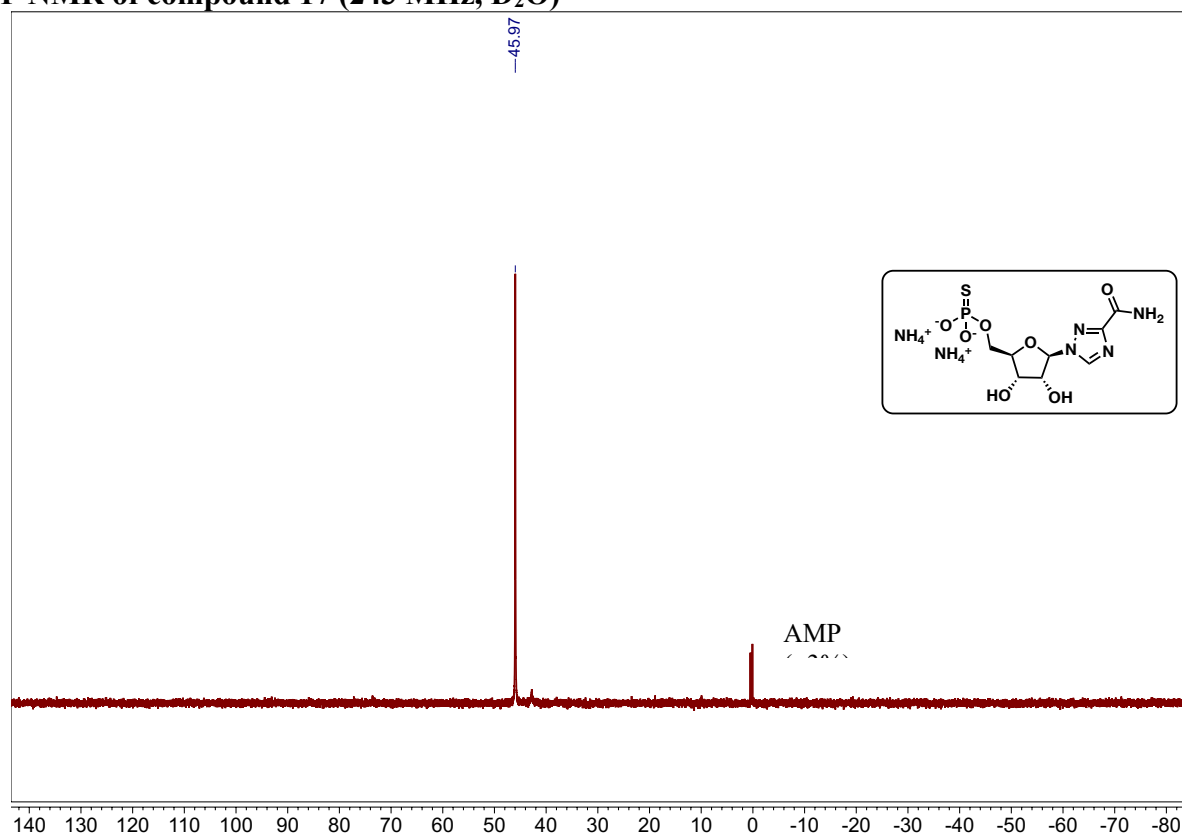
^1H NMR of compound 17 (600 MHz, D_2O)



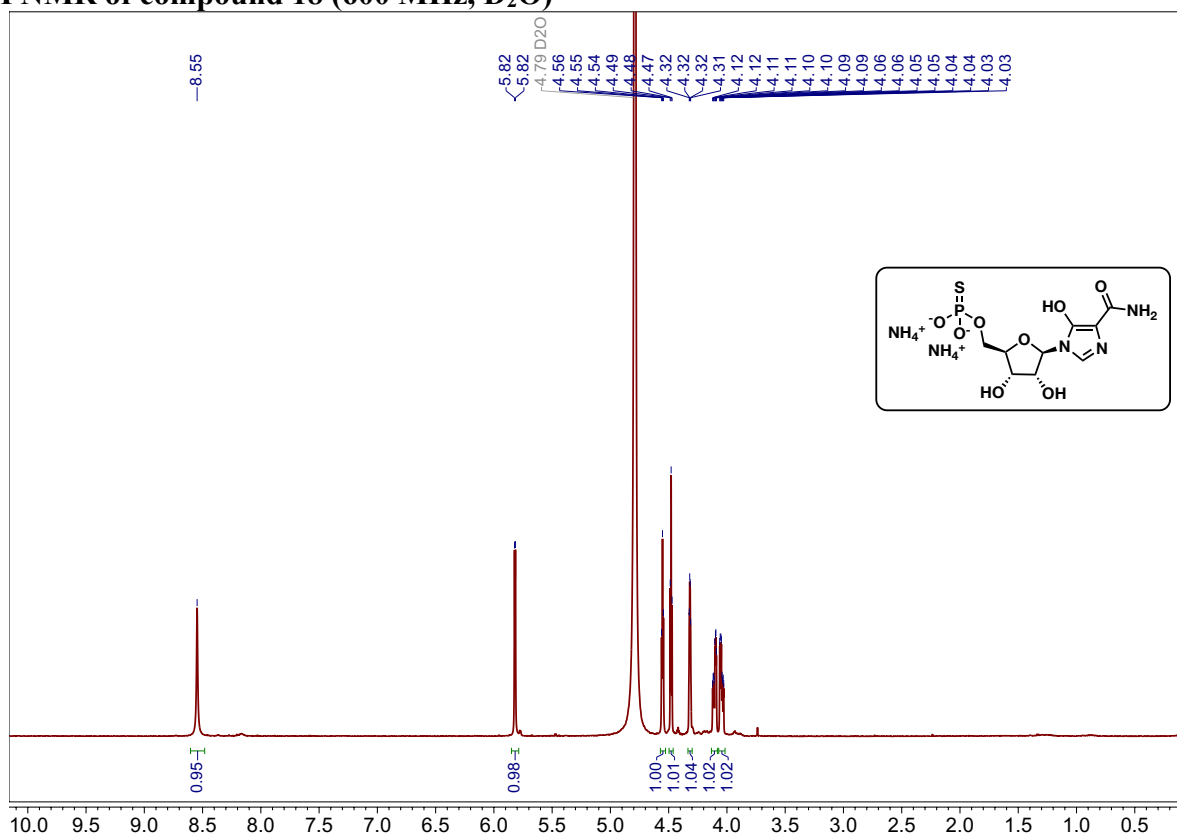
^{13}C NMR of compound 17 (151 MHz, D_2O)



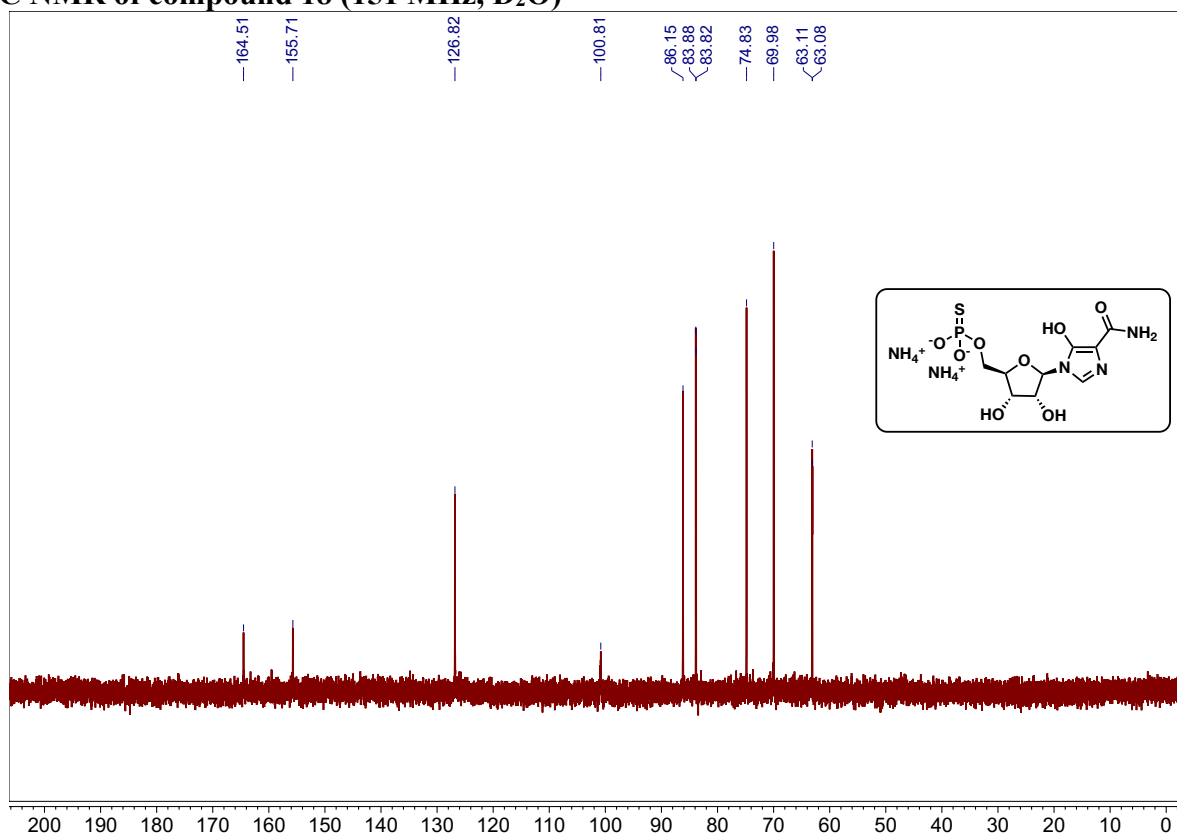
^{31}P NMR of compound 17 (243 MHz, D_2O)



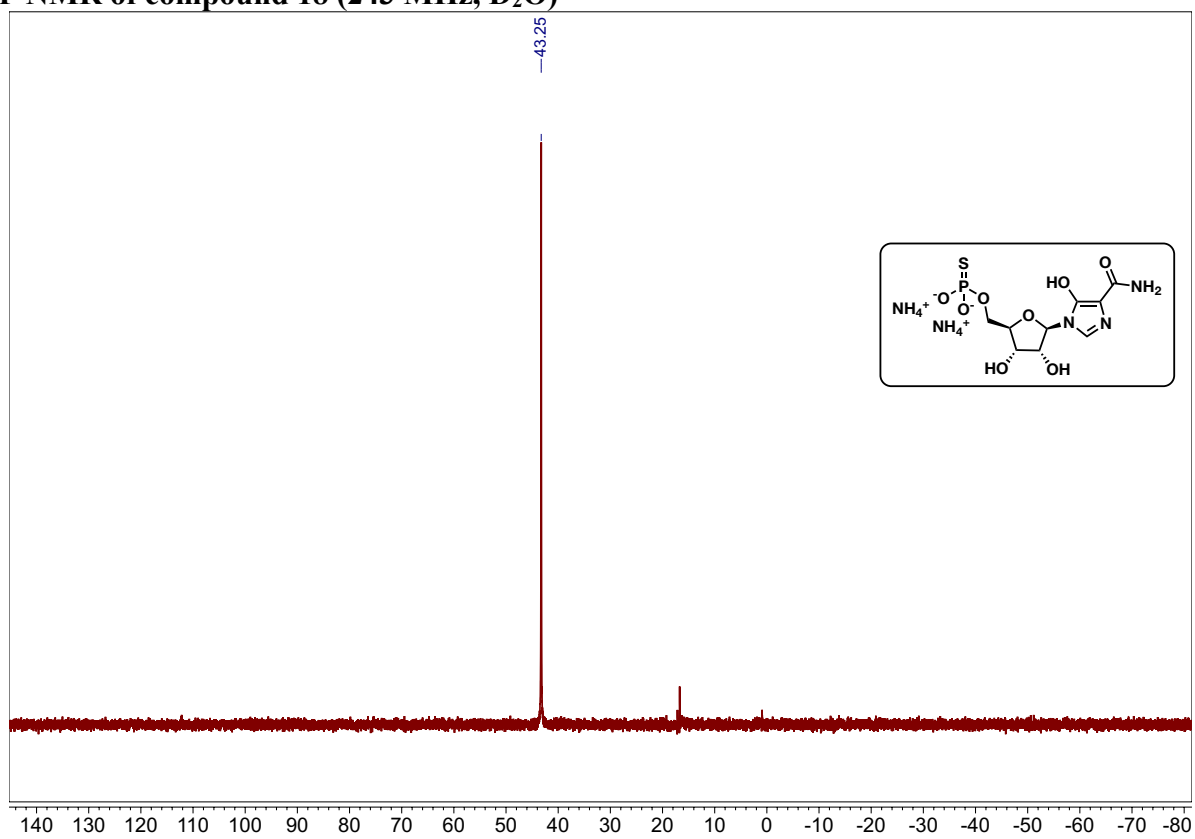
¹H NMR of compound 18 (600 MHz, D₂O)



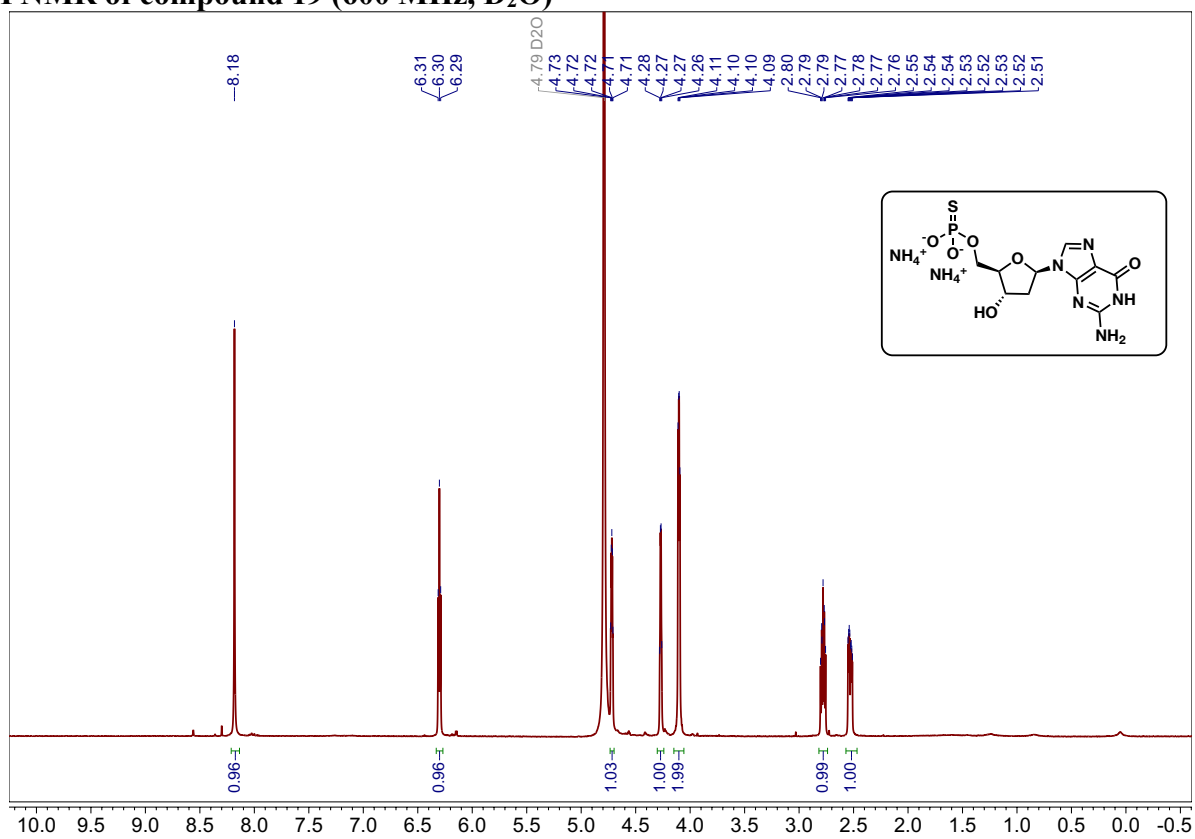
¹³C NMR of compound 18 (151 MHz, D₂O)



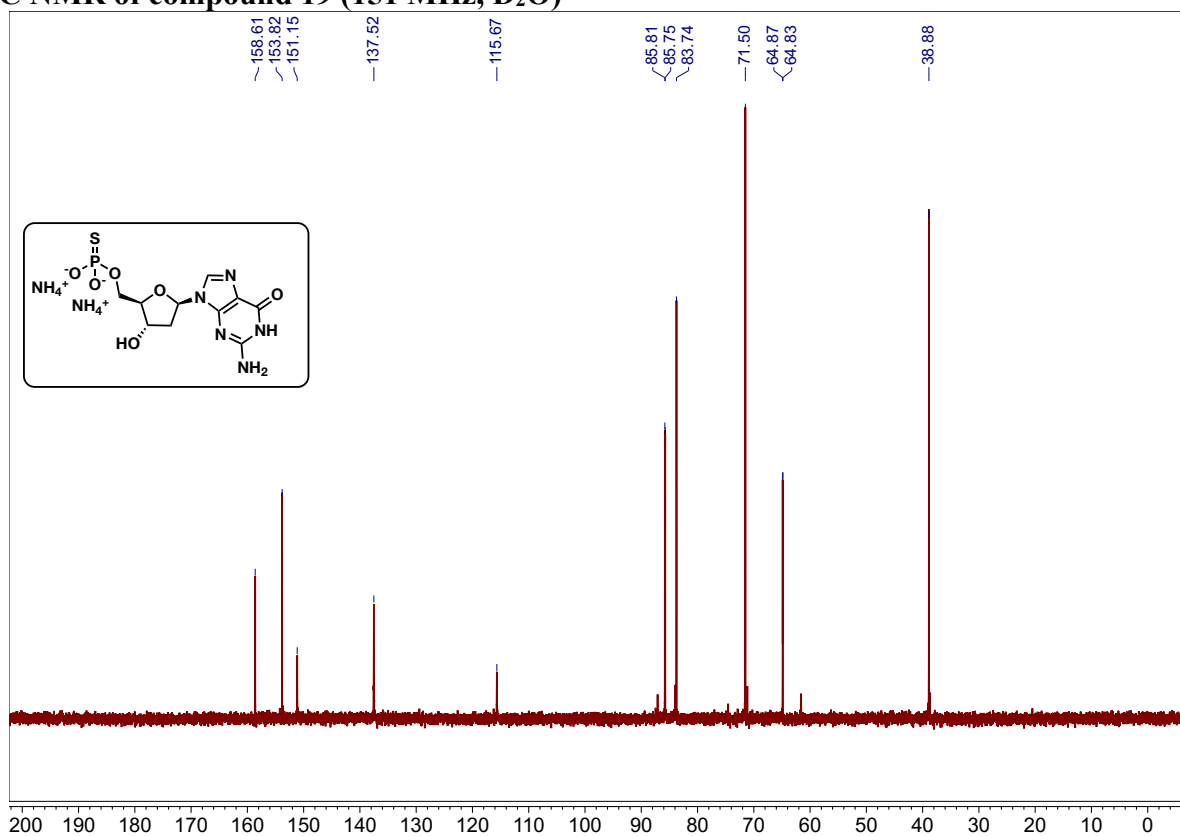
^{31}P NMR of compound 18 (243 MHz, D_2O)



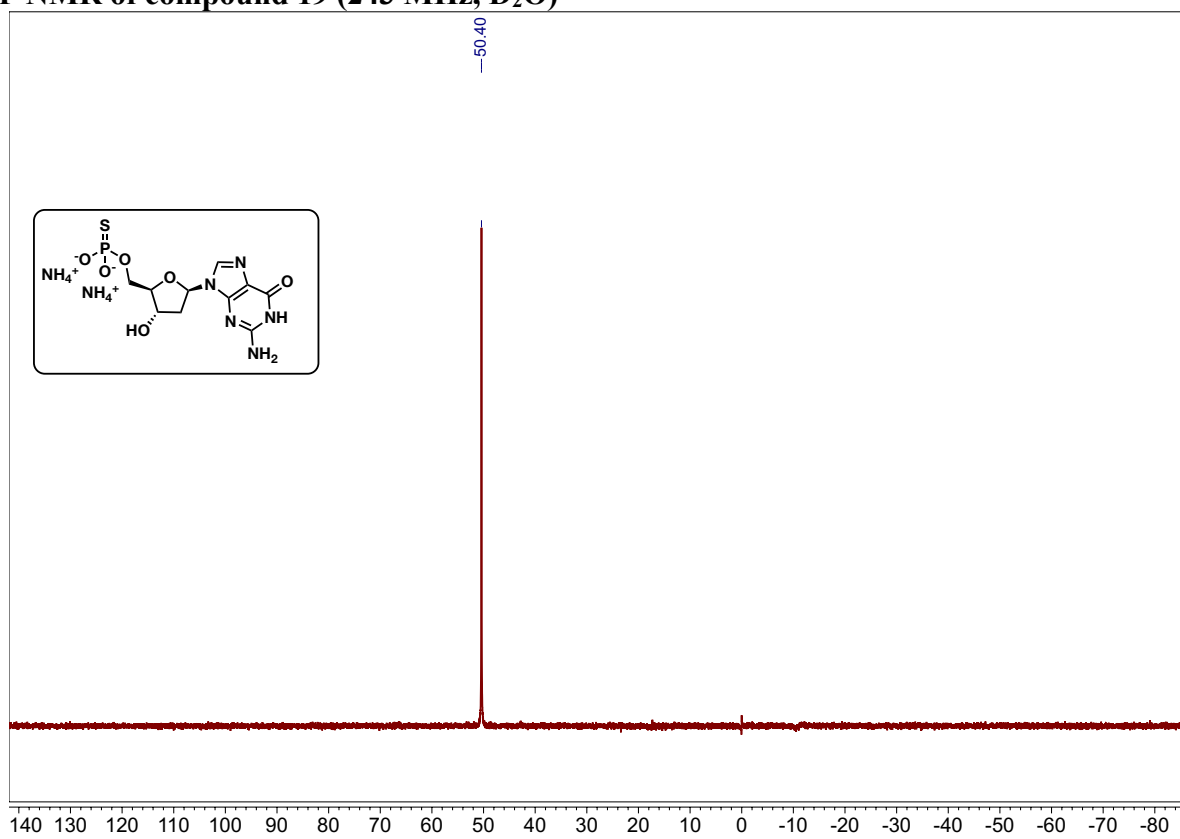
¹H NMR of compound 19 (600 MHz, D₂O)



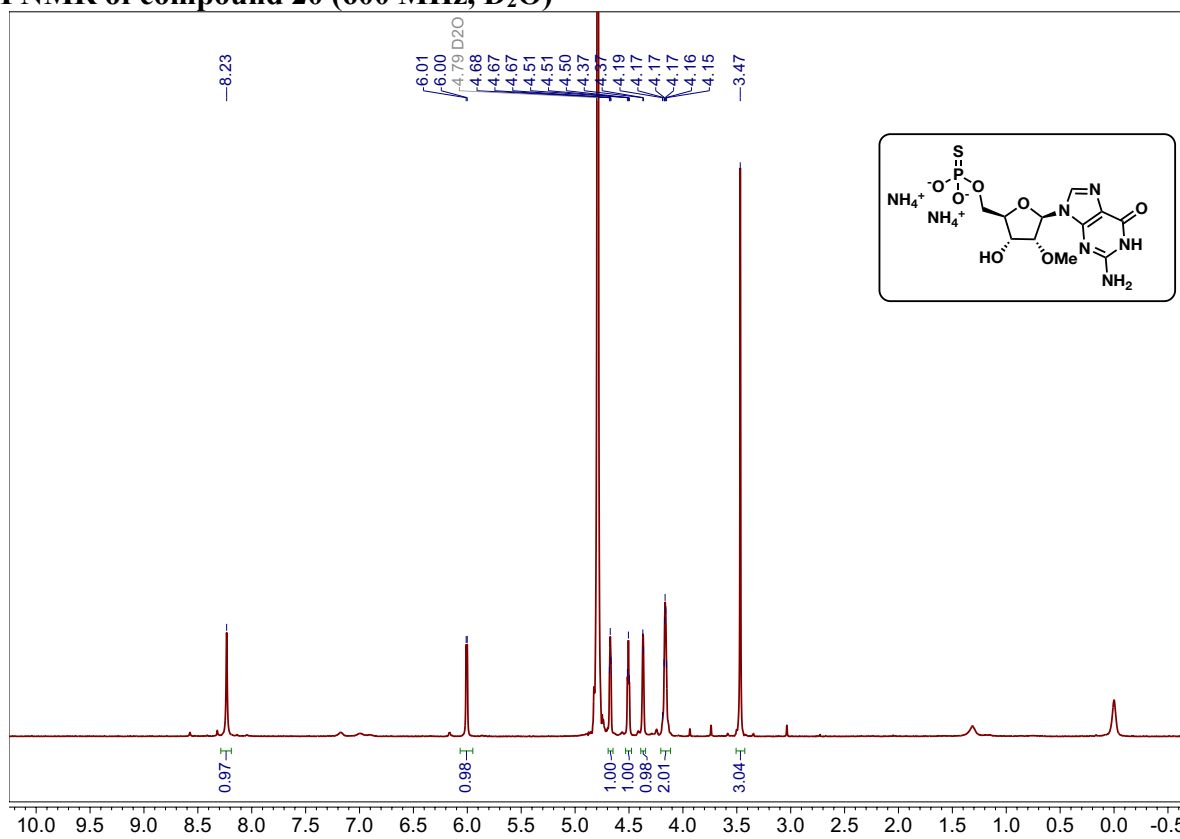
¹³C NMR of compound 19 (151 MHz, D₂O)



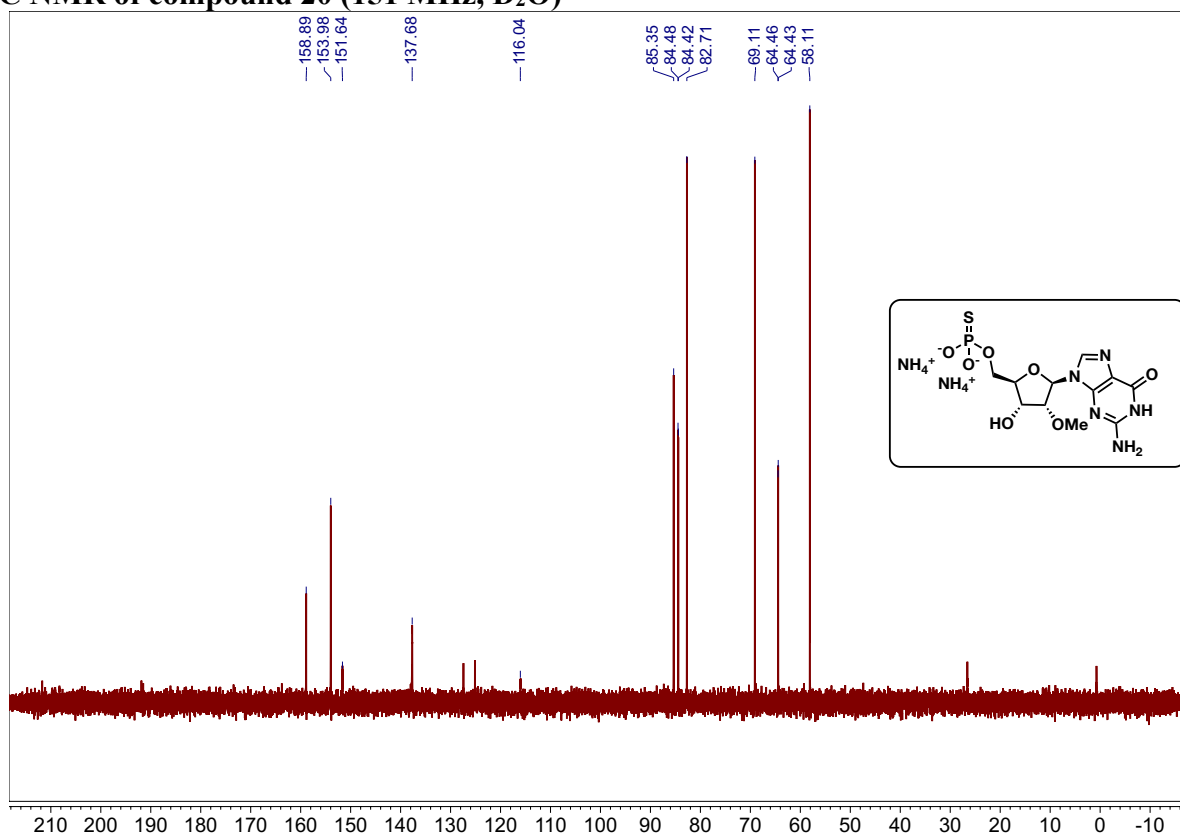
^{31}P NMR of compound 19 (243 MHz, D_2O)



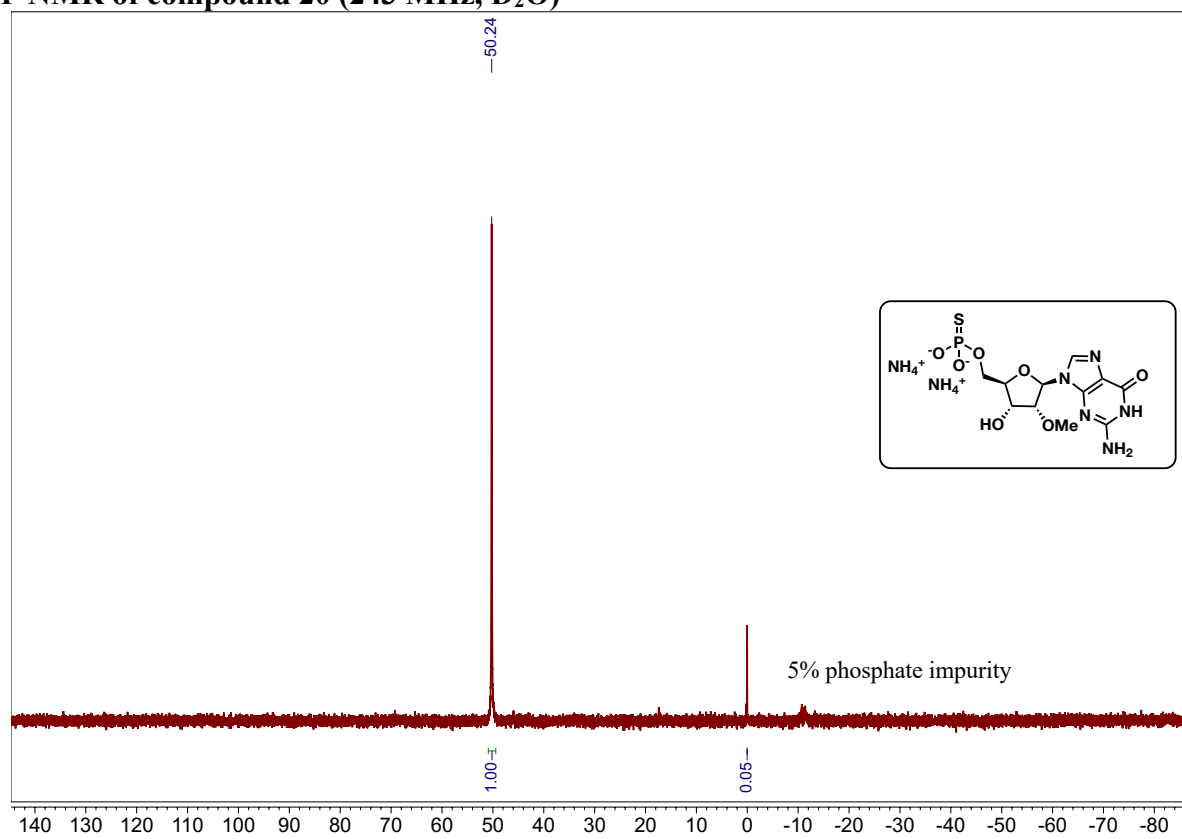
¹H NMR of compound 20 (600 MHz, D₂O)



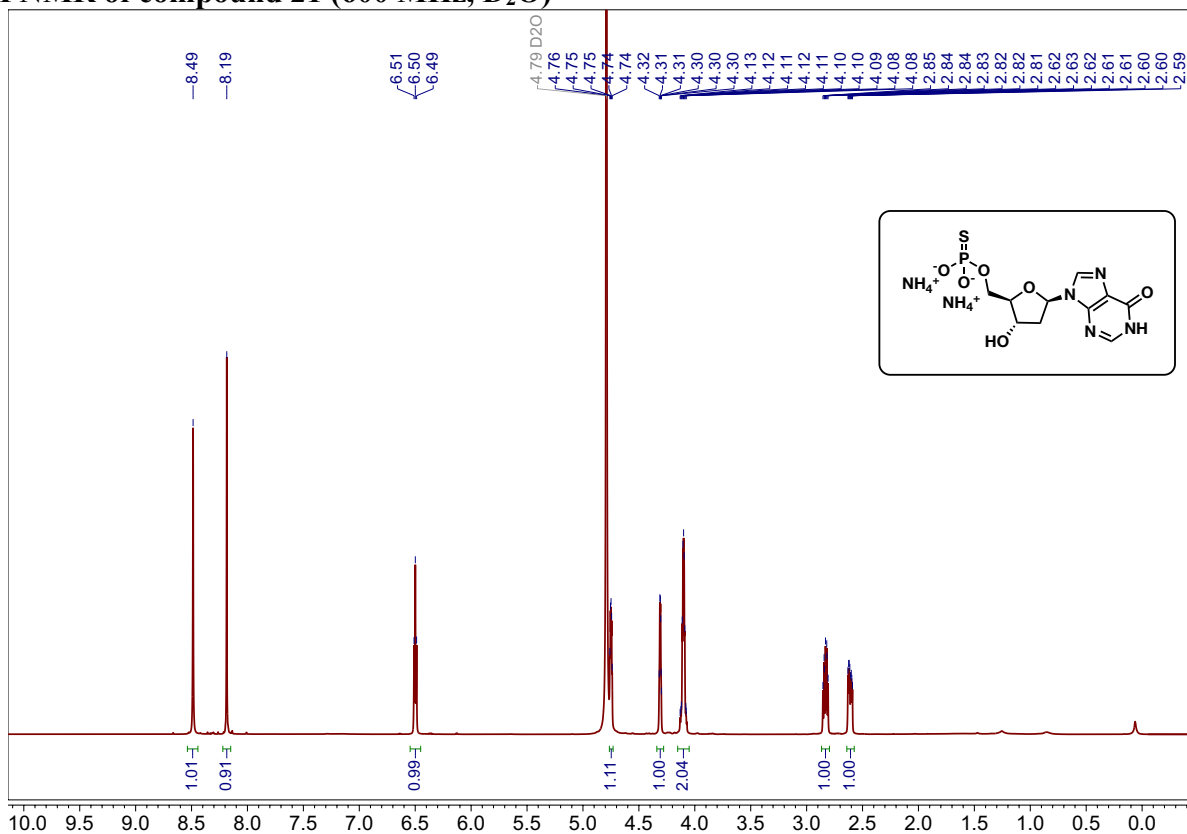
¹³C NMR of compound 20 (151 MHz, D₂O)



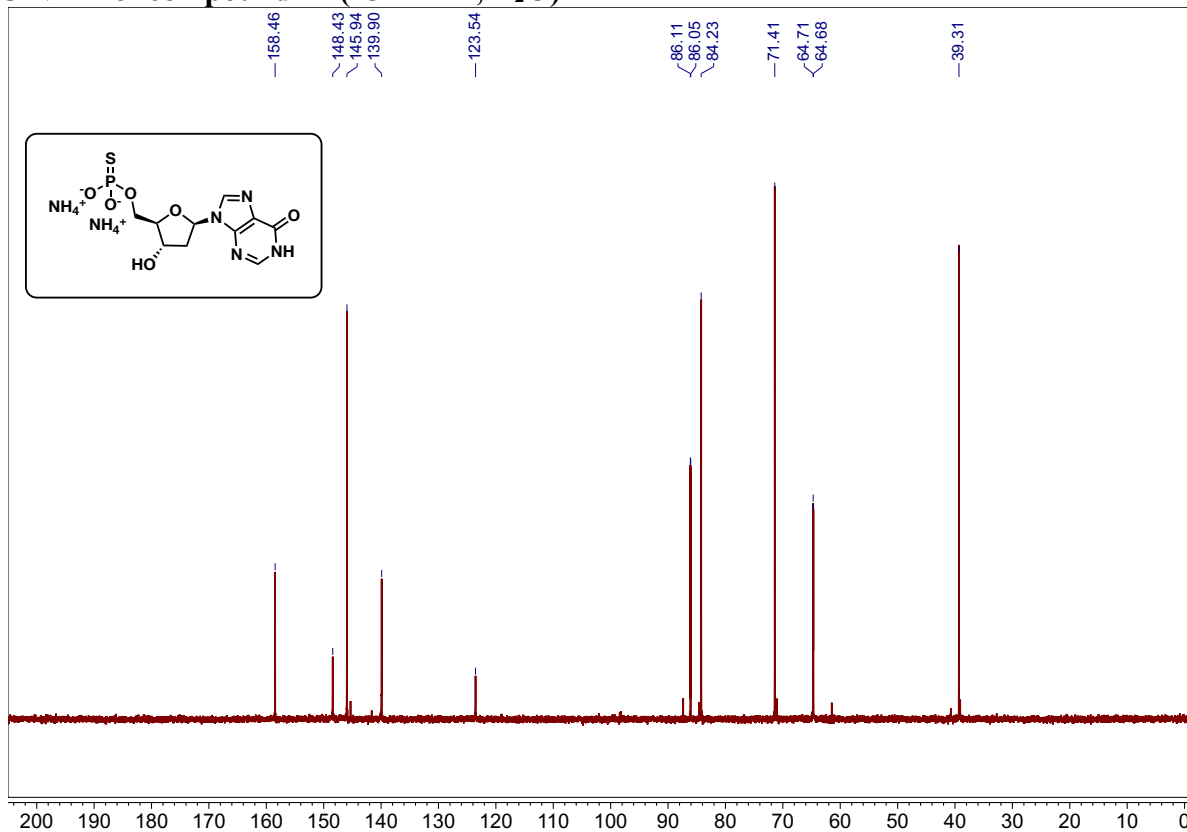
^{31}P NMR of compound 20 (243 MHz, D_2O)



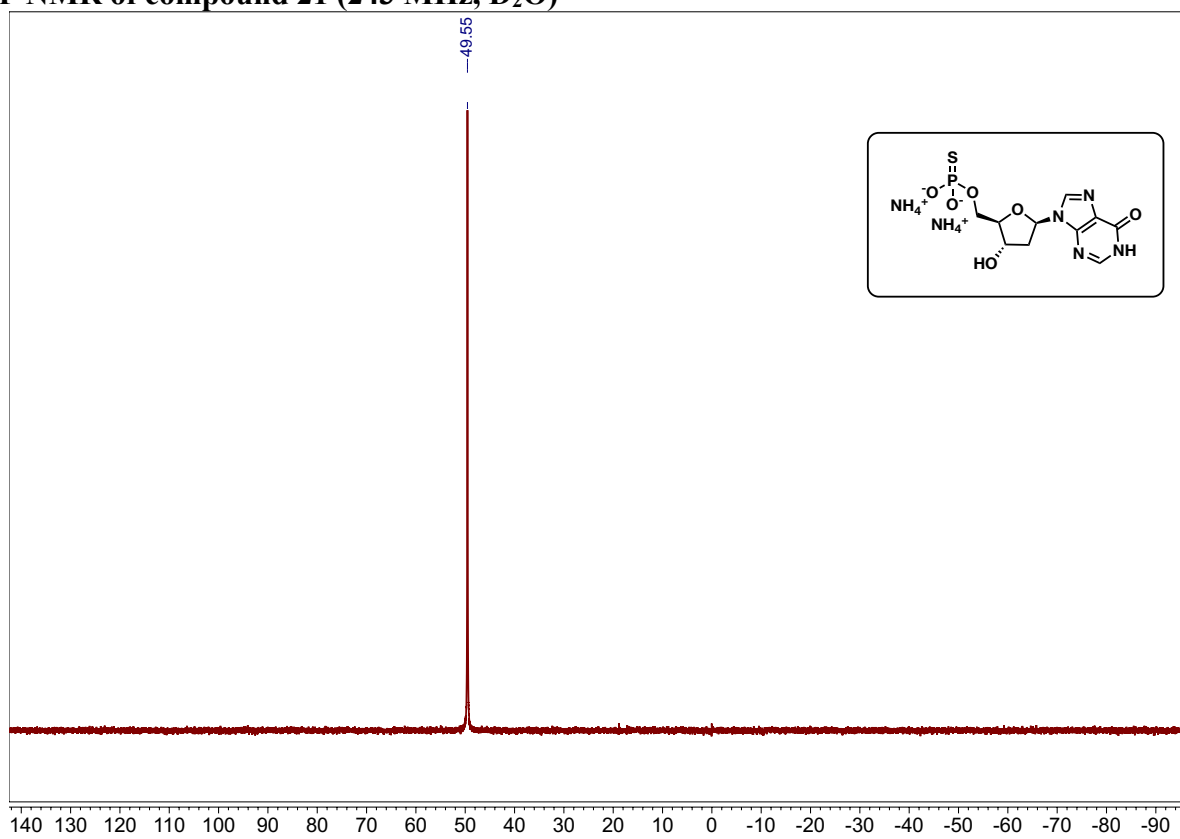
¹H NMR of compound 21 (600 MHz, D₂O)



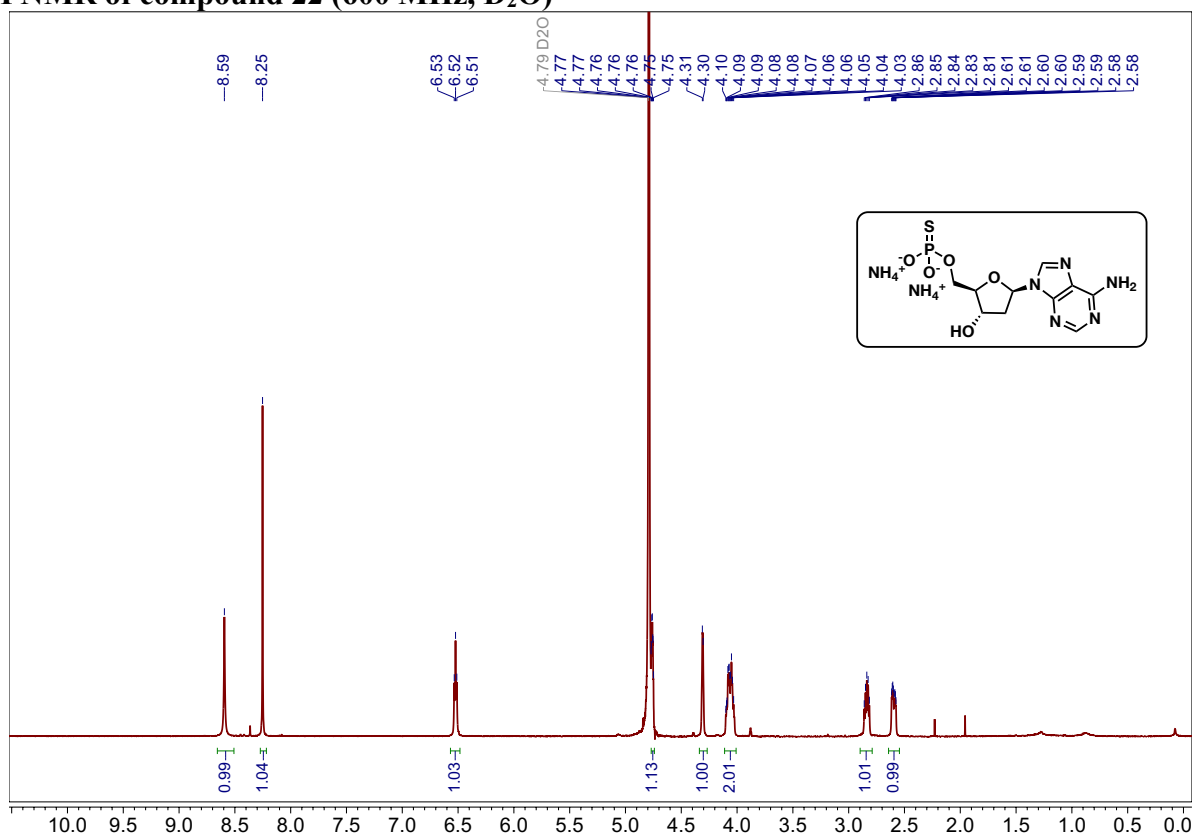
¹³C NMR of compound 21 (151 MHz, D₂O)



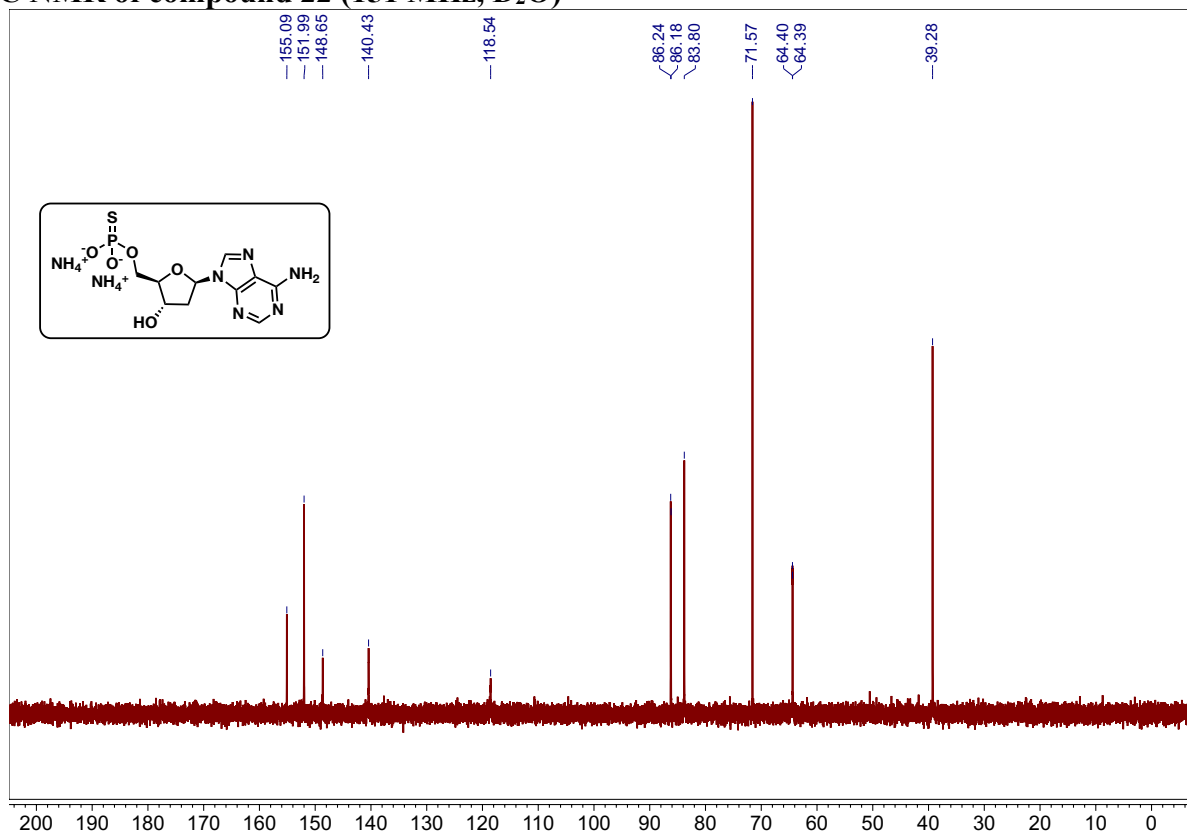
^{31}P NMR of compound 21 (243 MHz, D_2O)



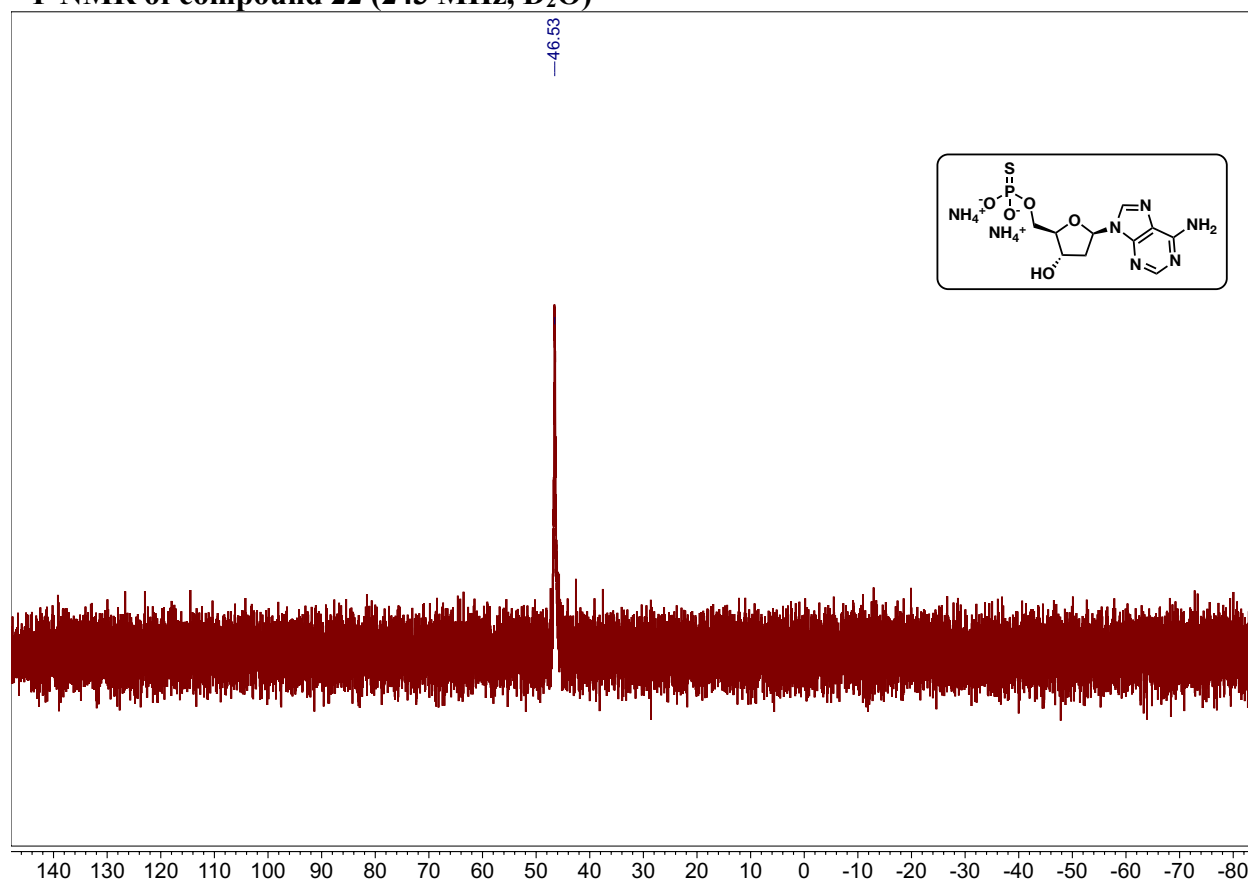
¹H NMR of compound 22 (600 MHz, D₂O)



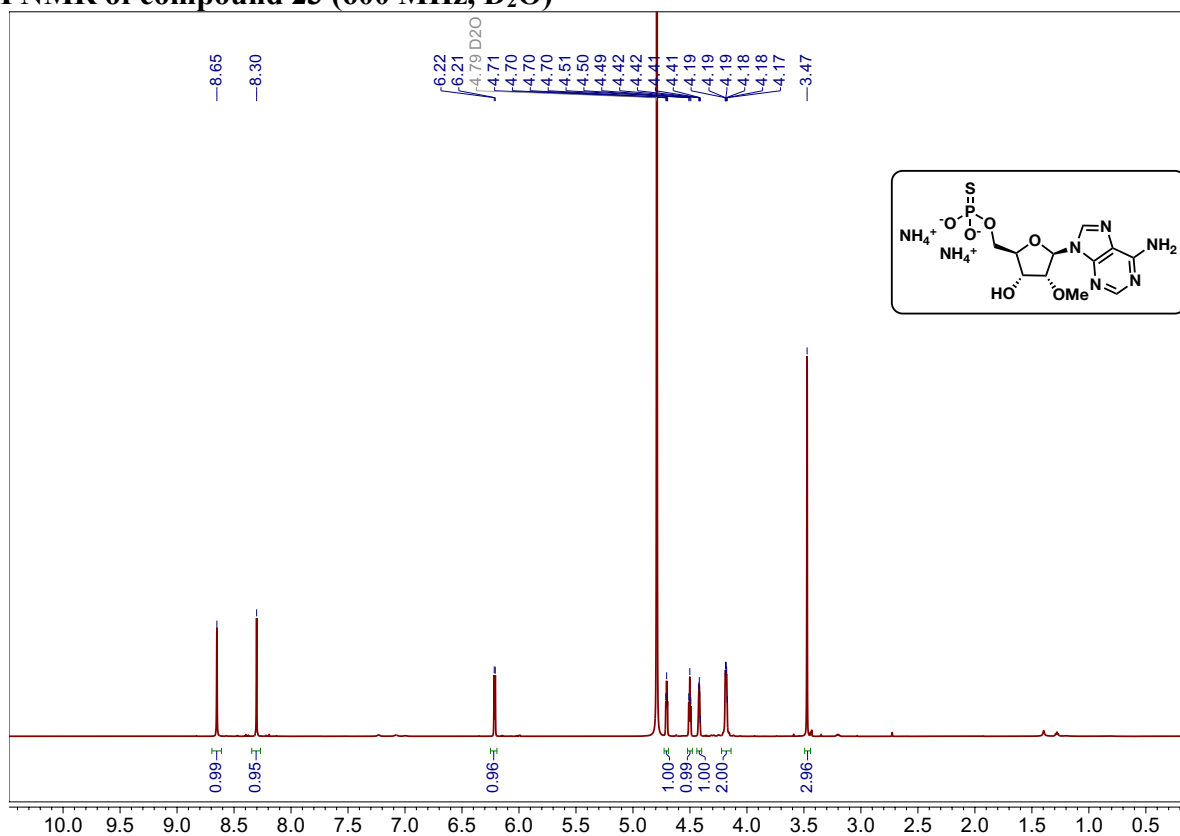
¹³C NMR of compound 22 (151 MHz, D₂O)



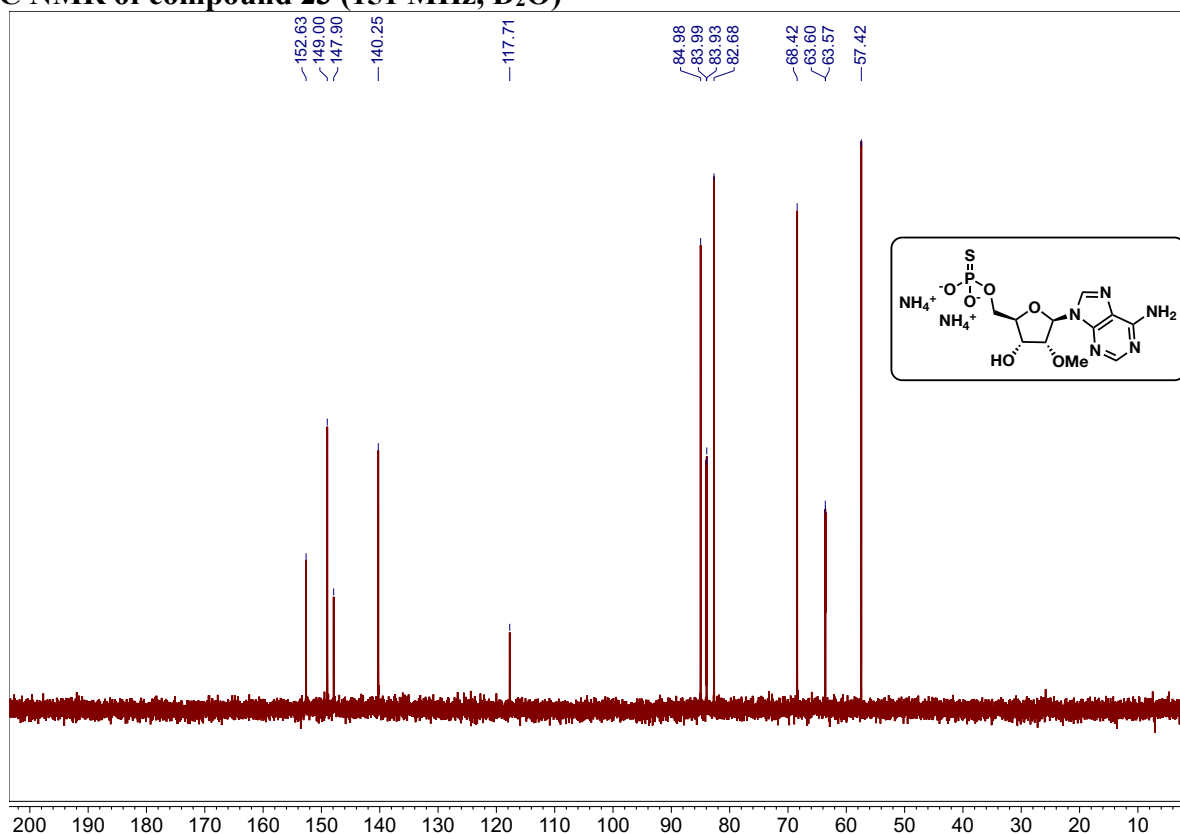
^{31}P NMR of compound 22 (243 MHz, D_2O)



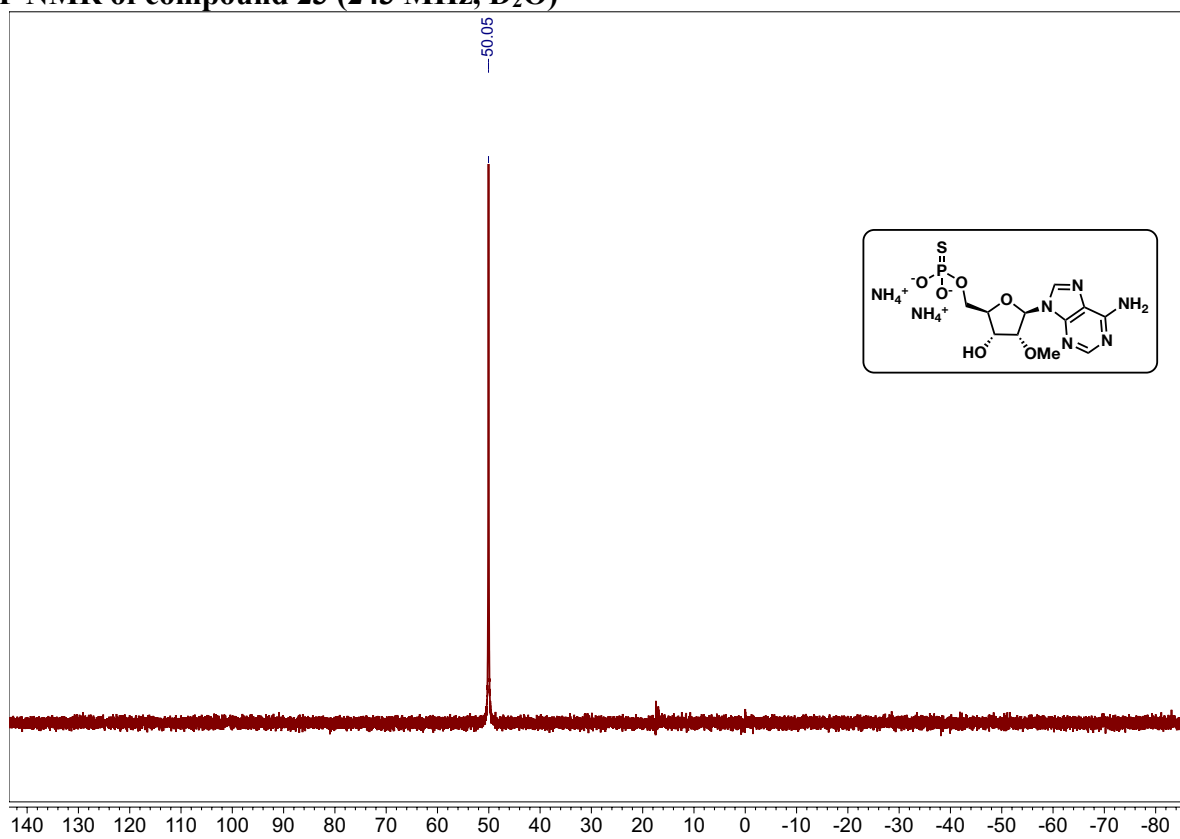
¹H NMR of compound 23 (600 MHz, D₂O)



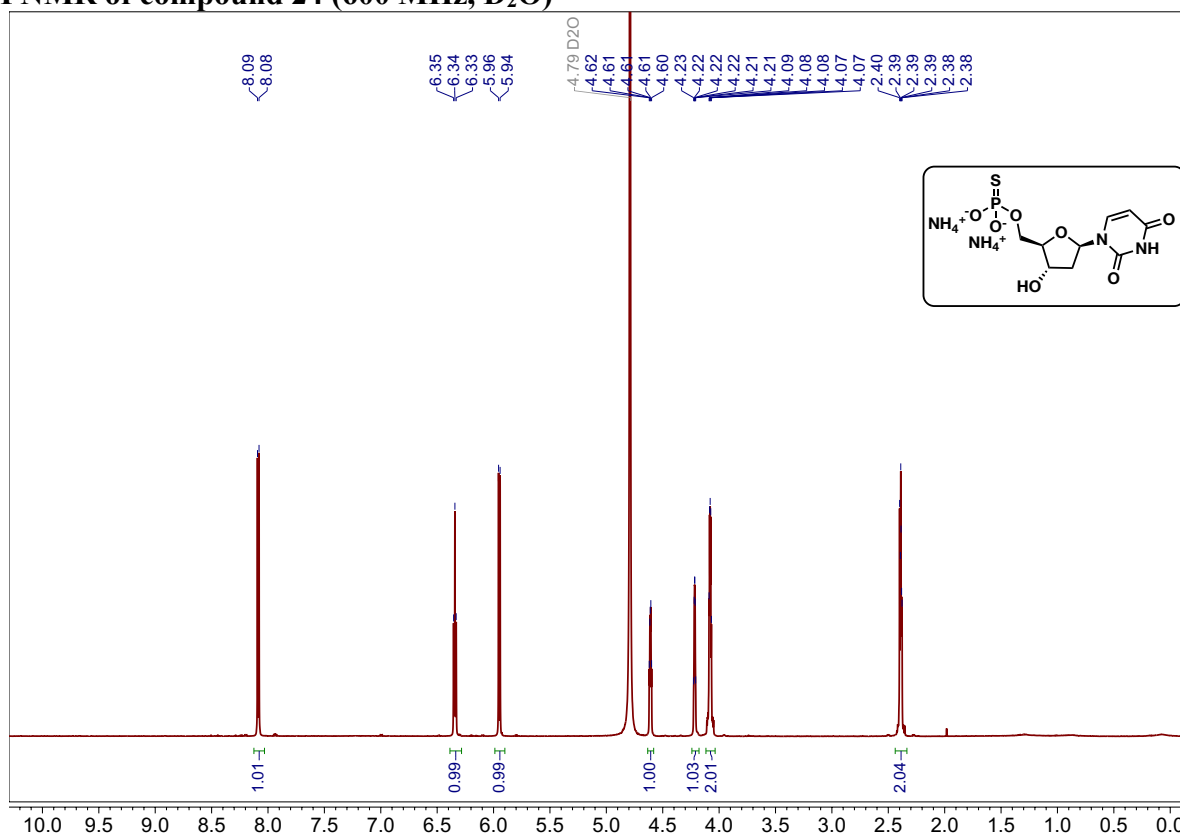
¹³C NMR of compound 23 (151 MHz, D₂O)



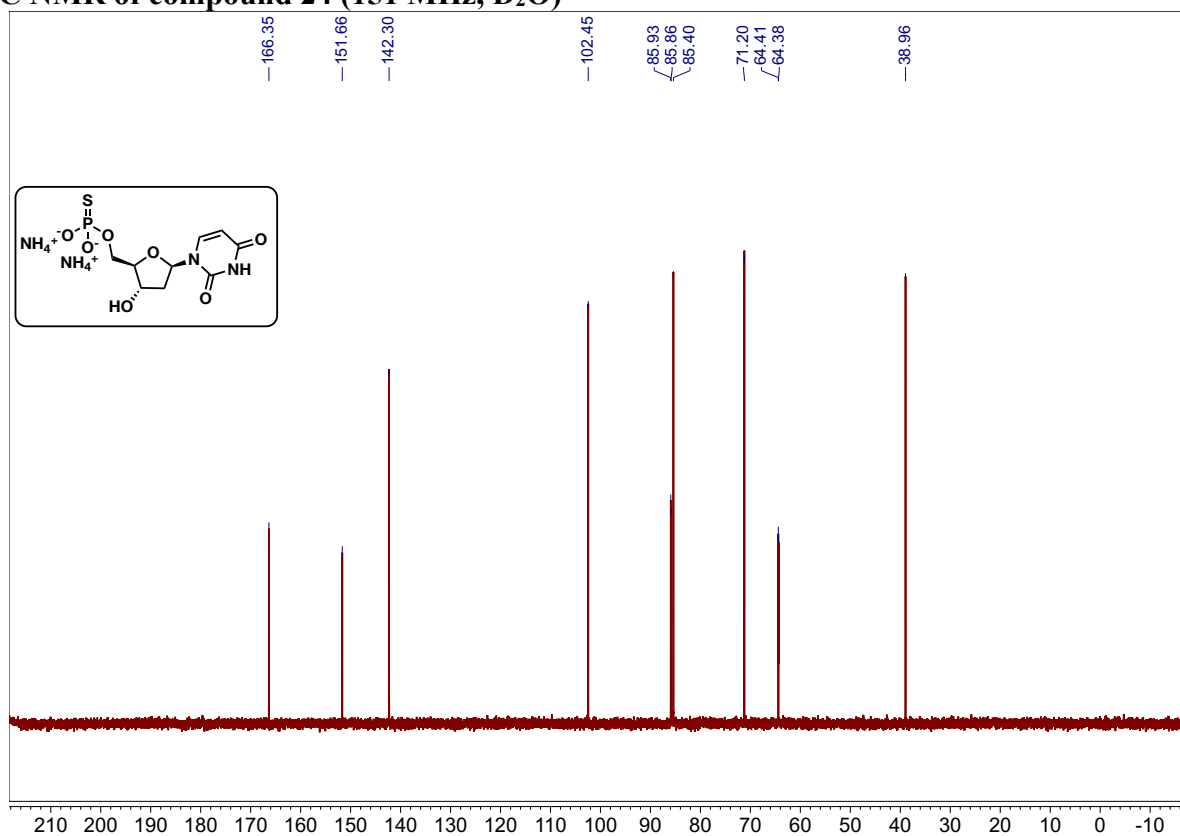
^{31}P NMR of compound 23 (243 MHz, D_2O)



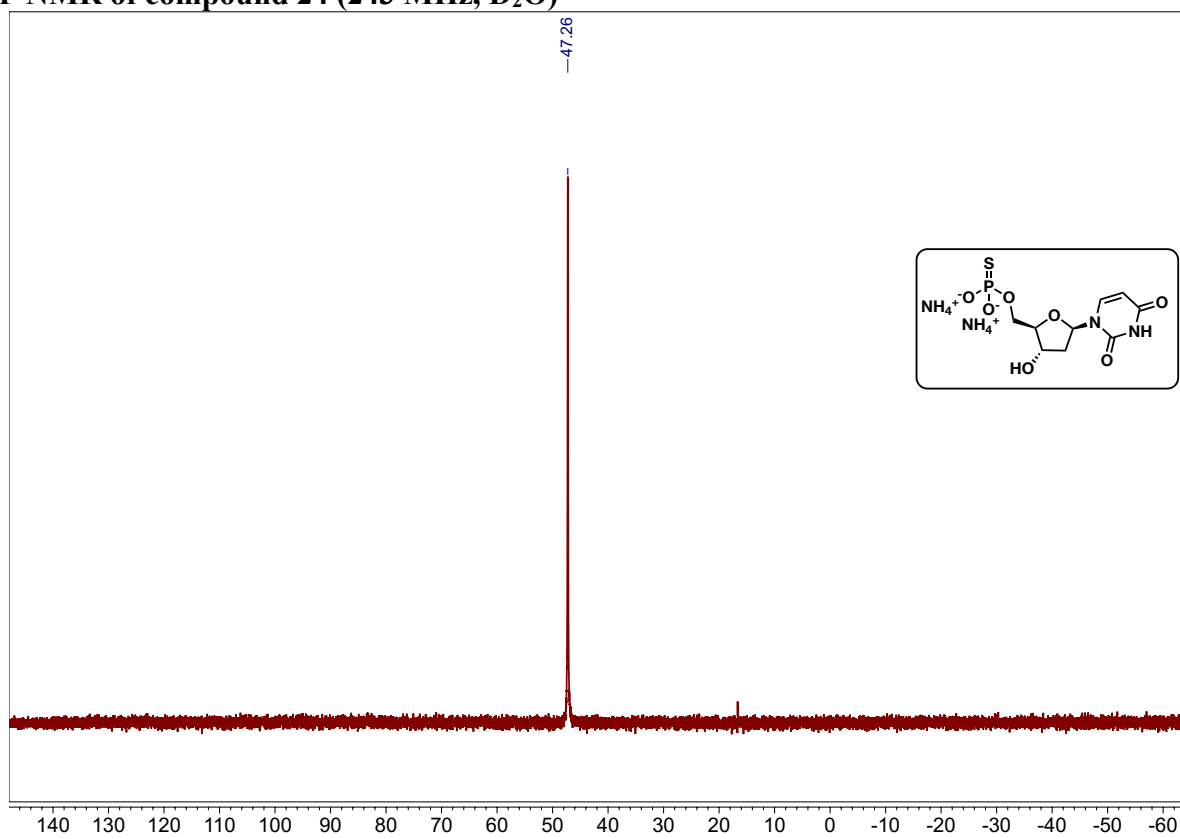
^1H NMR of compound 24 (600 MHz, D_2O)



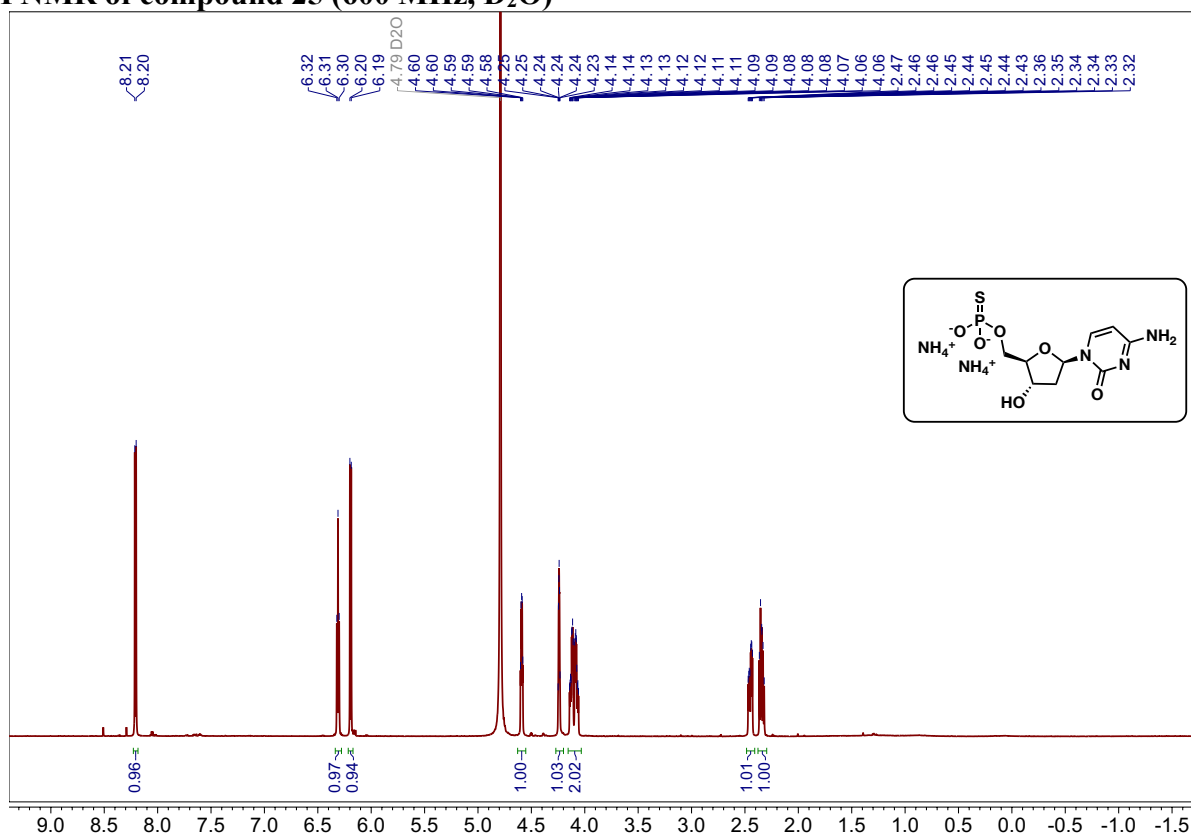
^{13}C NMR of compound 24 (151 MHz, D_2O)



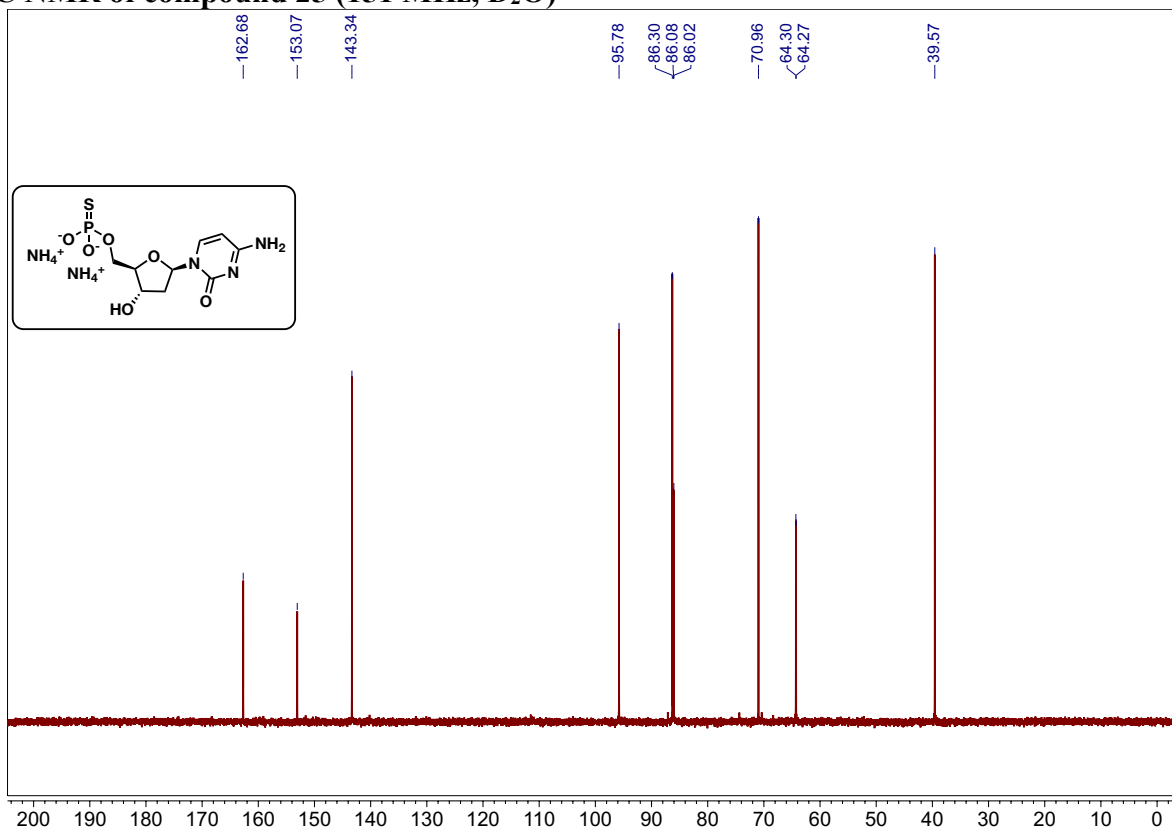
^{31}P NMR of compound 24 (243 MHz, D_2O)



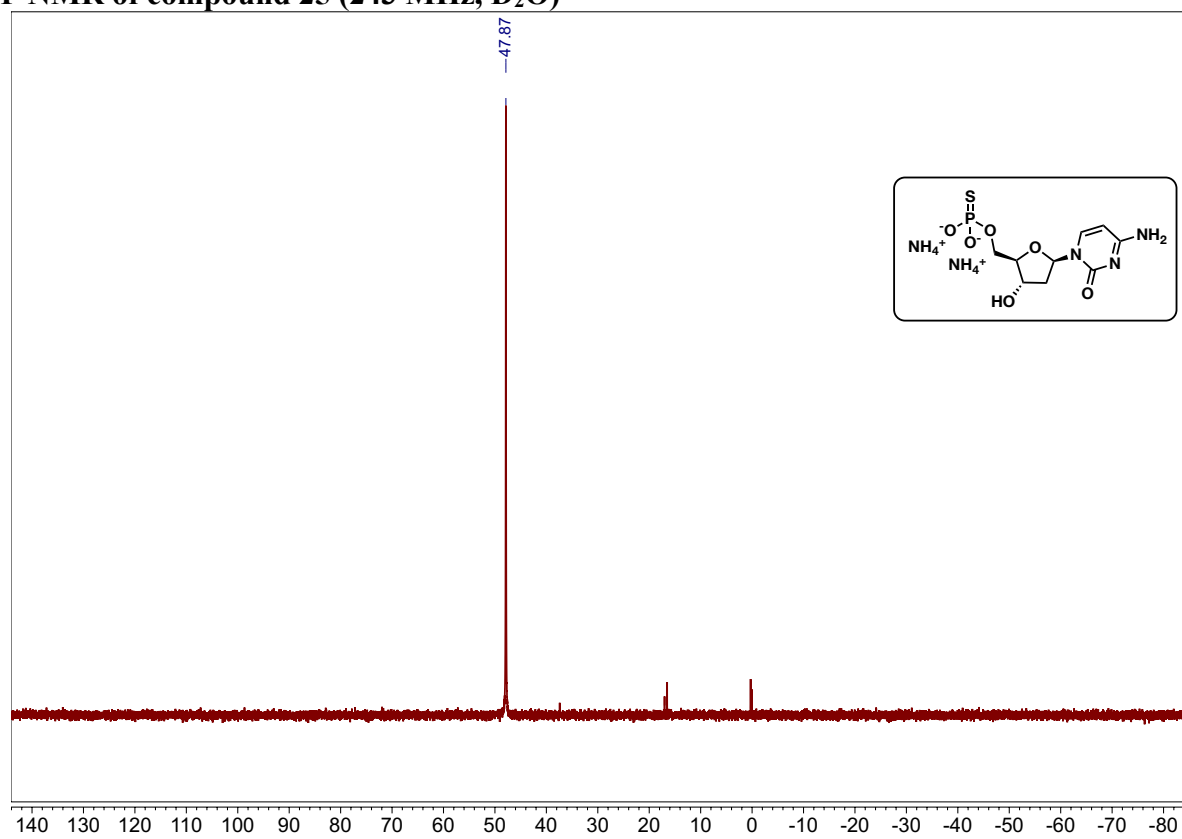
¹H NMR of compound 25 (600 MHz, D₂O)



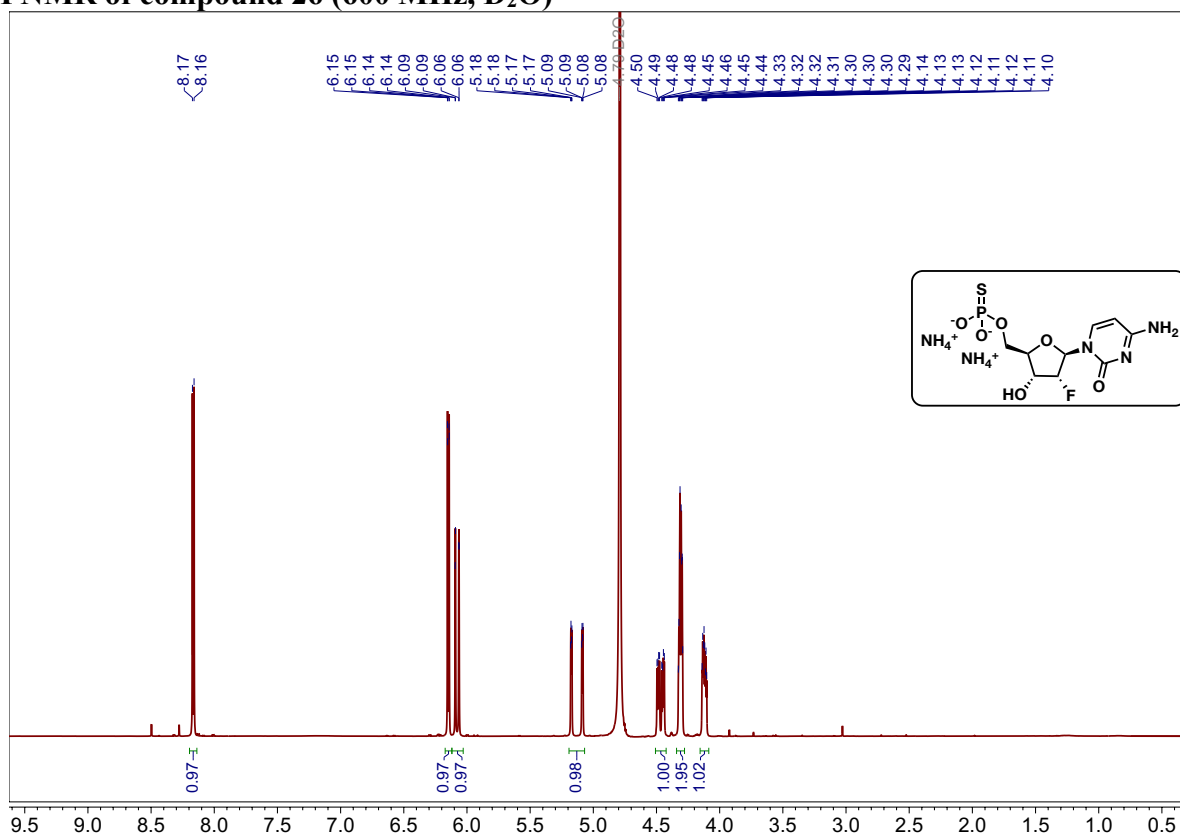
¹³C NMR of compound 25 (151 MHz, D₂O)



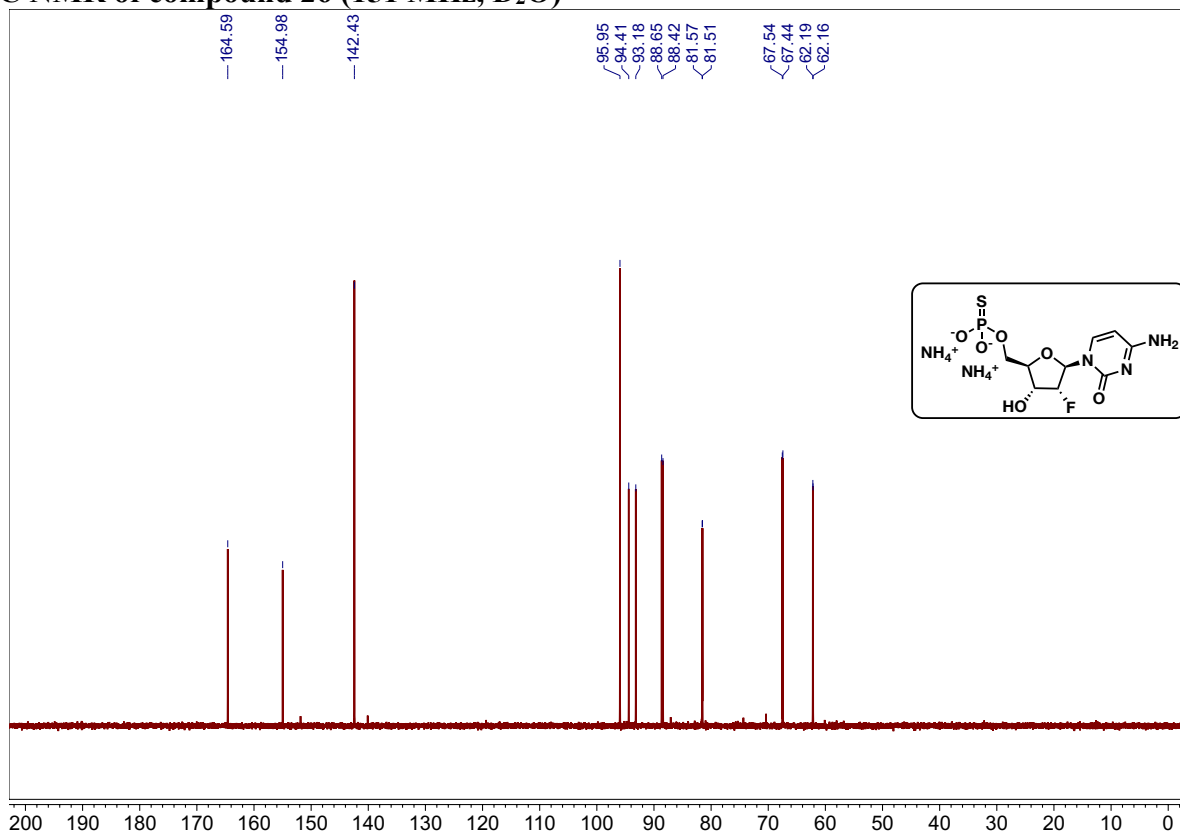
^{31}P NMR of compound 25 (243 MHz, D_2O)



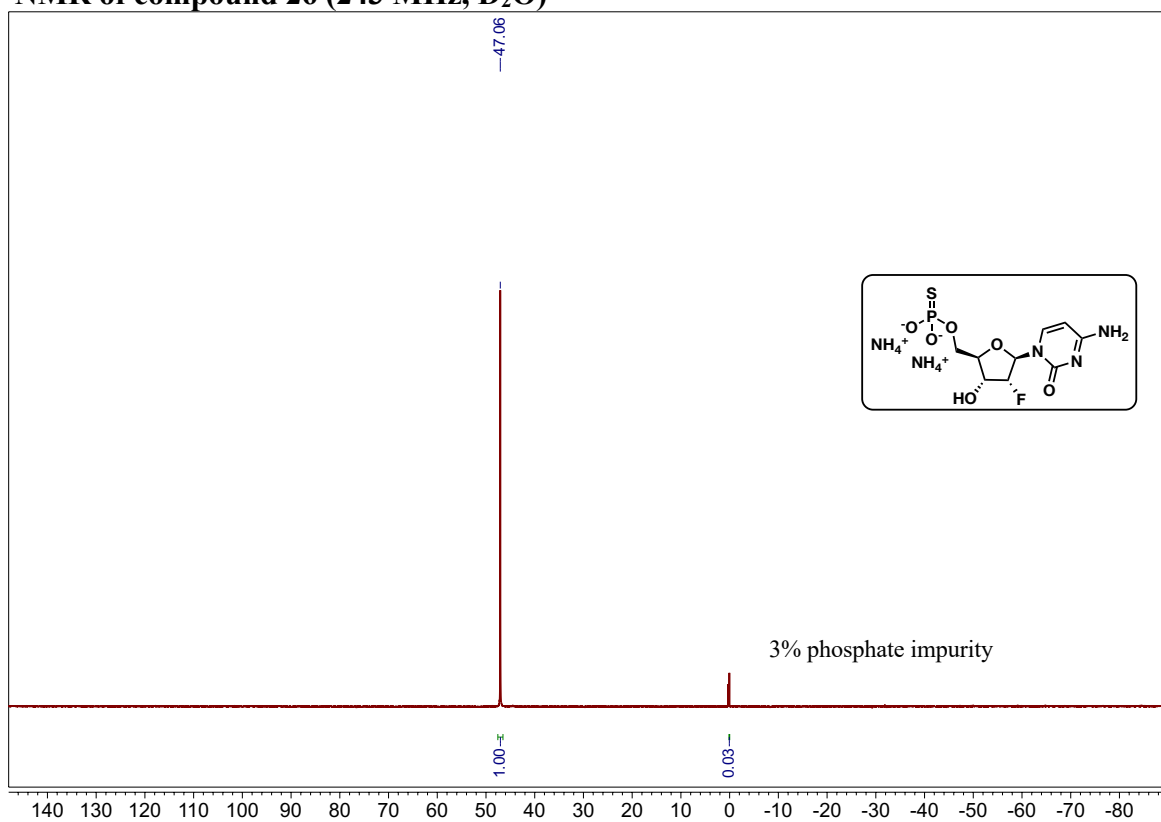
¹H NMR of compound 26 (600 MHz, D₂O)



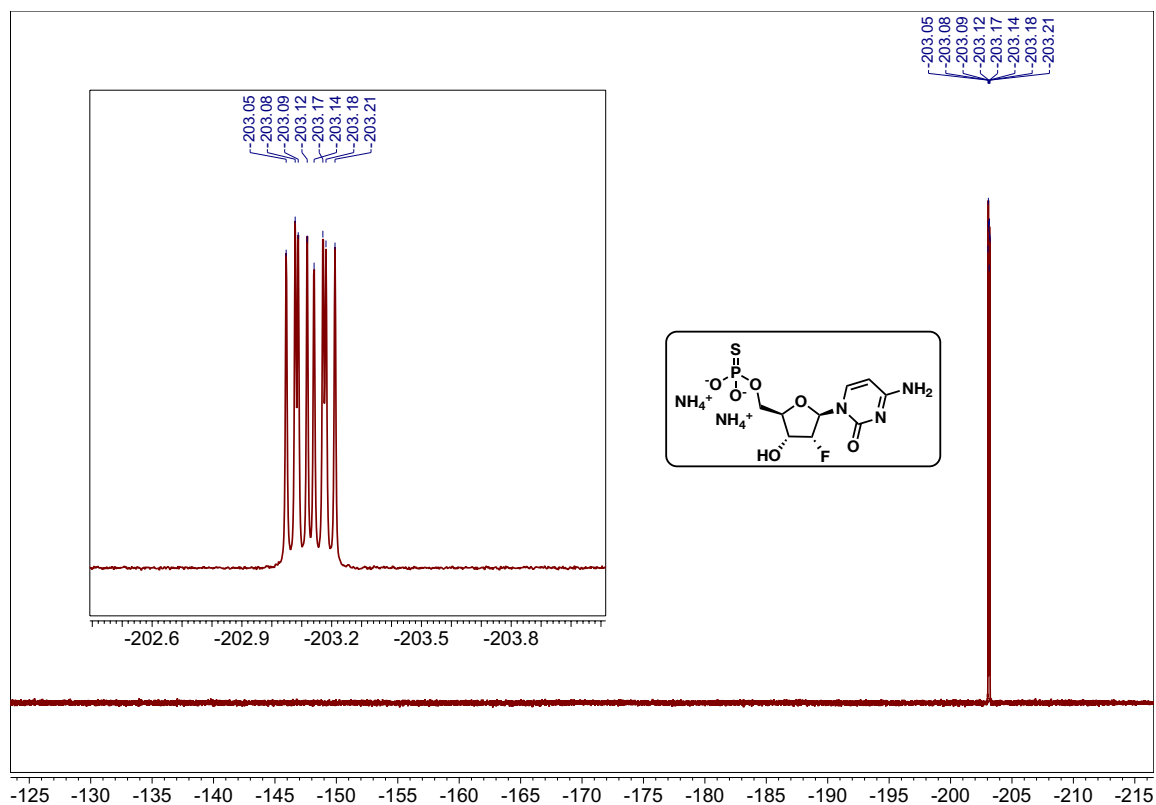
¹³C NMR of compound 26 (151 MHz, D₂O)



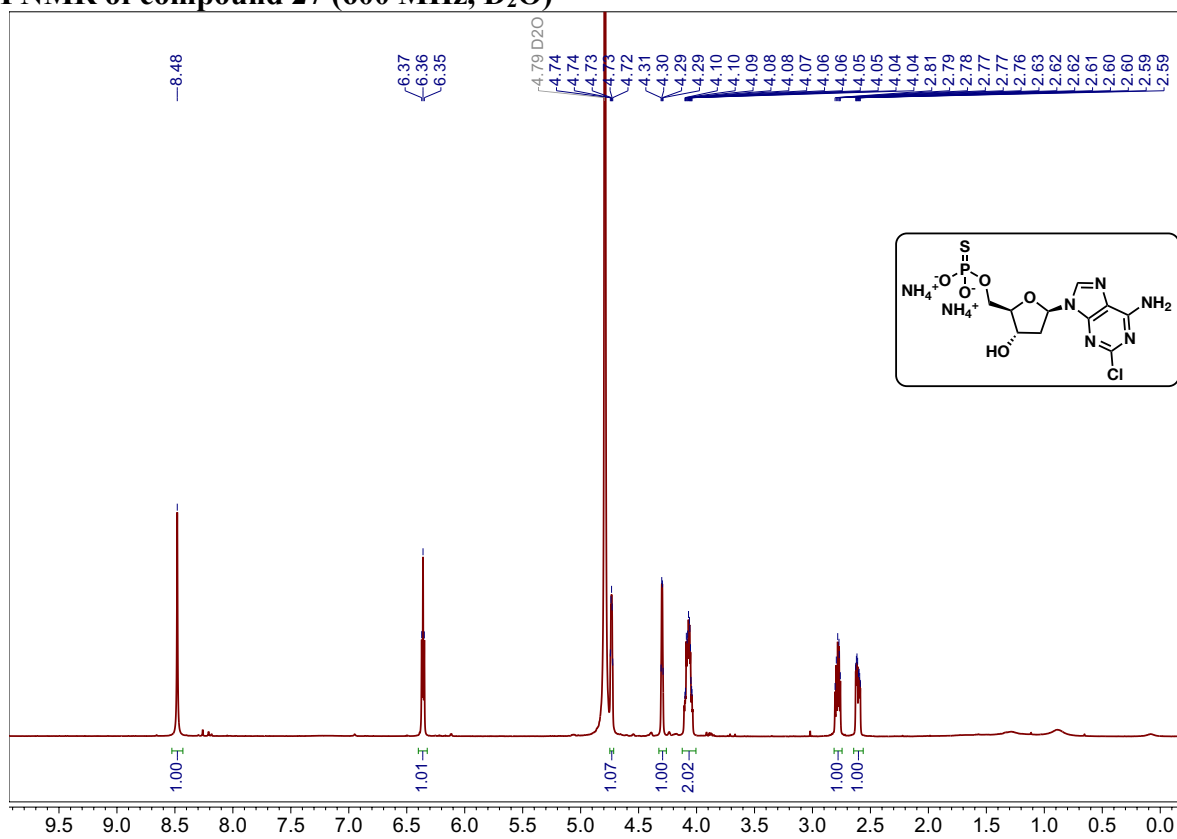
^{31}P NMR of compound 26 (243 MHz, D_2O)



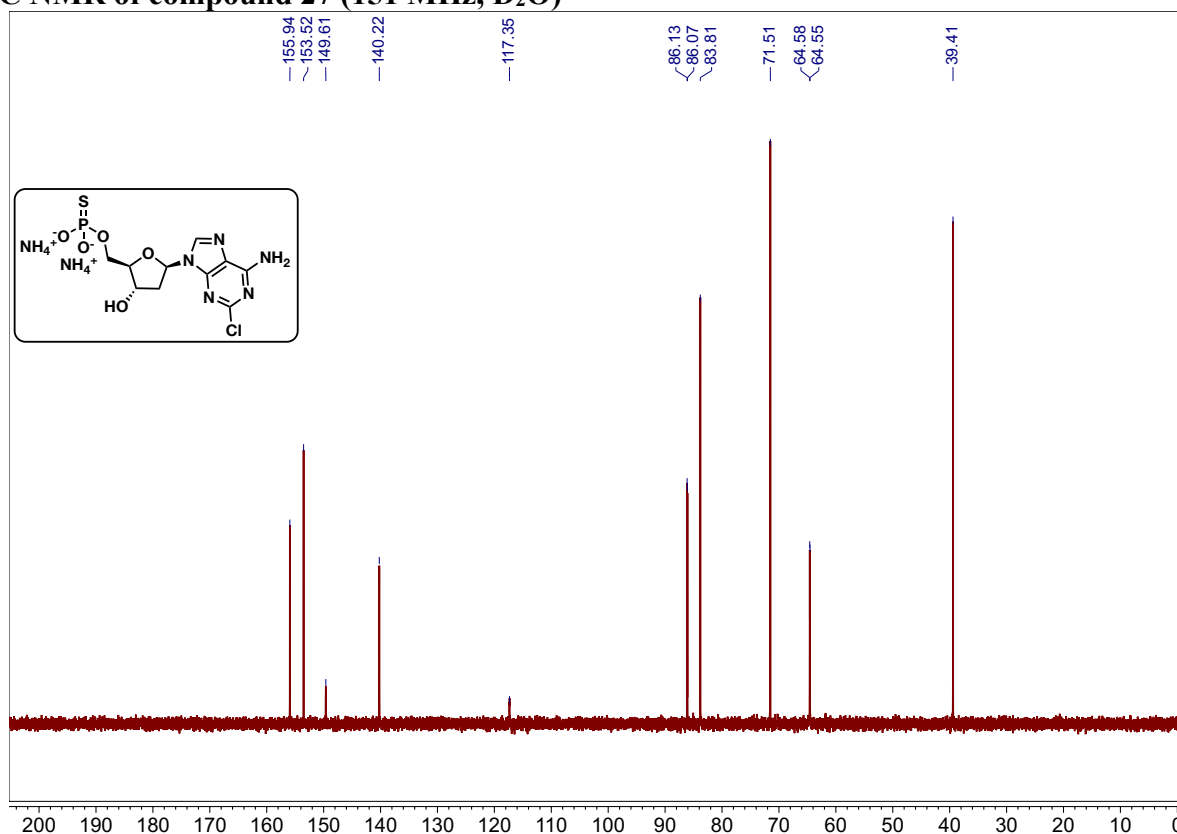
^{19}F NMR of compound 26 (565 MHz, D_2O)



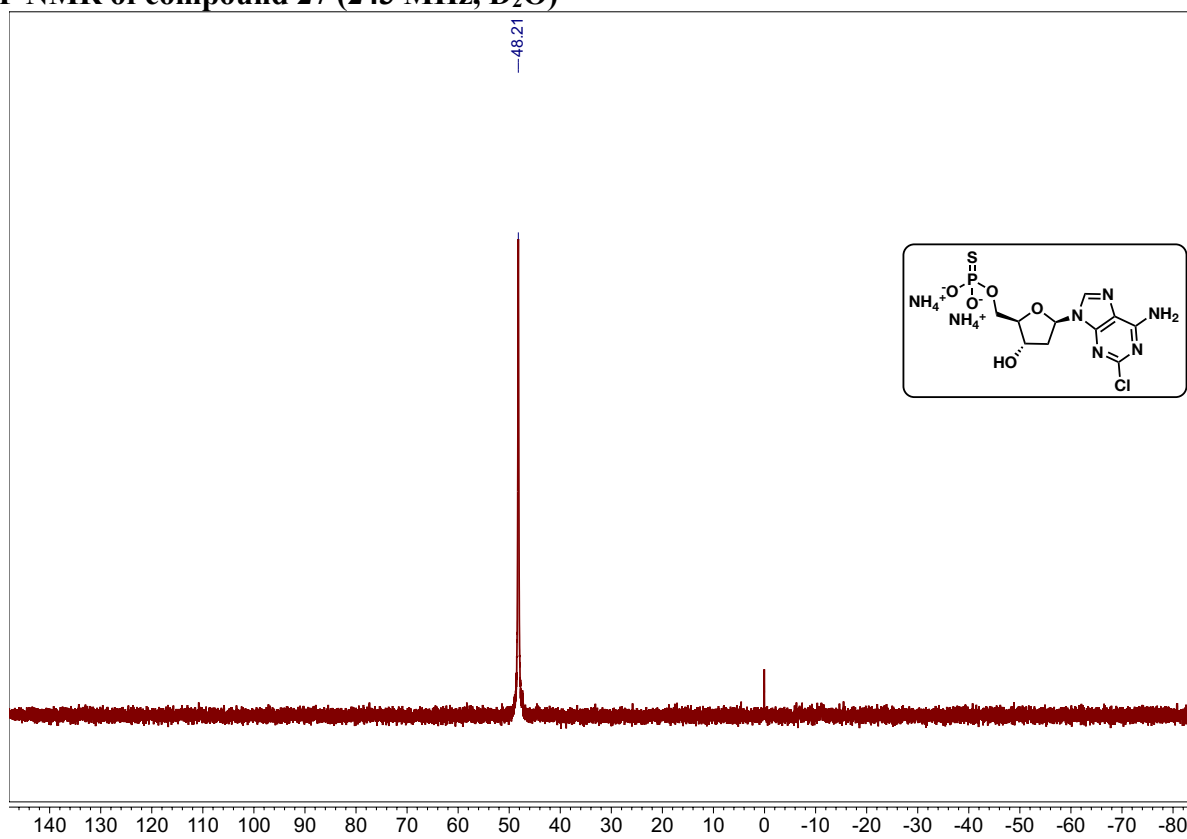
^1H NMR of compound 27 (600 MHz, D_2O)



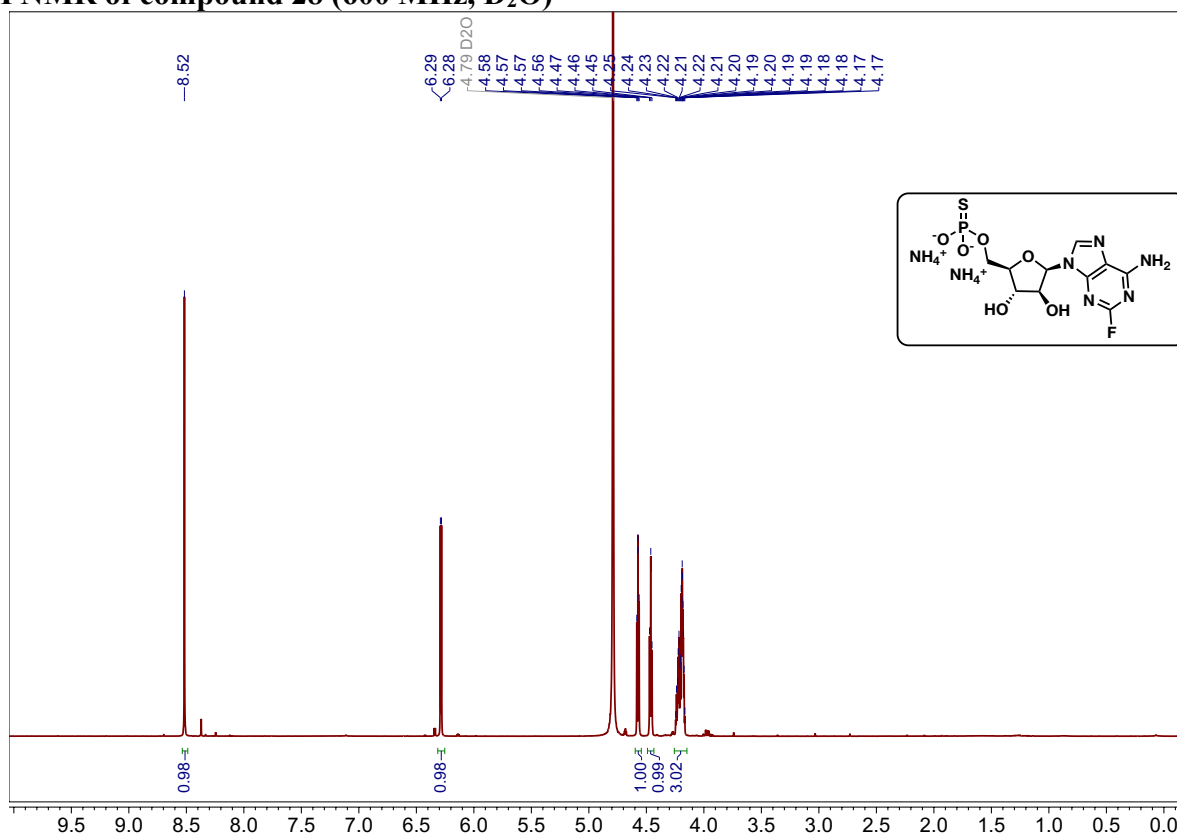
^{13}C NMR of compound 27 (151 MHz, D_2O)



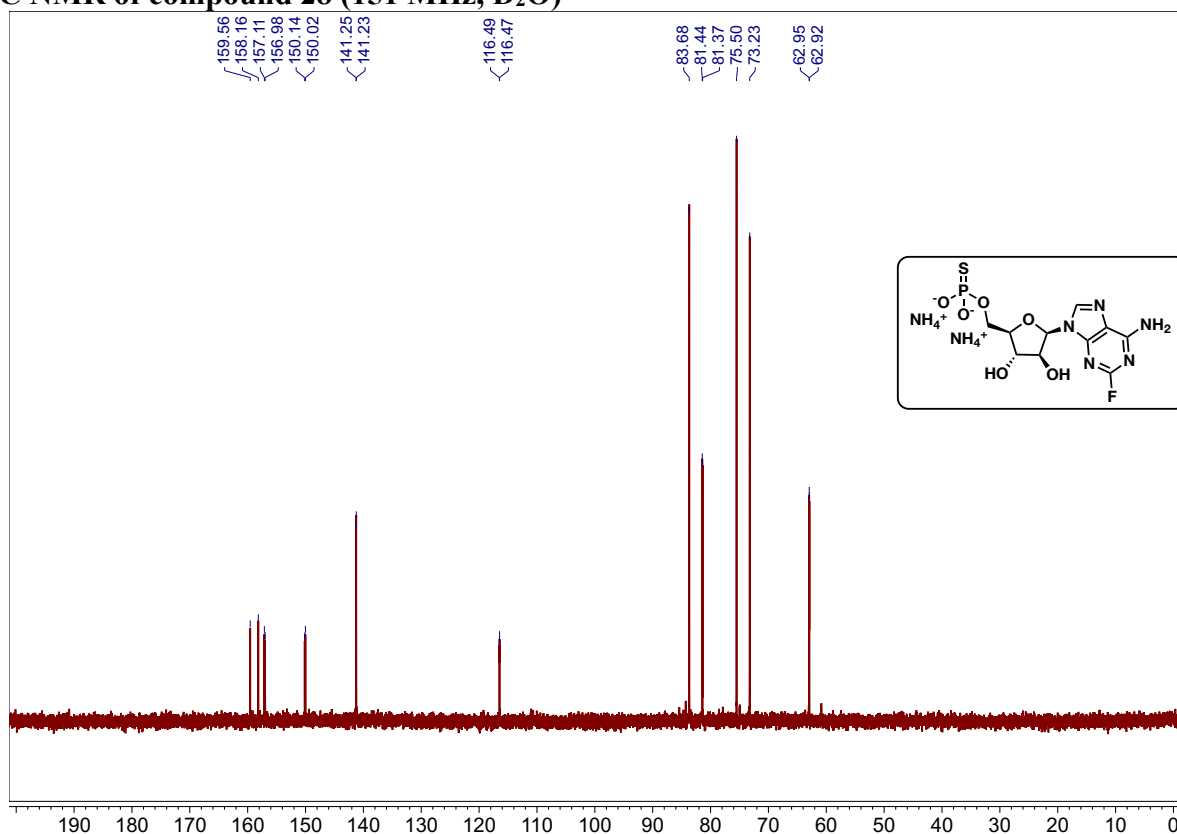
^{31}P NMR of compound 27 (243 MHz, D_2O)



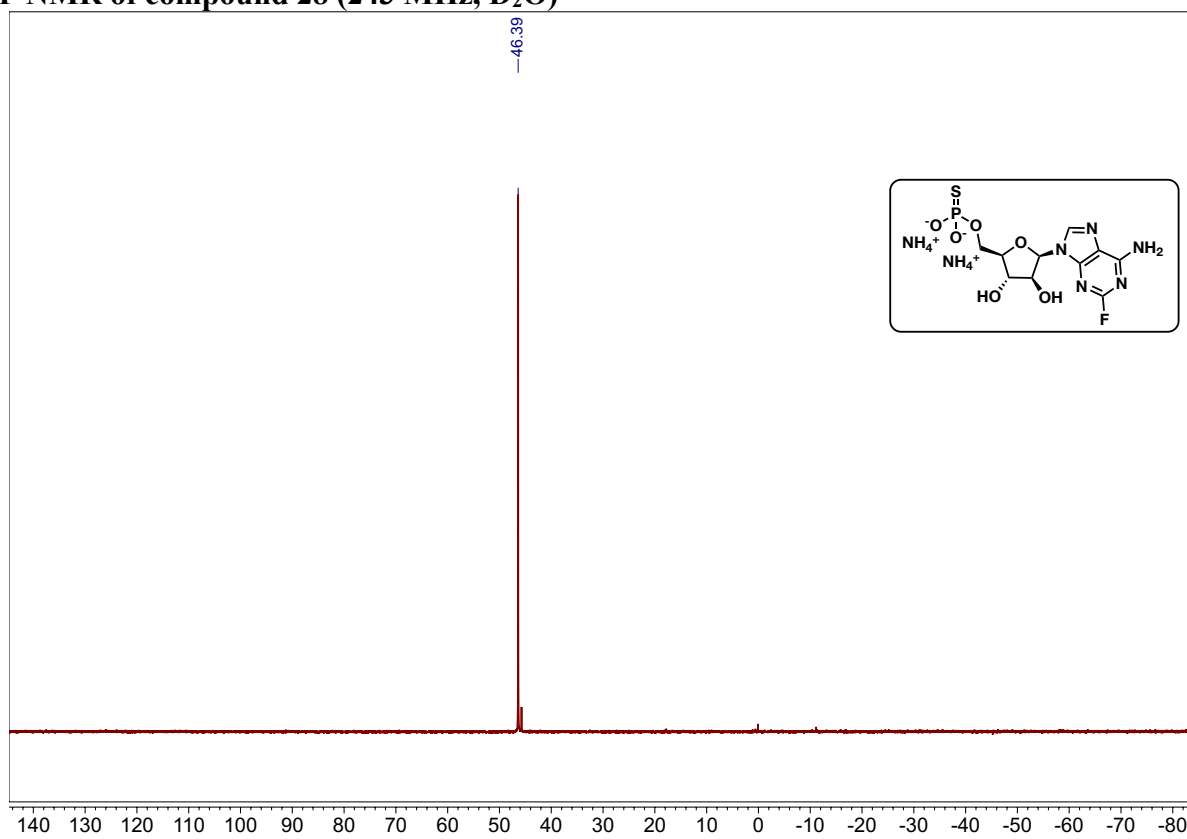
¹H NMR of compound 28 (600 MHz, D₂O)



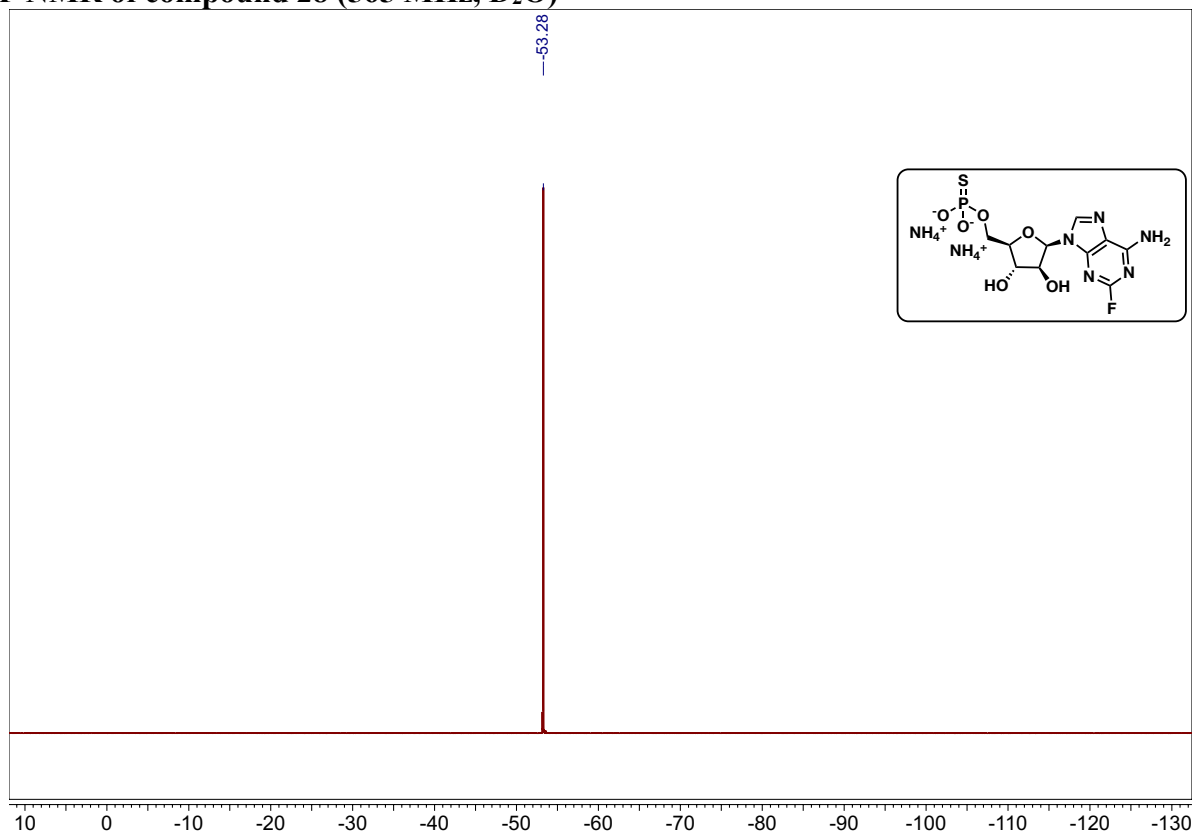
¹³C NMR of compound 28 (151 MHz, D₂O)



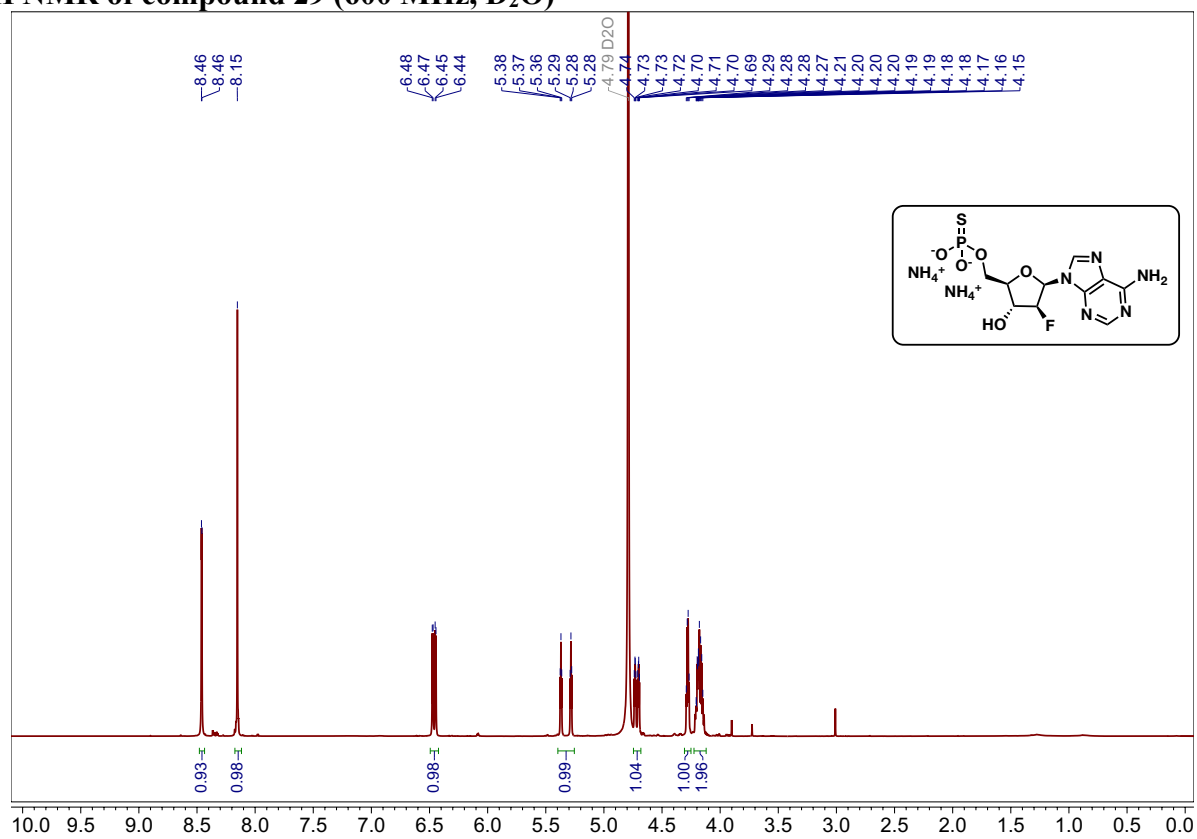
^{31}P NMR of compound 28 (243 MHz, D_2O)



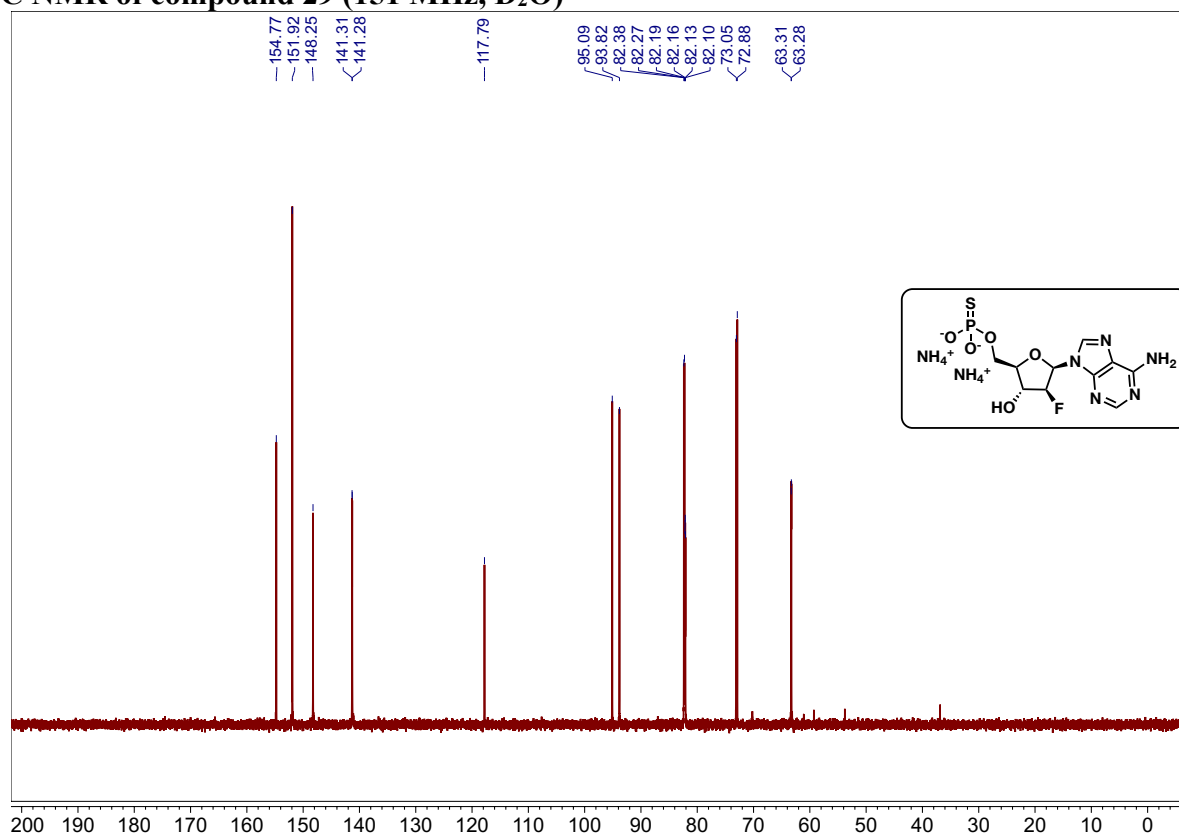
^{19}F NMR of compound 28 (565 MHz, D_2O)



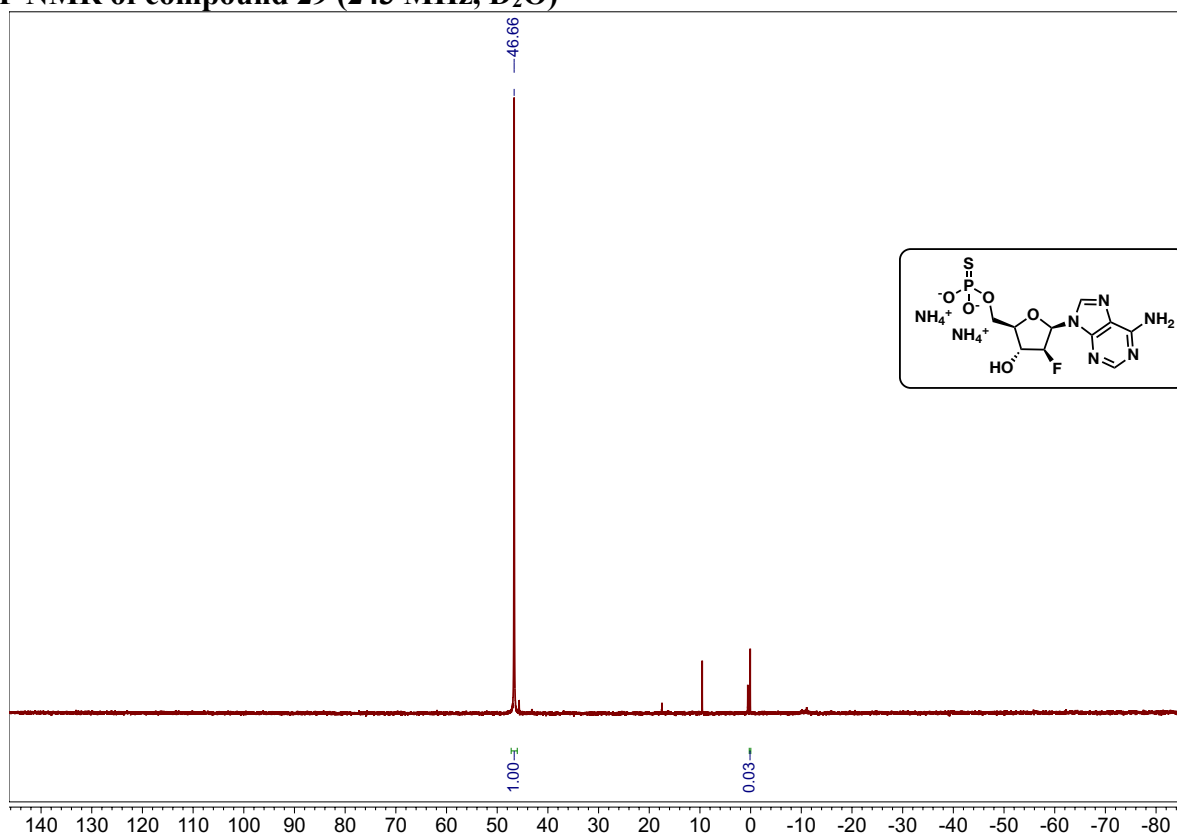
^1H NMR of compound 29 (600 MHz, D_2O)



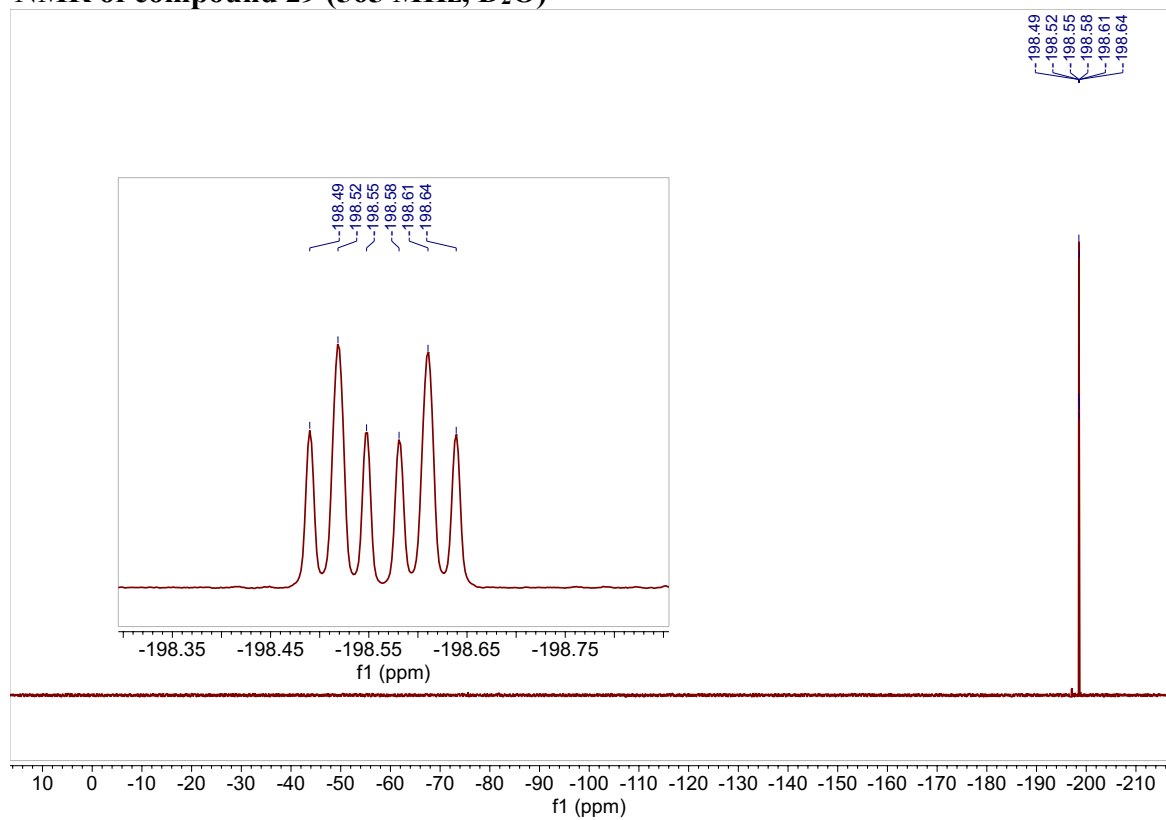
^{13}C NMR of compound 29 (151 MHz, D_2O)



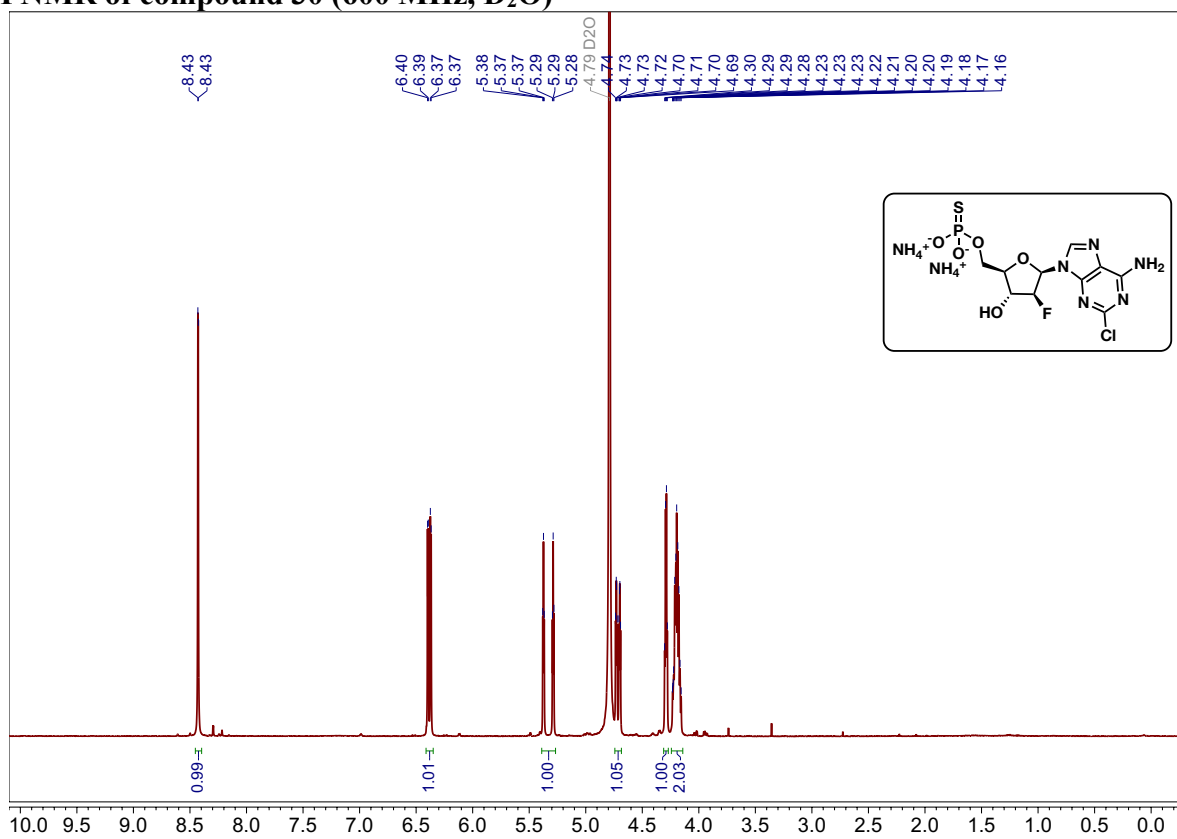
^{31}P NMR of compound 29 (243 MHz, D_2O)



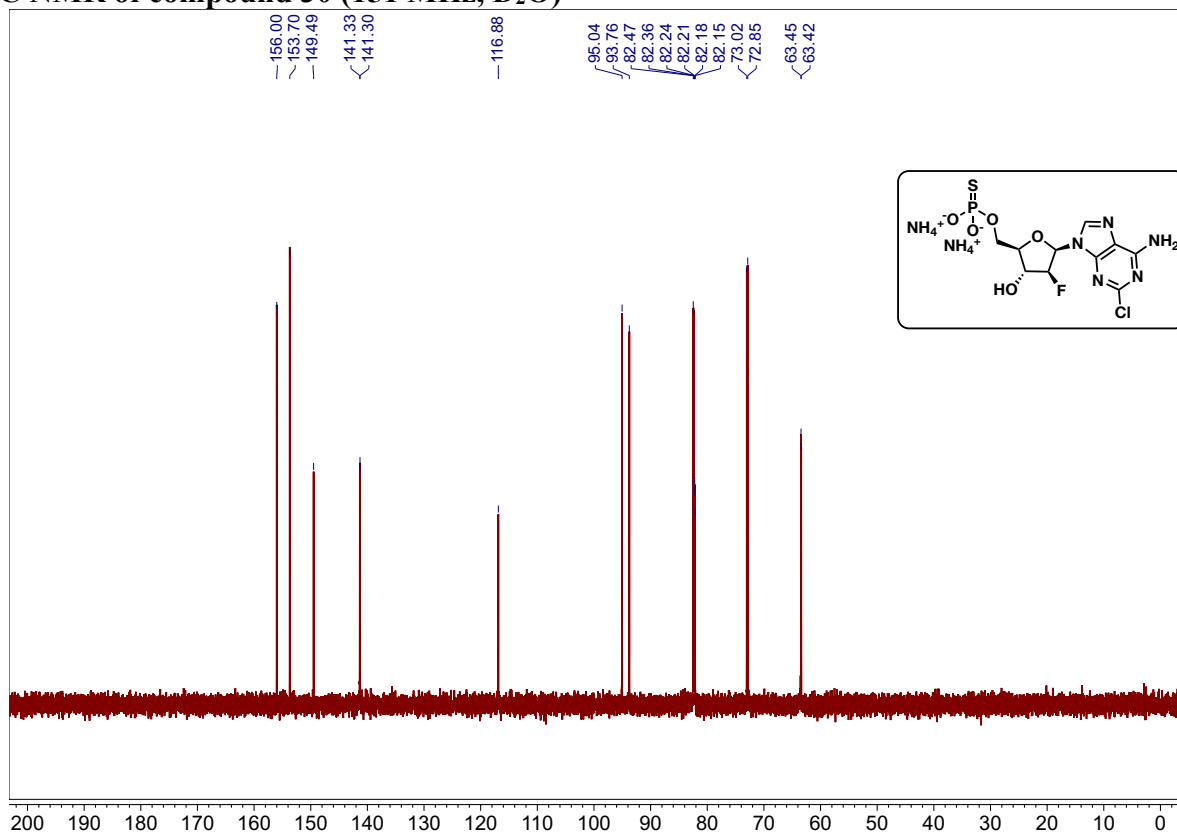
^{19}F NMR of compound 29 (565 MHz, D_2O)



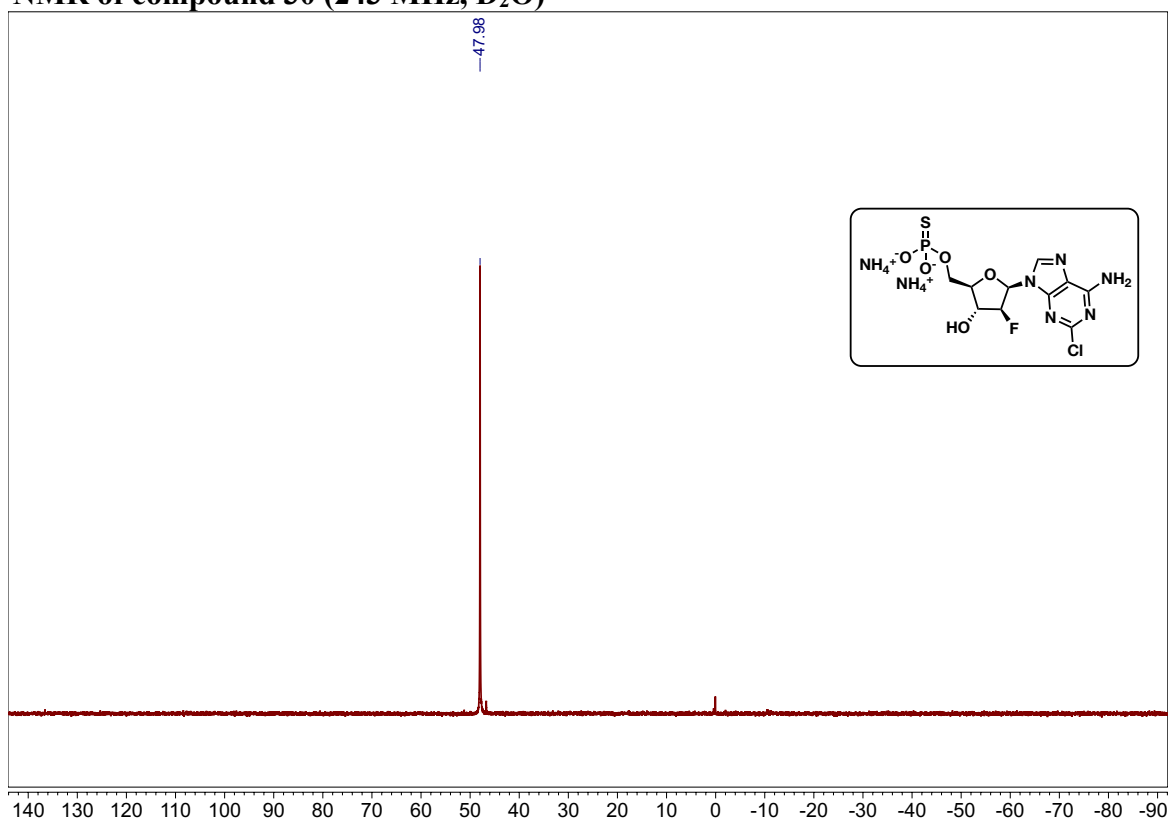
¹H NMR of compound 30 (600 MHz, D₂O)



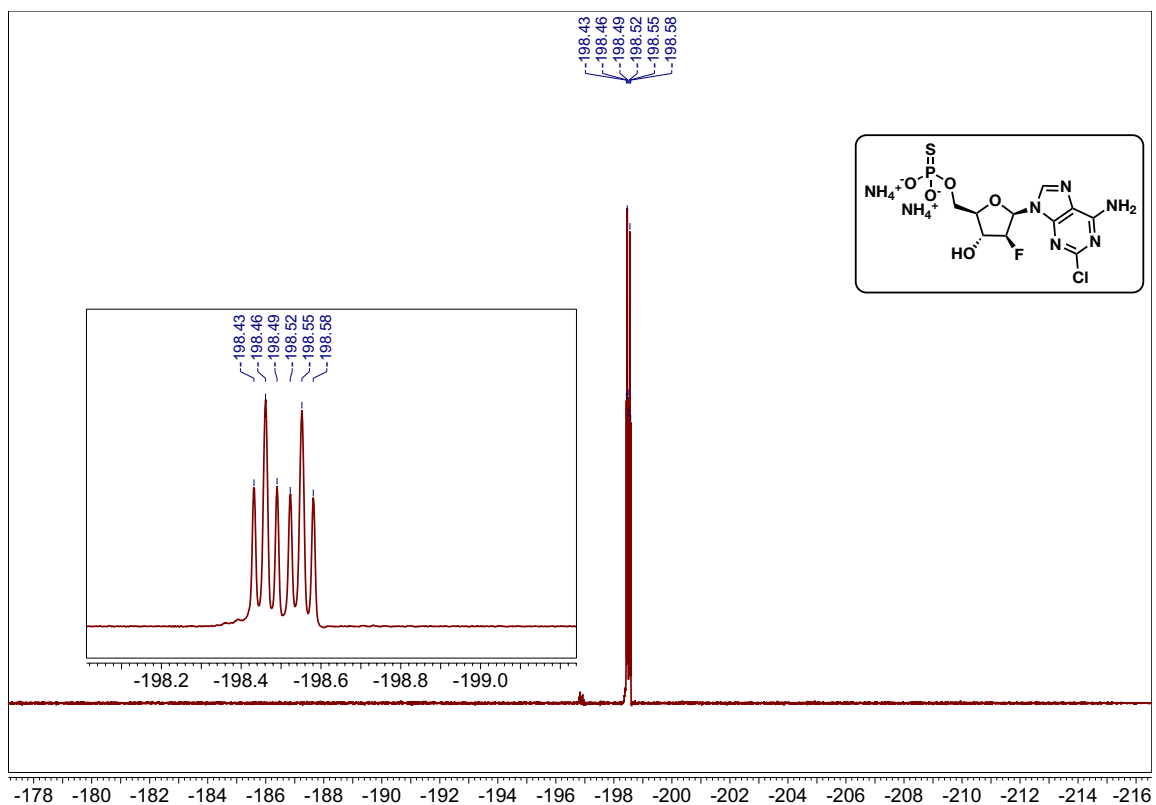
¹³C NMR of compound 30 (151 MHz, D₂O)



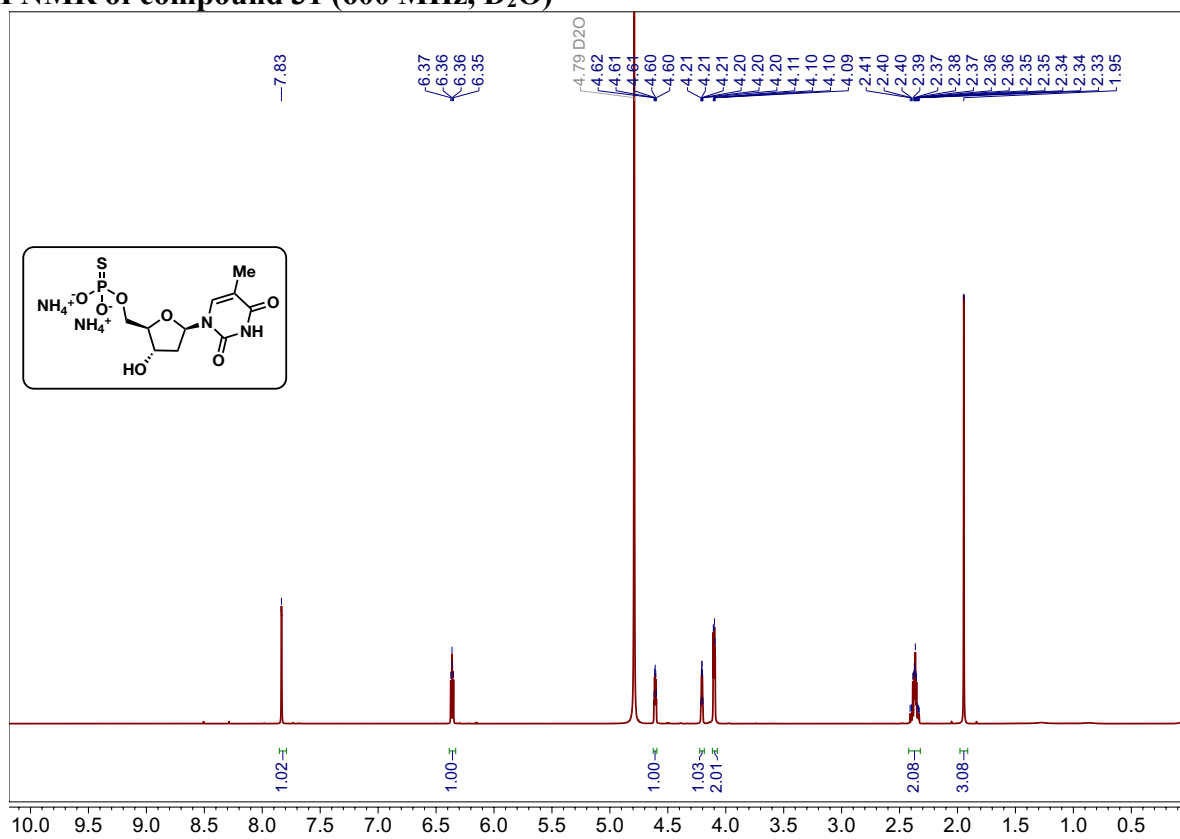
^{31}P NMR of compound 30 (243 MHz, D_2O)



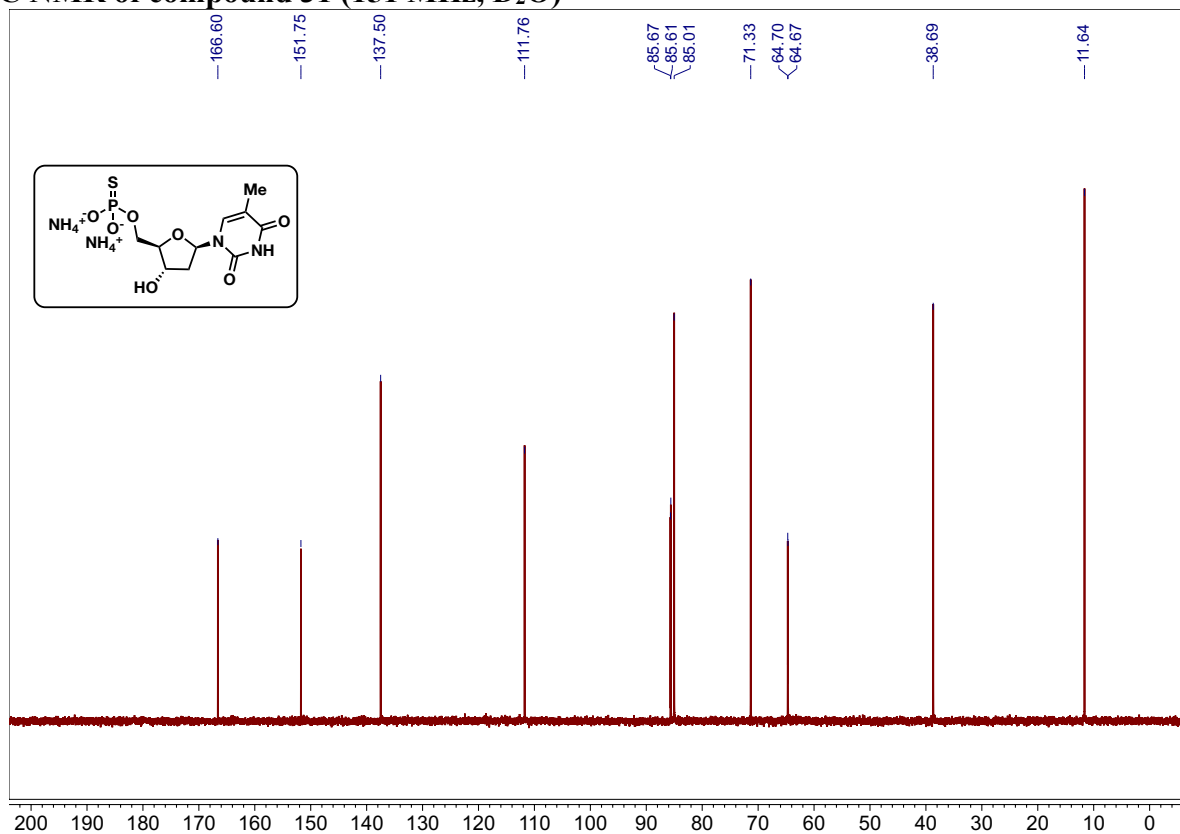
^{19}F NMR of compound 30 (565 MHz, D_2O)



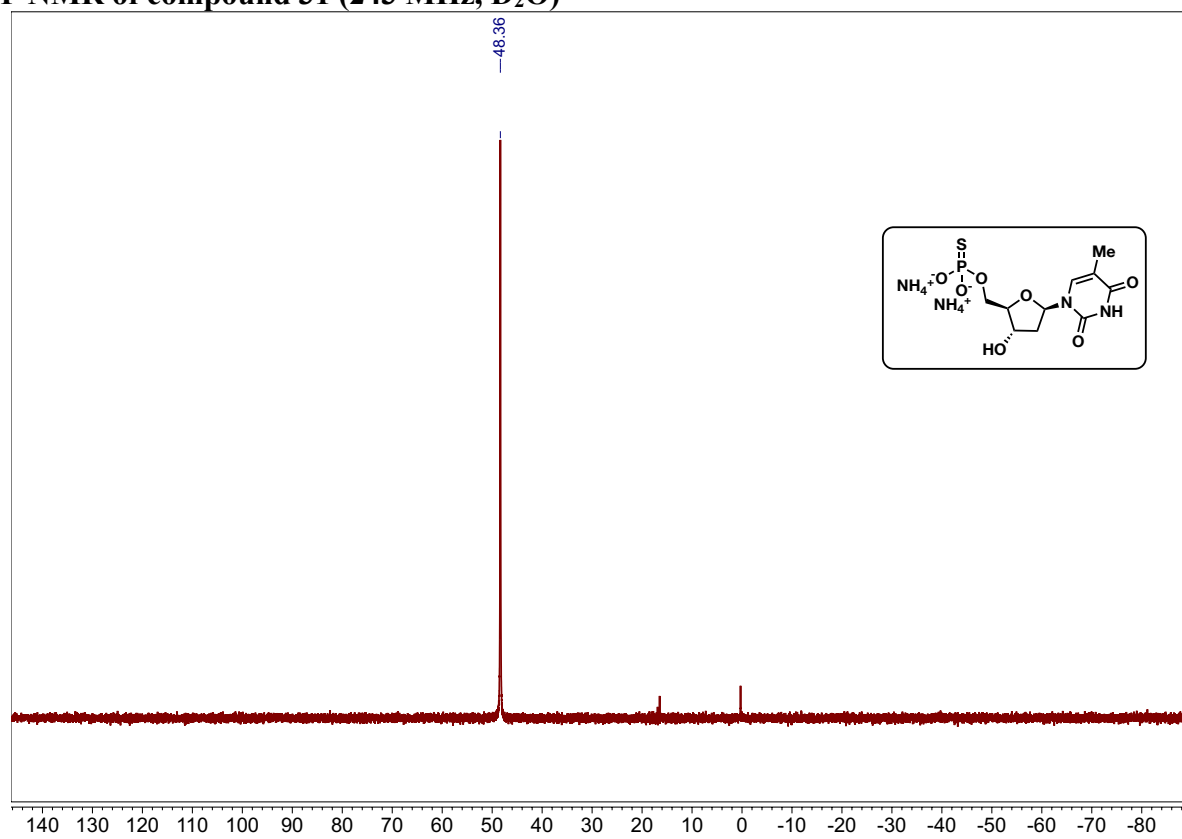
¹H NMR of compound 31 (600 MHz, D₂O)



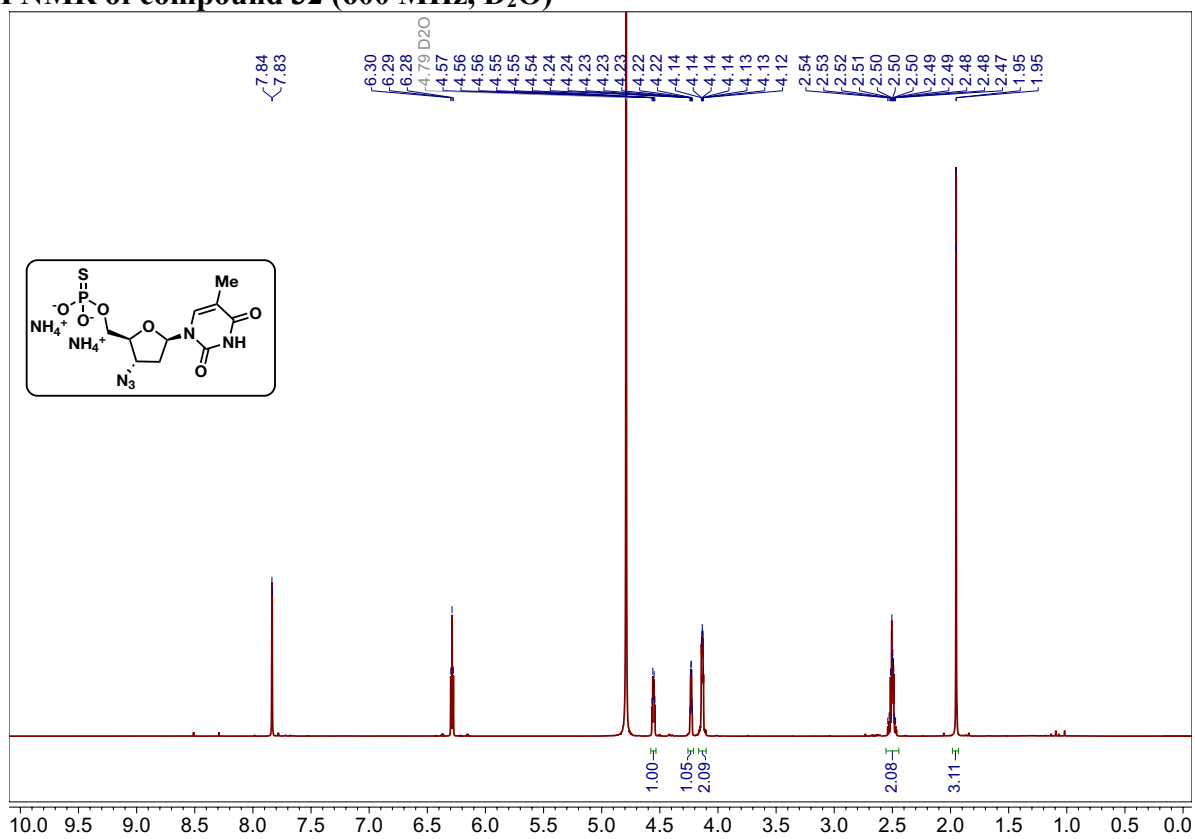
¹³C NMR of compound 31 (151 MHz, D₂O)



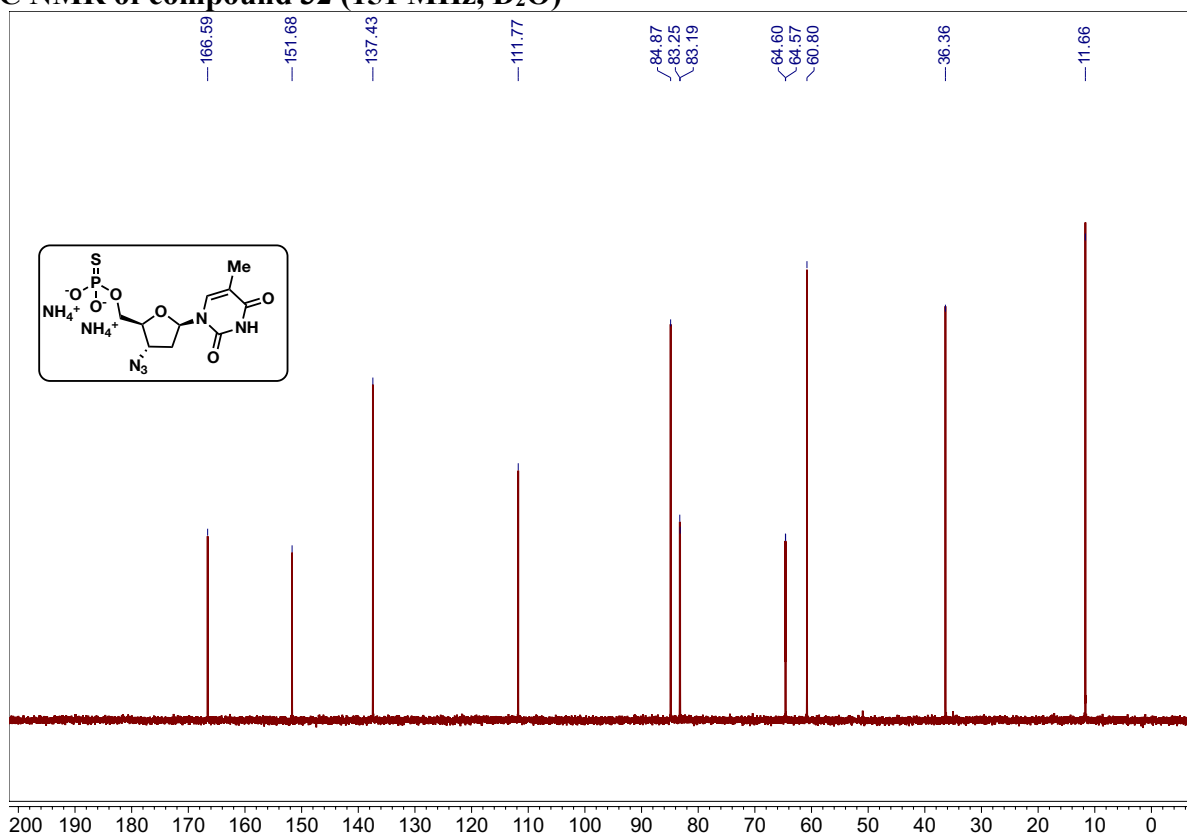
^{31}P NMR of compound 31 (243 MHz, D_2O)



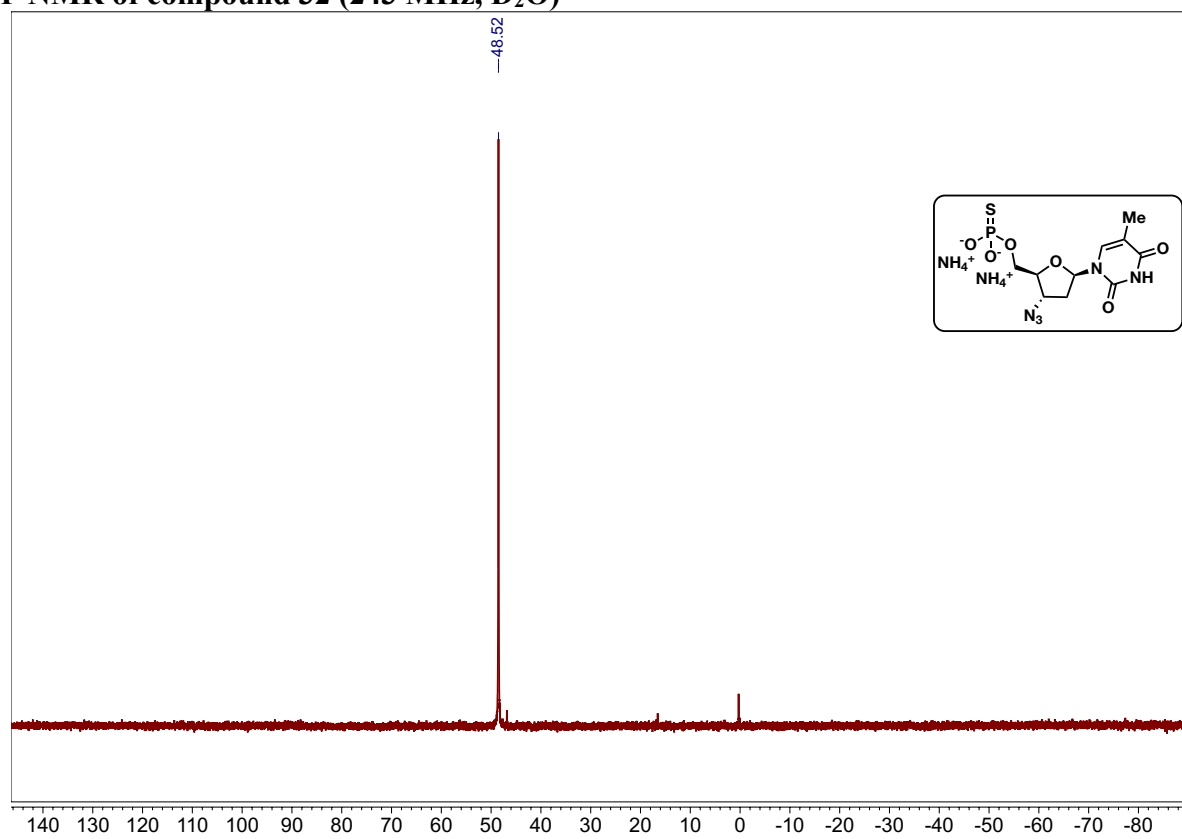
¹H NMR of compound 32 (600 MHz, D₂O)



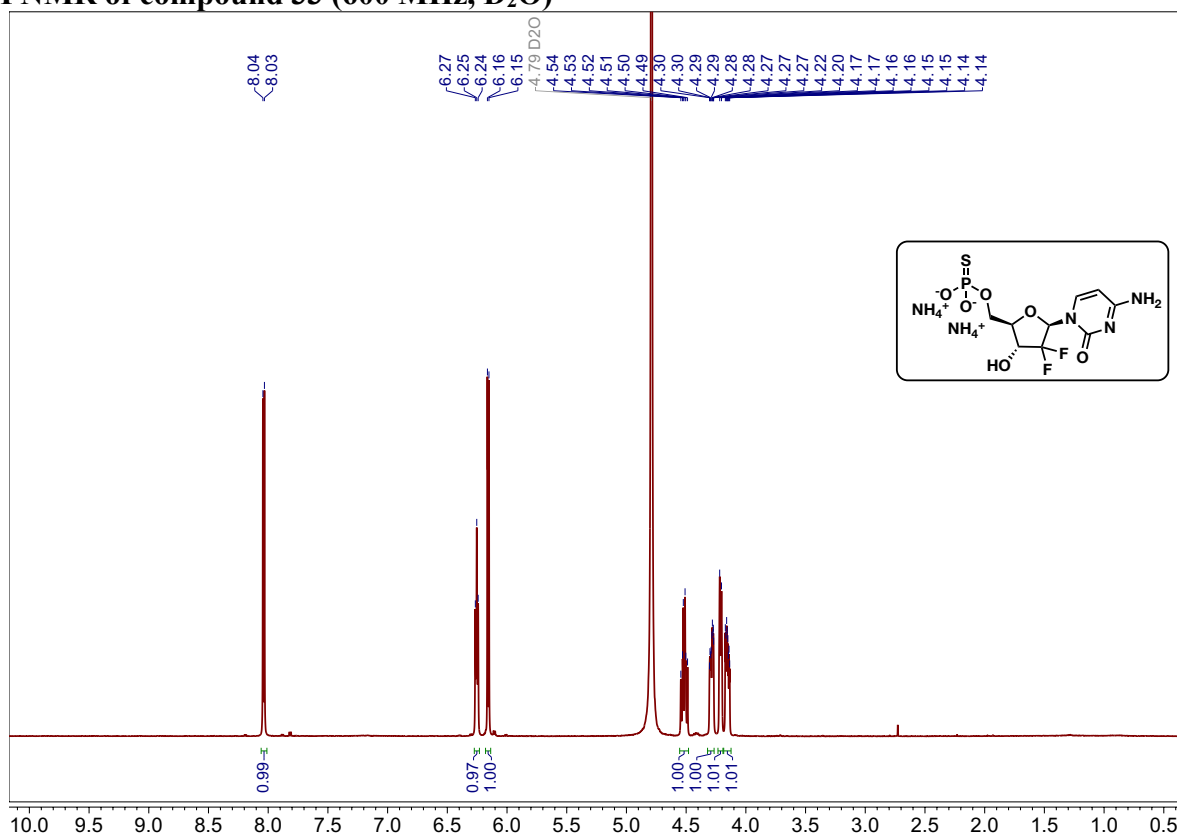
¹³C NMR of compound 32 (151 MHz, D₂O)



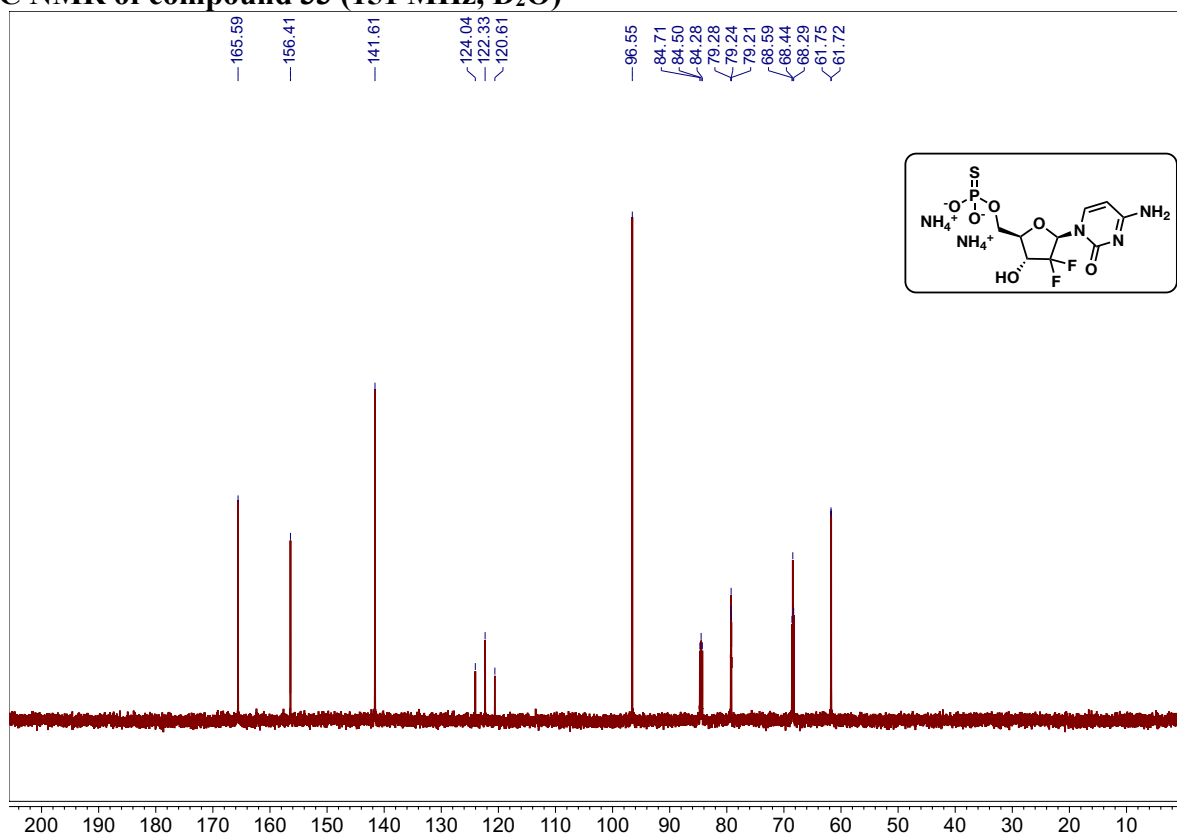
^{31}P NMR of compound 32 (243 MHz, D_2O)



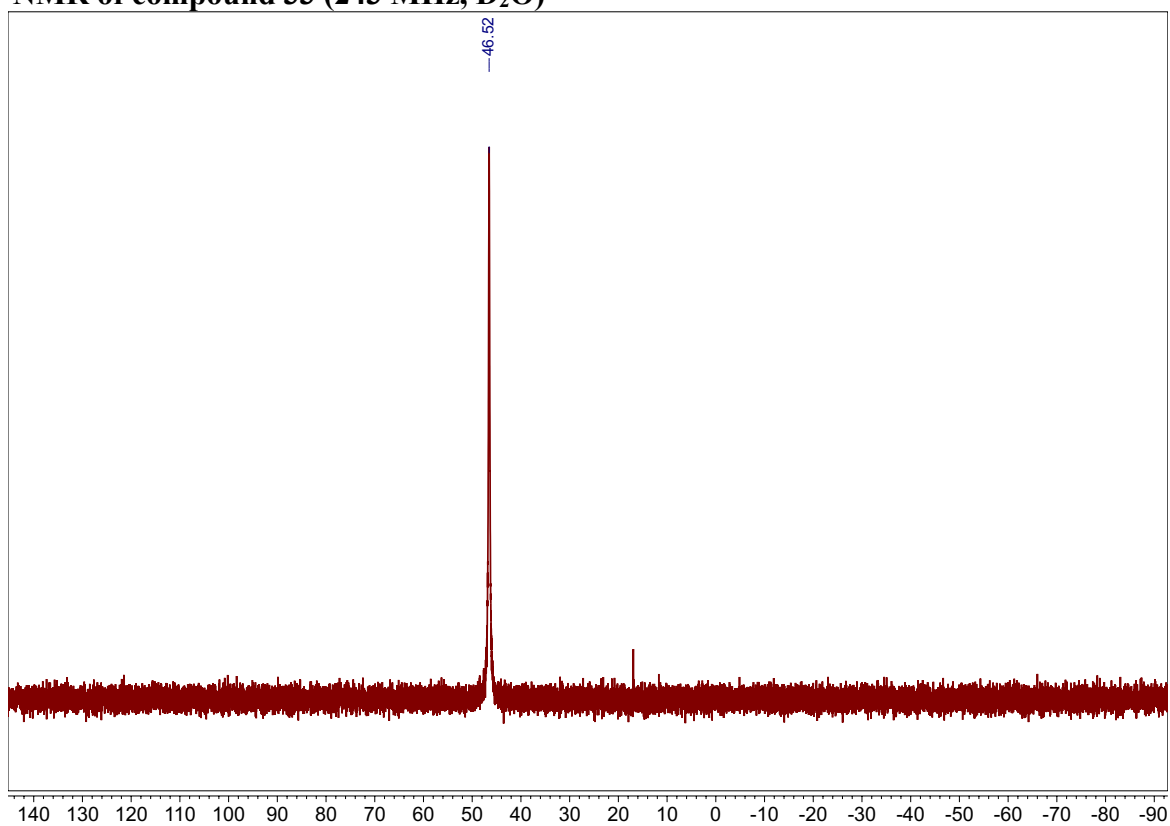
¹H NMR of compound 33 (600 MHz, D₂O)



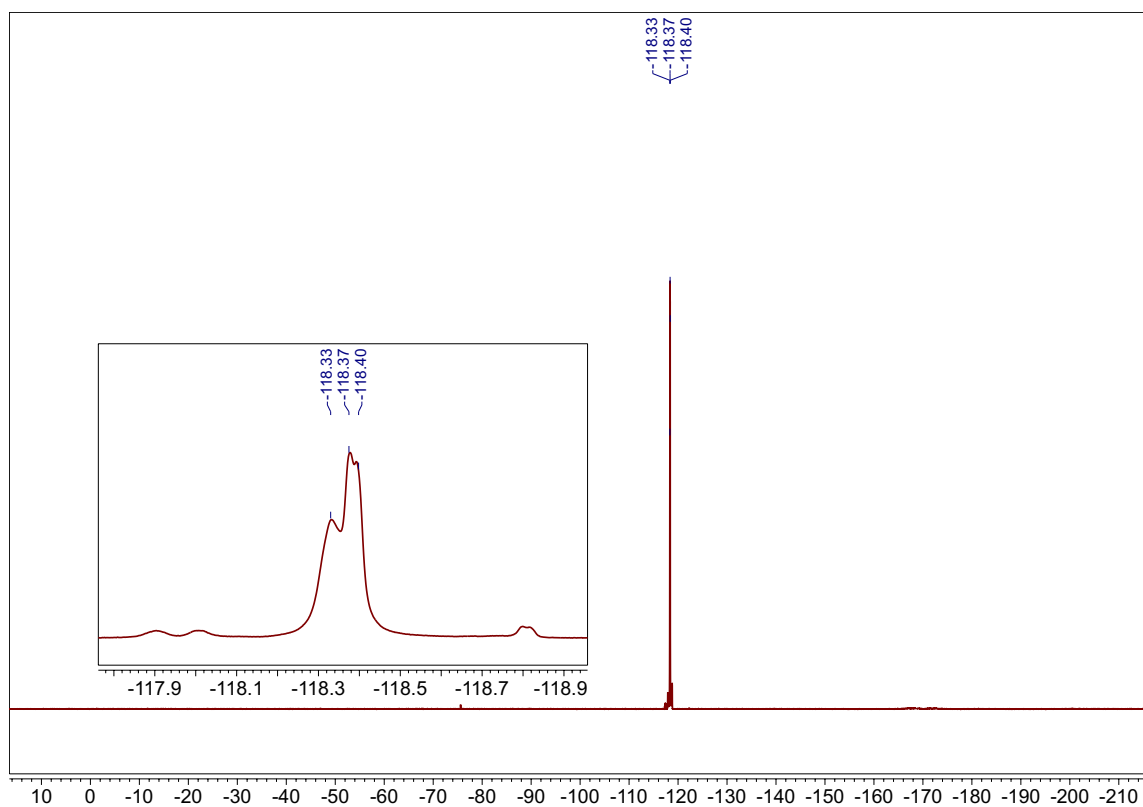
¹³C NMR of compound 33 (151 MHz, D₂O)



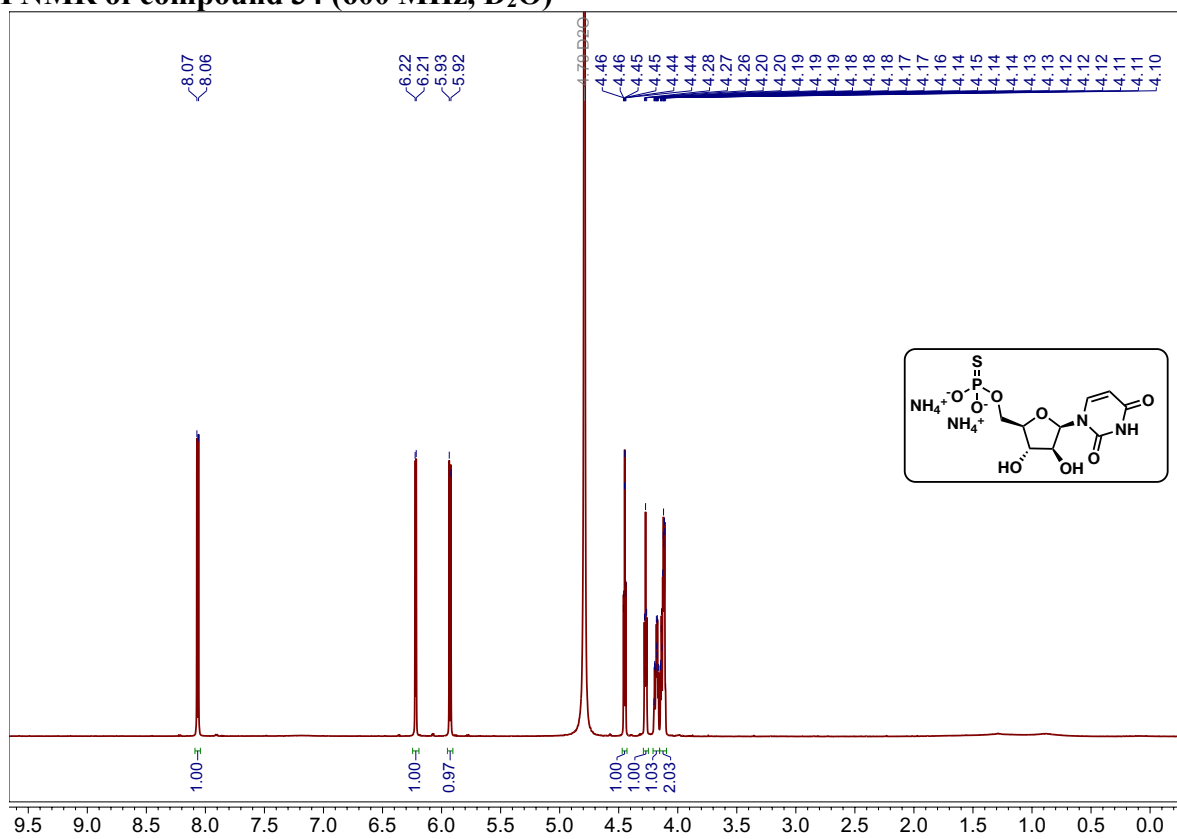
^{31}P NMR of compound 33 (243 MHz, D_2O)



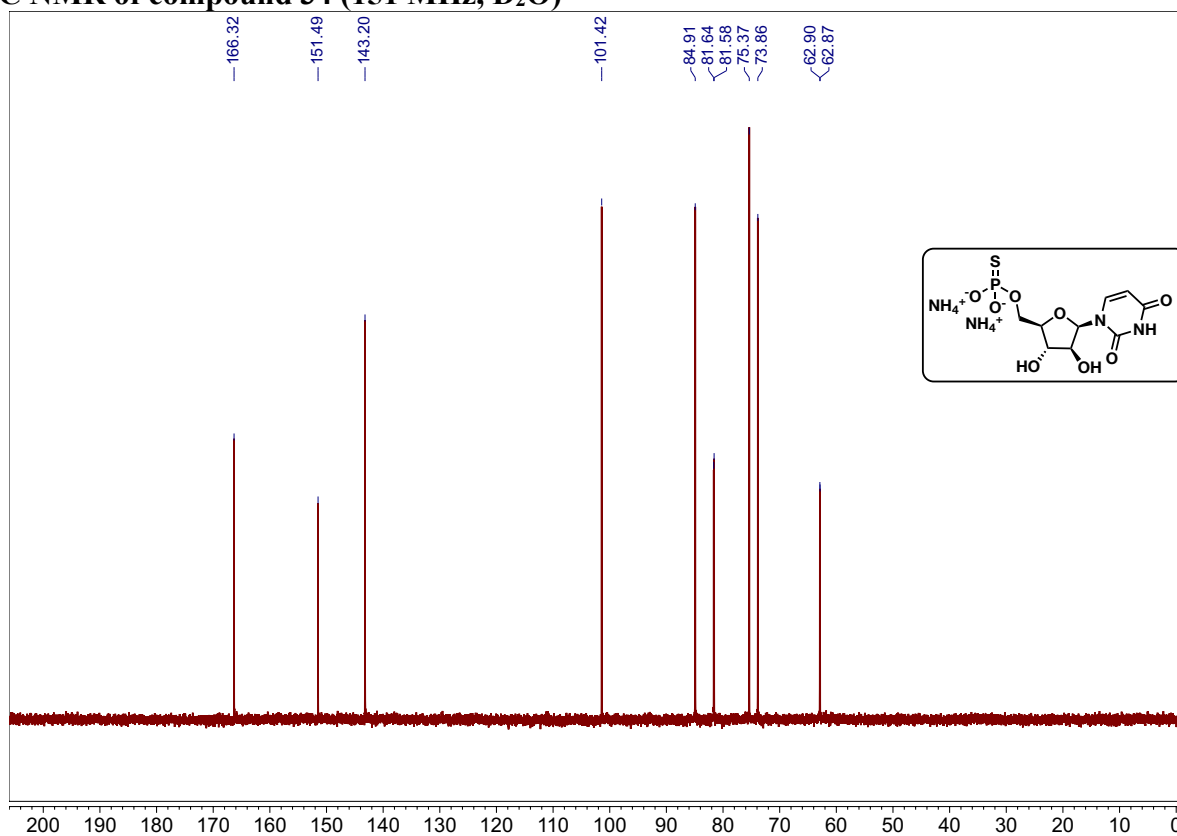
^{19}F NMR of compound 33 (565 MHz, D_2O)



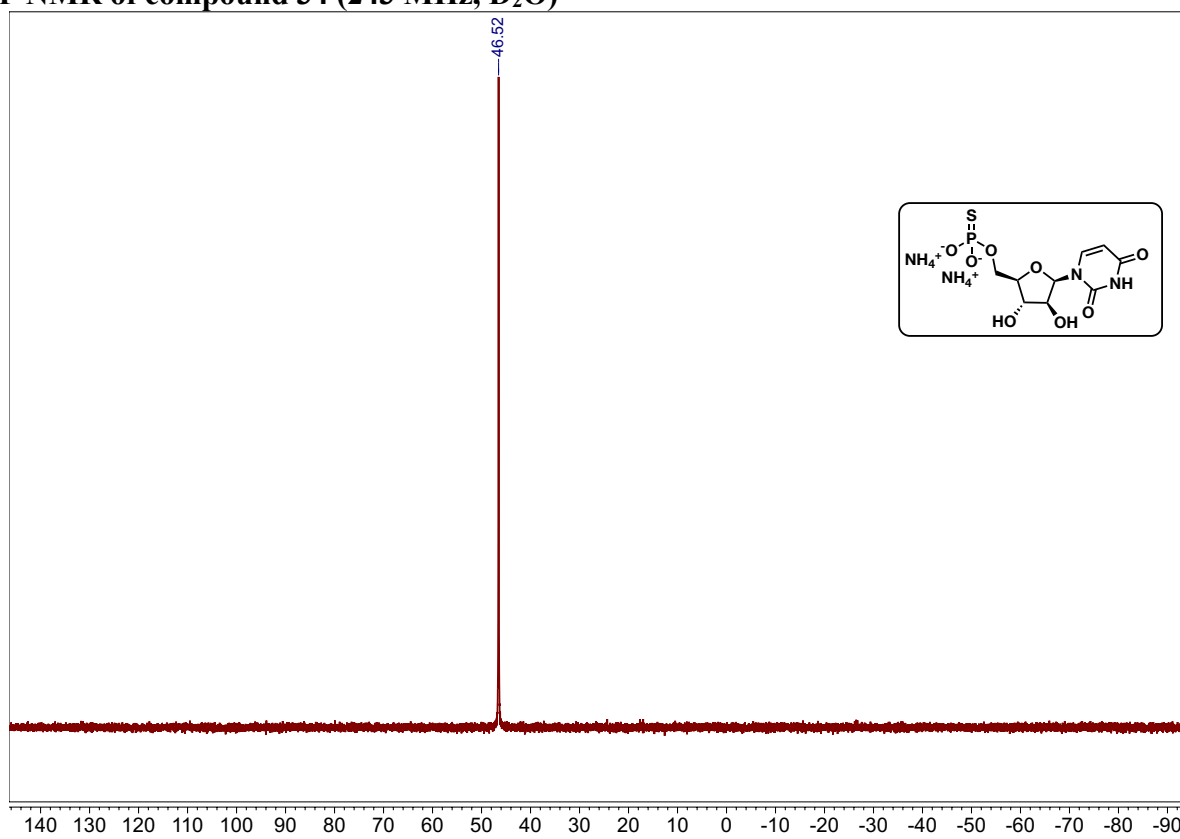
¹H NMR of compound 34 (600 MHz, D₂O)



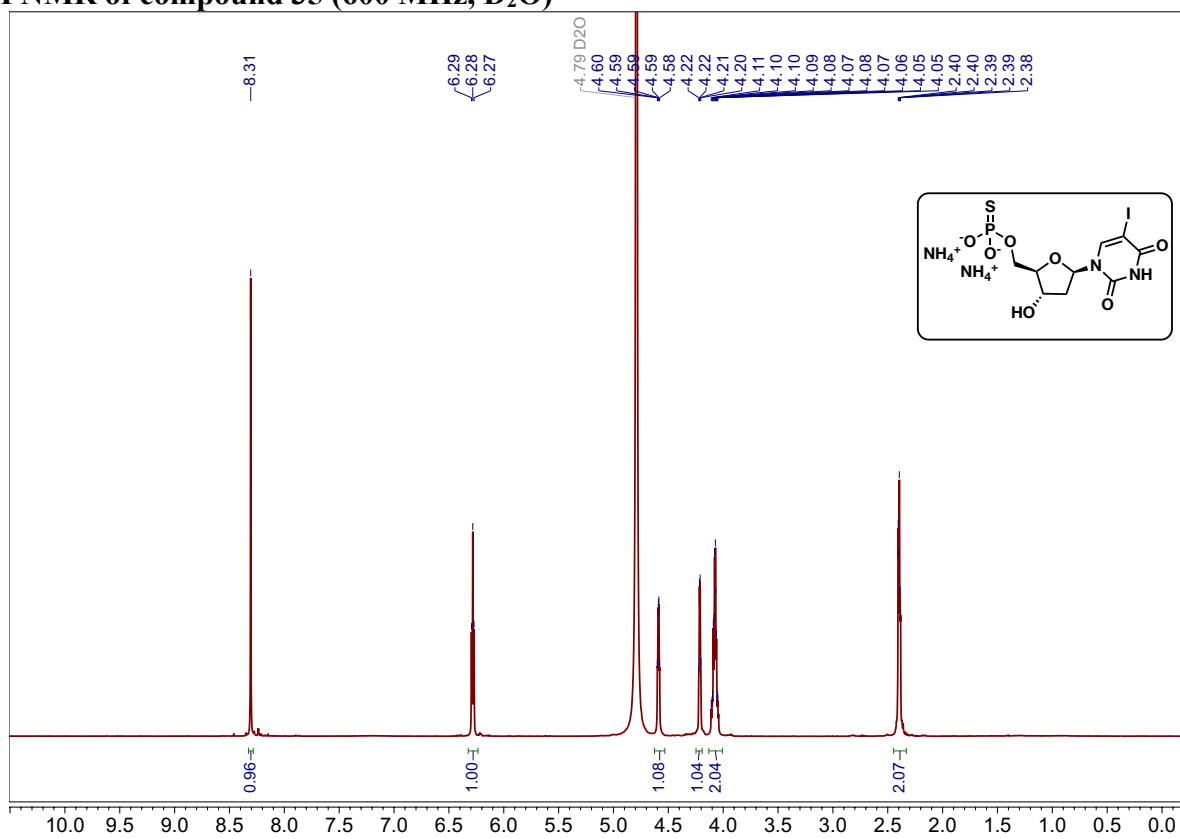
¹³C NMR of compound 34 (151 MHz, D₂O)



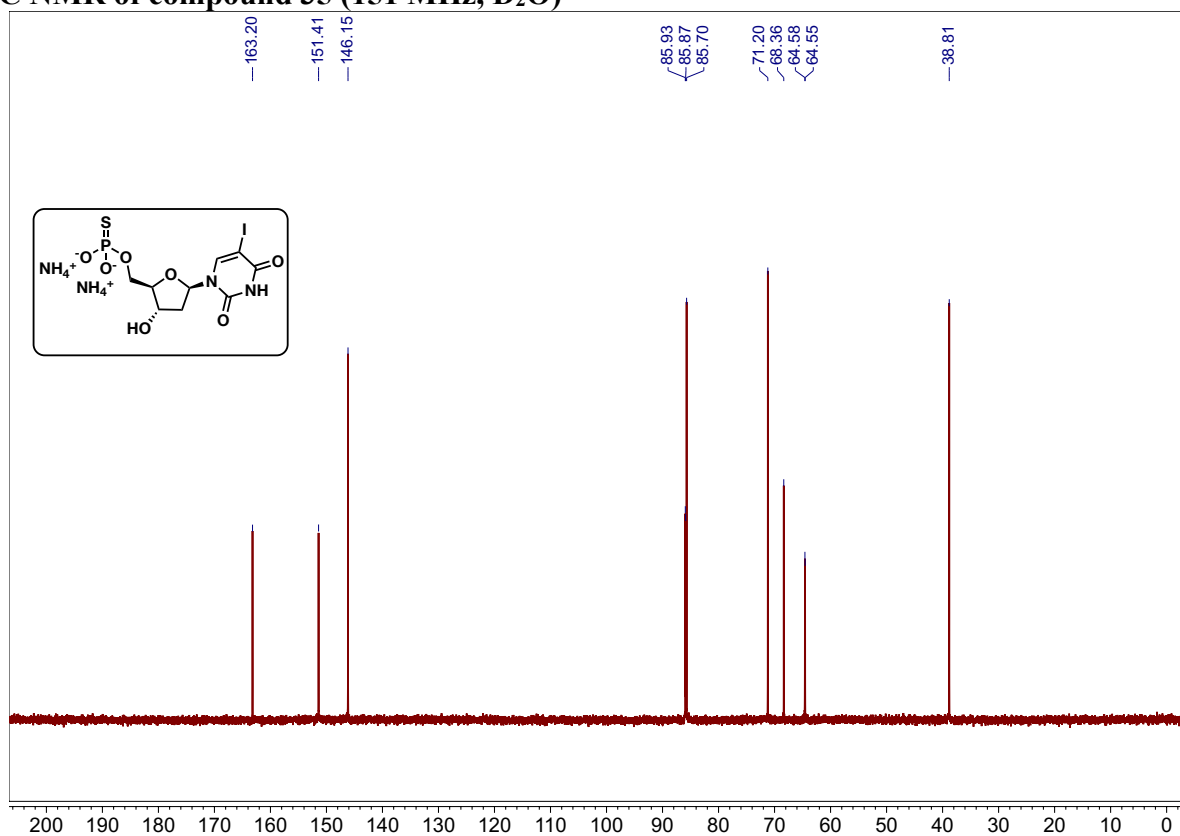
^{31}P NMR of compound 34 (243 MHz, D_2O)



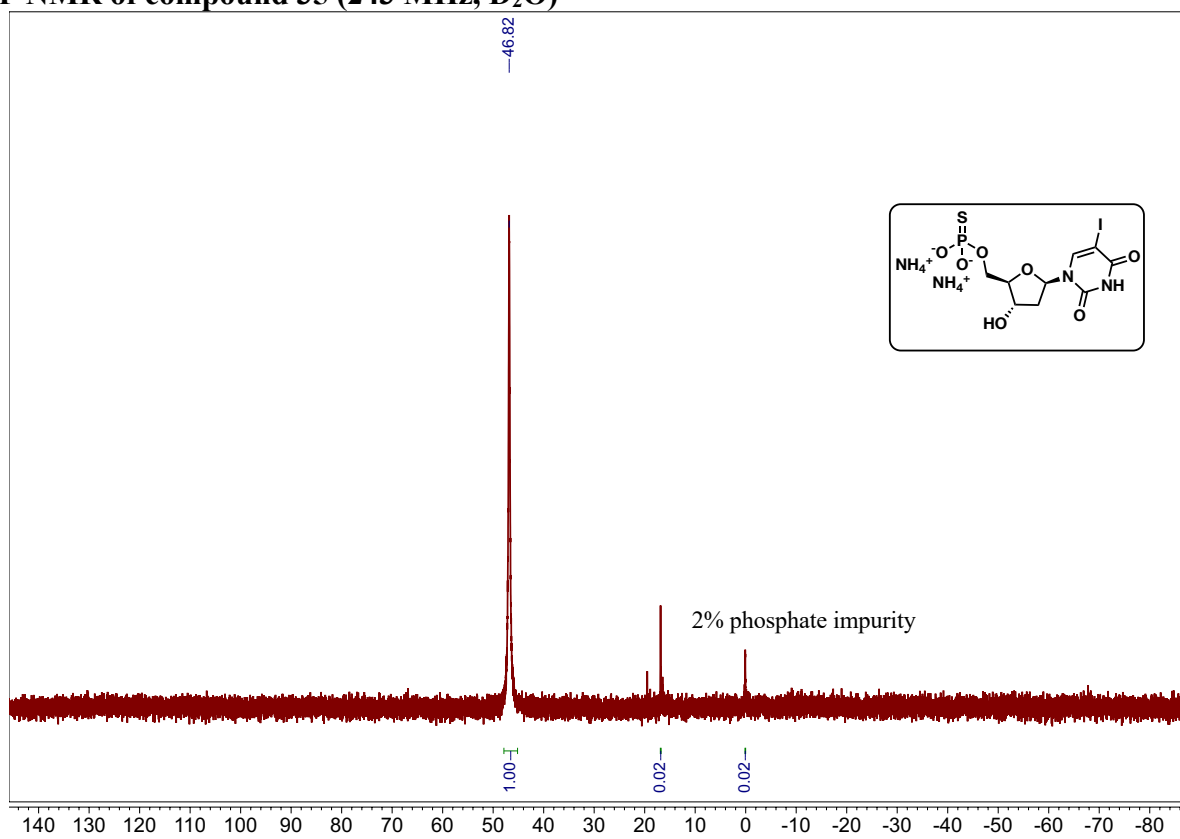
^1H NMR of compound 35 (600 MHz, D_2O)



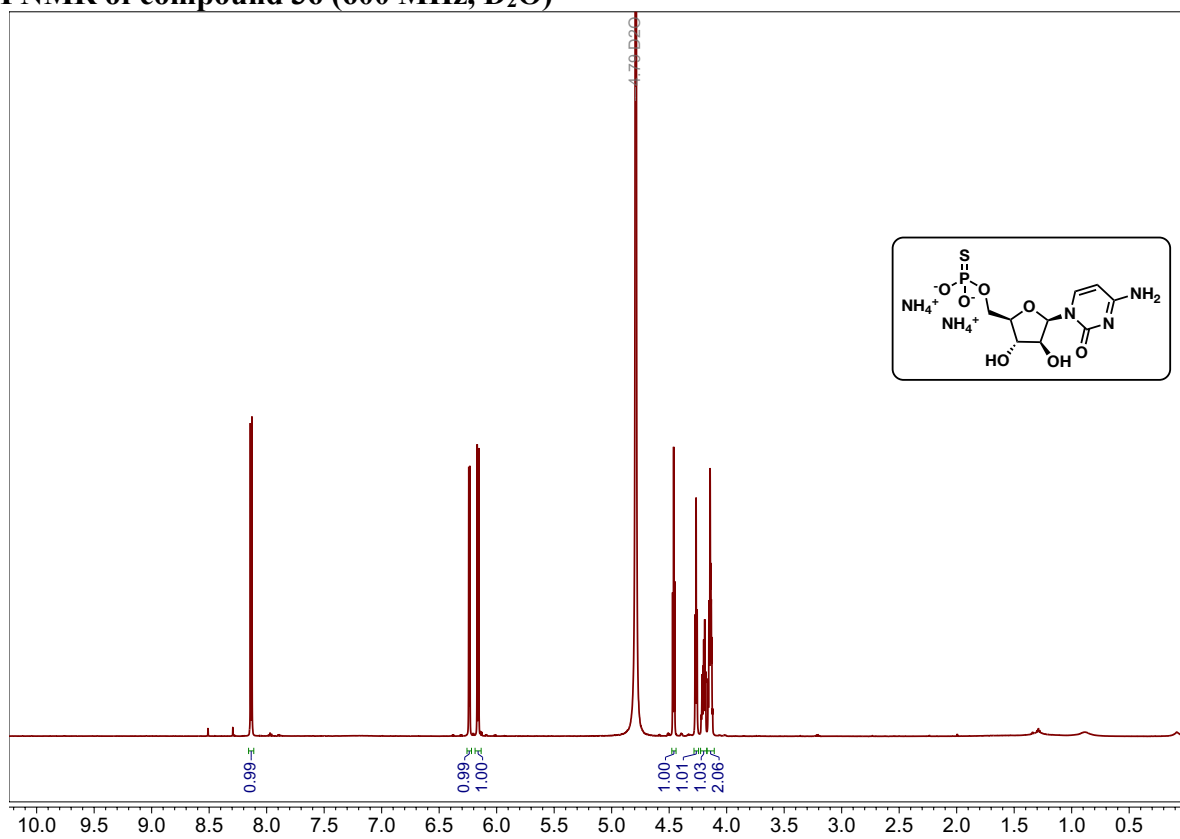
^{13}C NMR of compound 35 (151 MHz, D_2O)



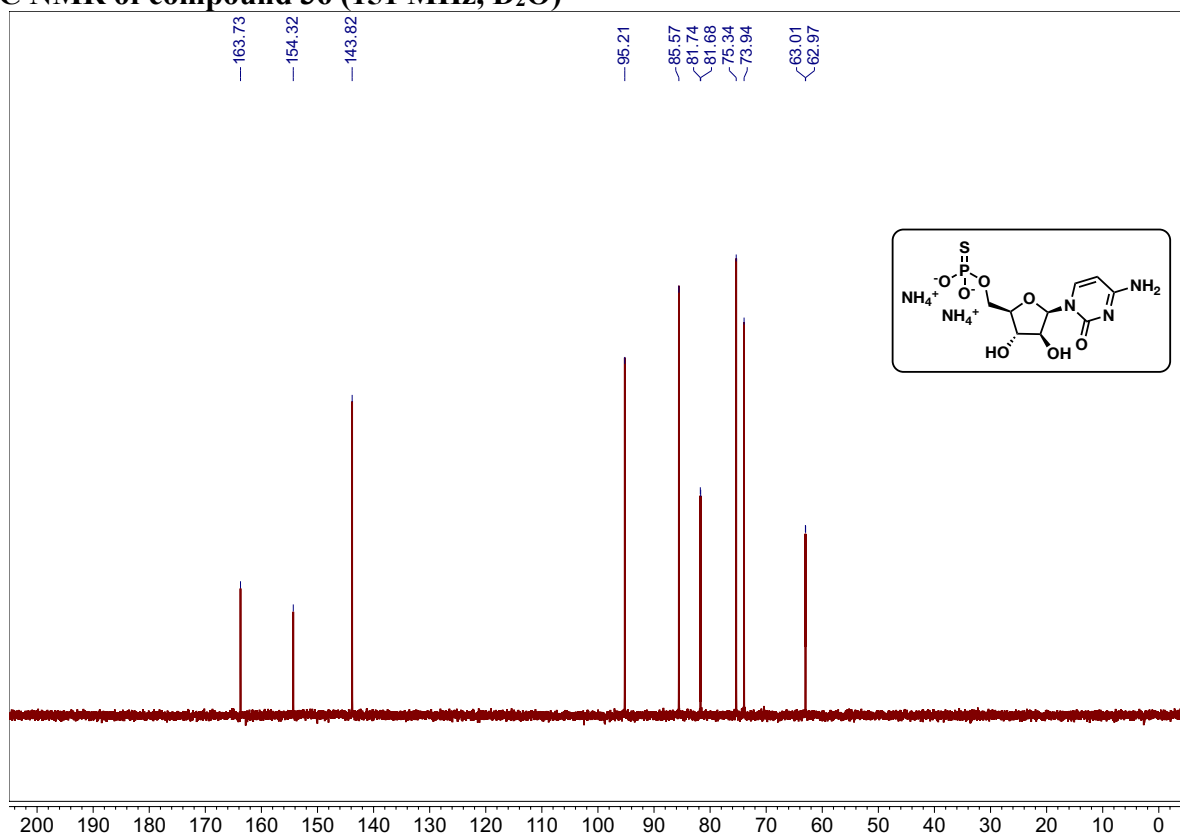
^{31}P NMR of compound 35 (243 MHz, D_2O)



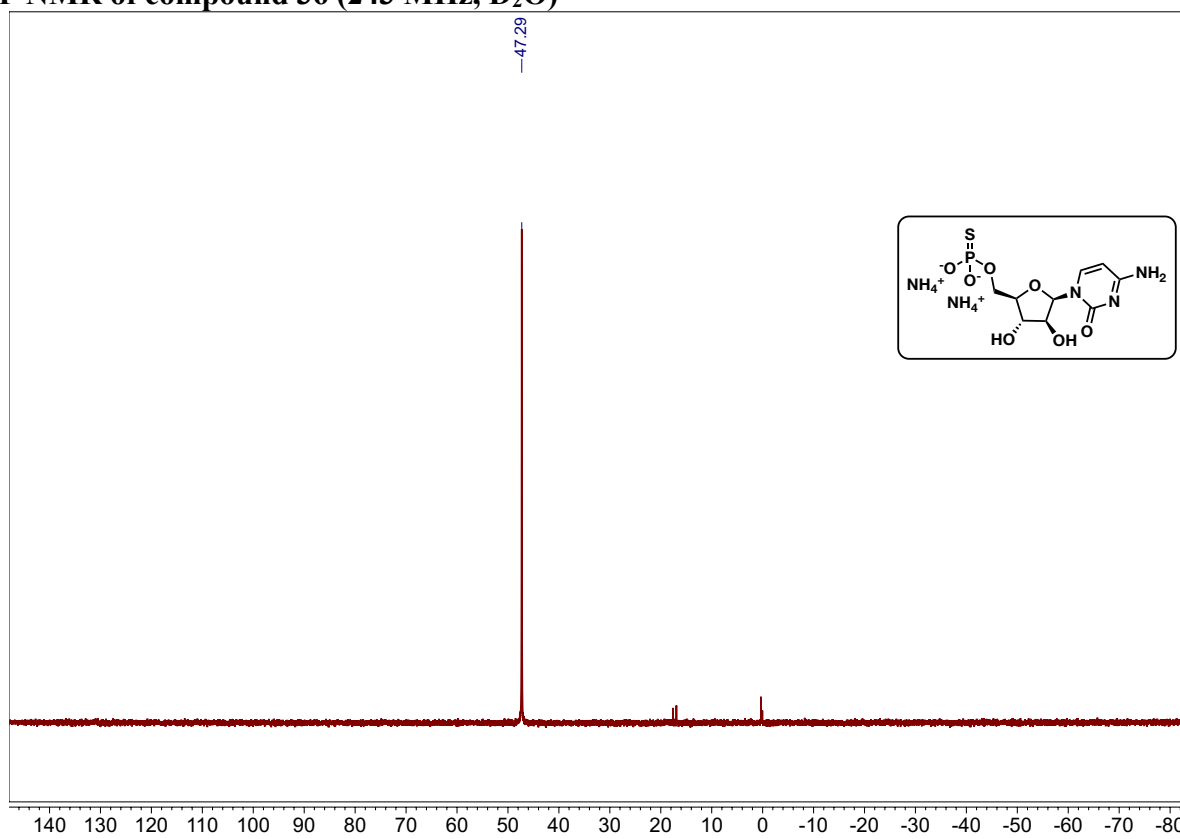
^1H NMR of compound 36 (600 MHz, D_2O)



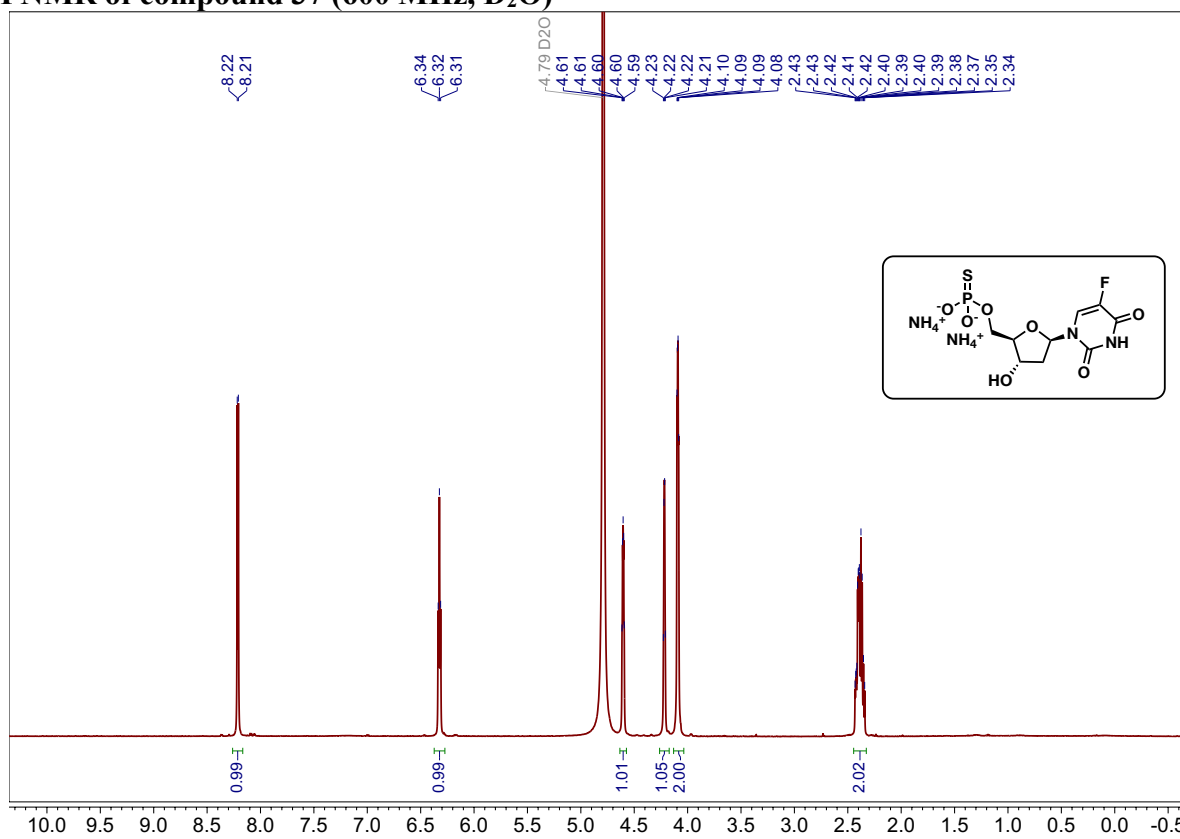
^{13}C NMR of compound 36 (151 MHz, D_2O)



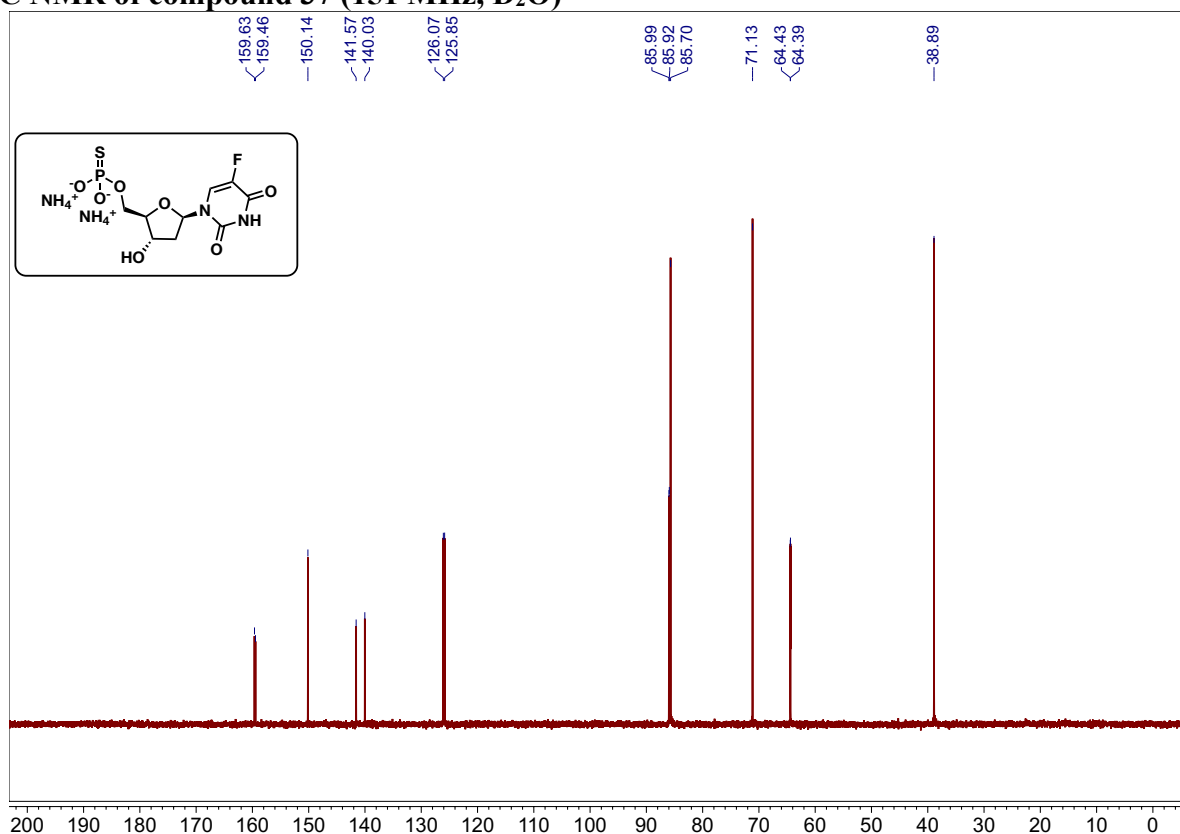
^{31}P NMR of compound 36 (243 MHz, D_2O)



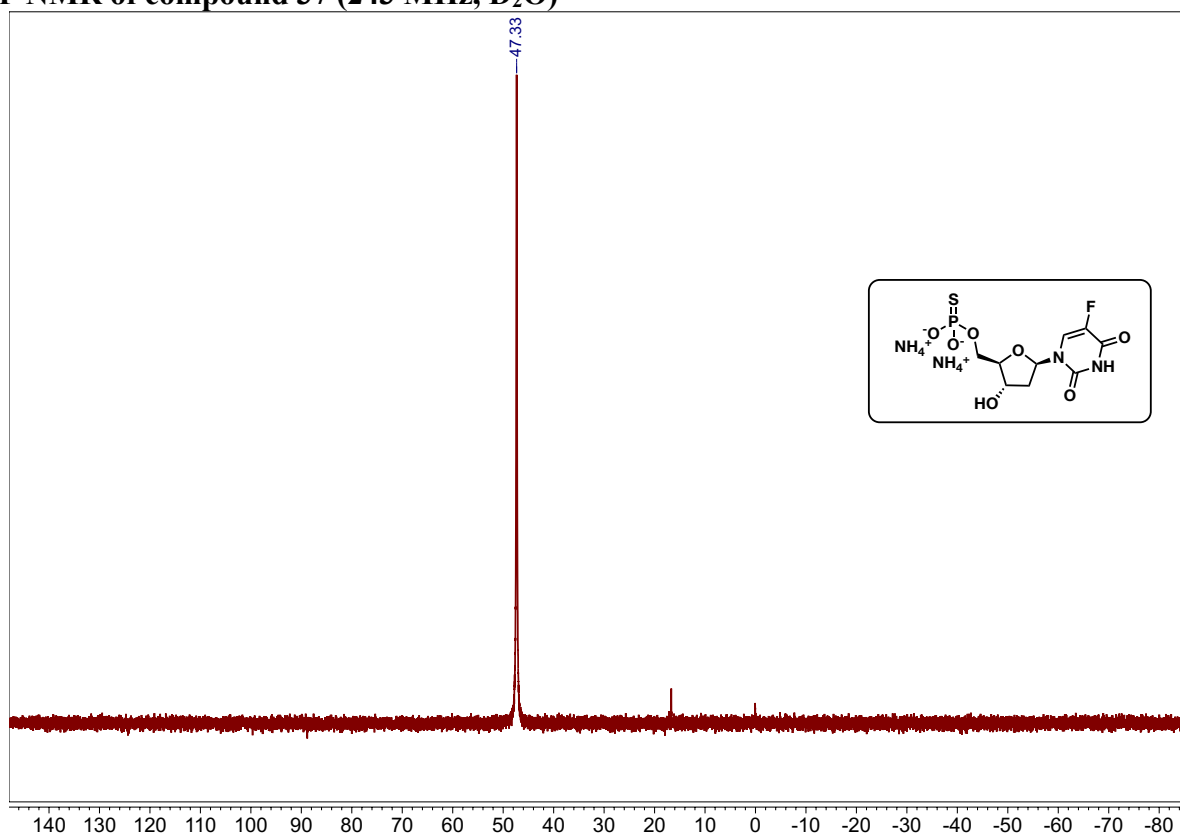
¹H NMR of compound 37 (600 MHz, D₂O)



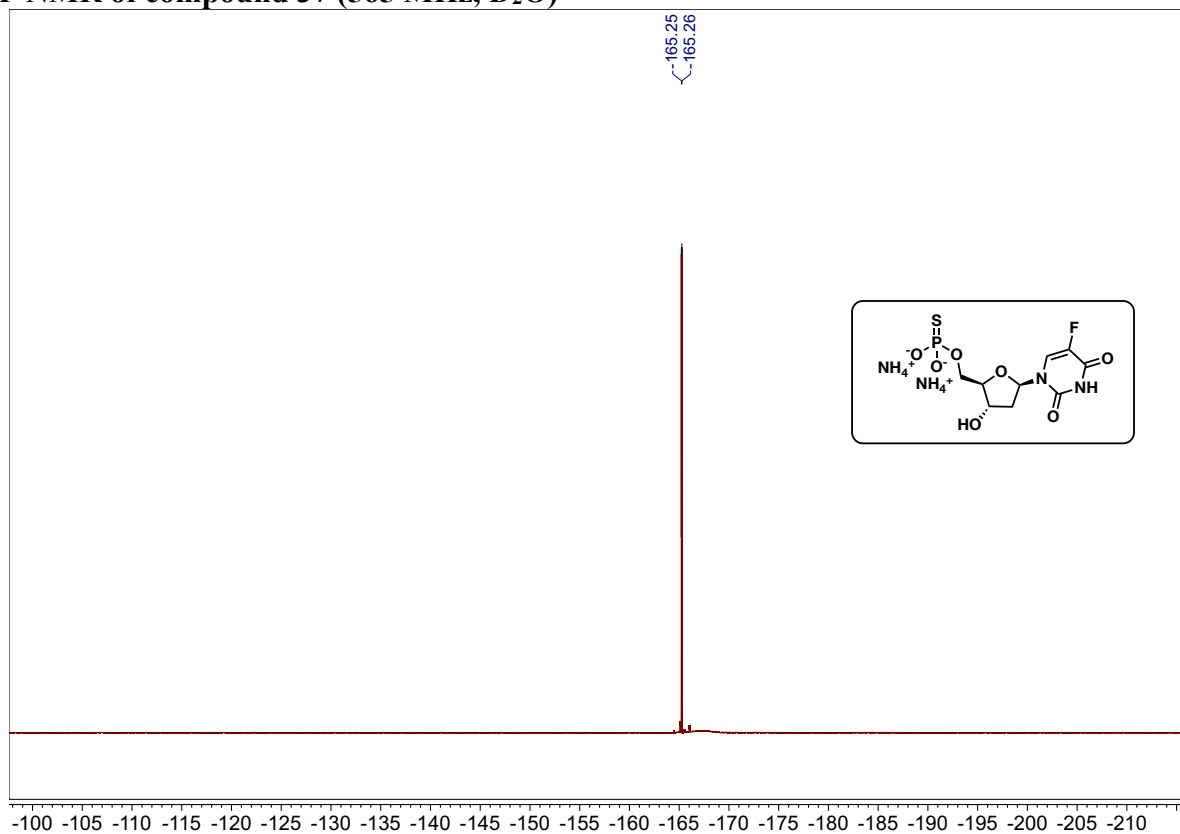
¹³C NMR of compound 37 (151 MHz, D₂O)



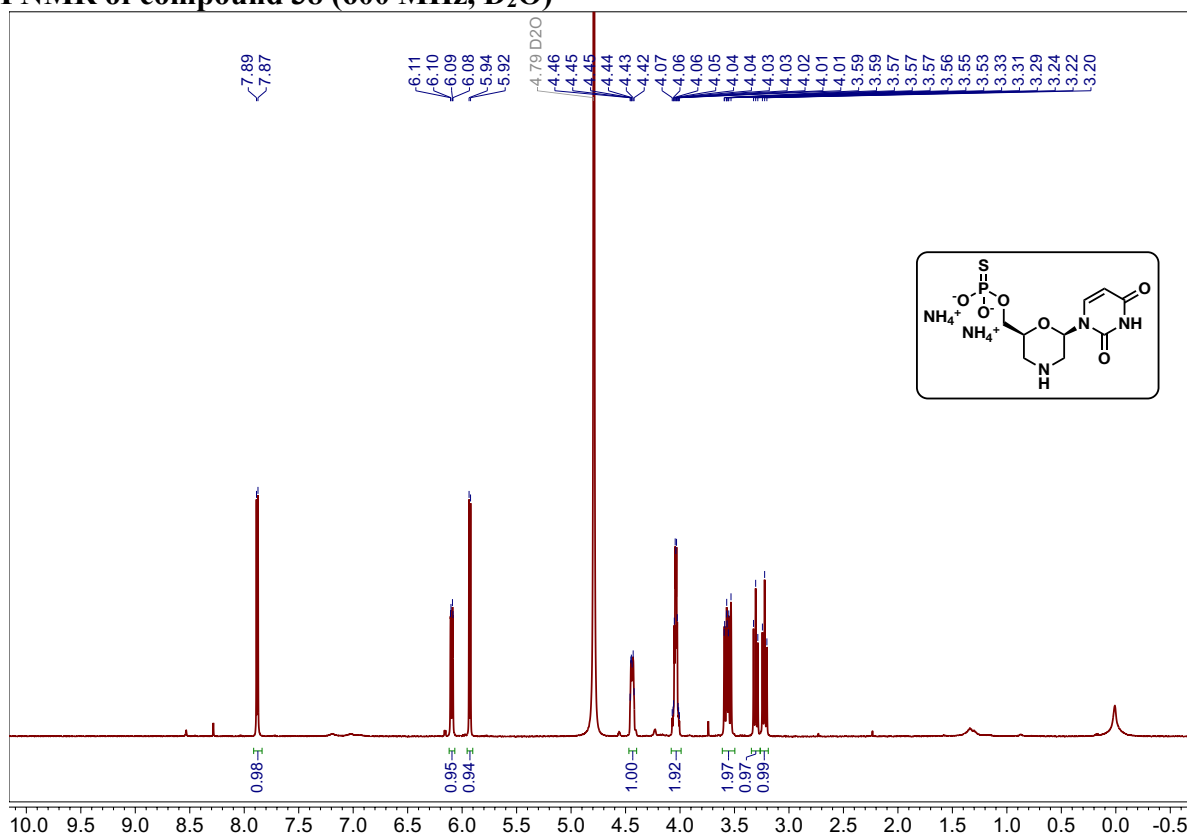
^{31}P NMR of compound 37 (243 MHz, D_2O)



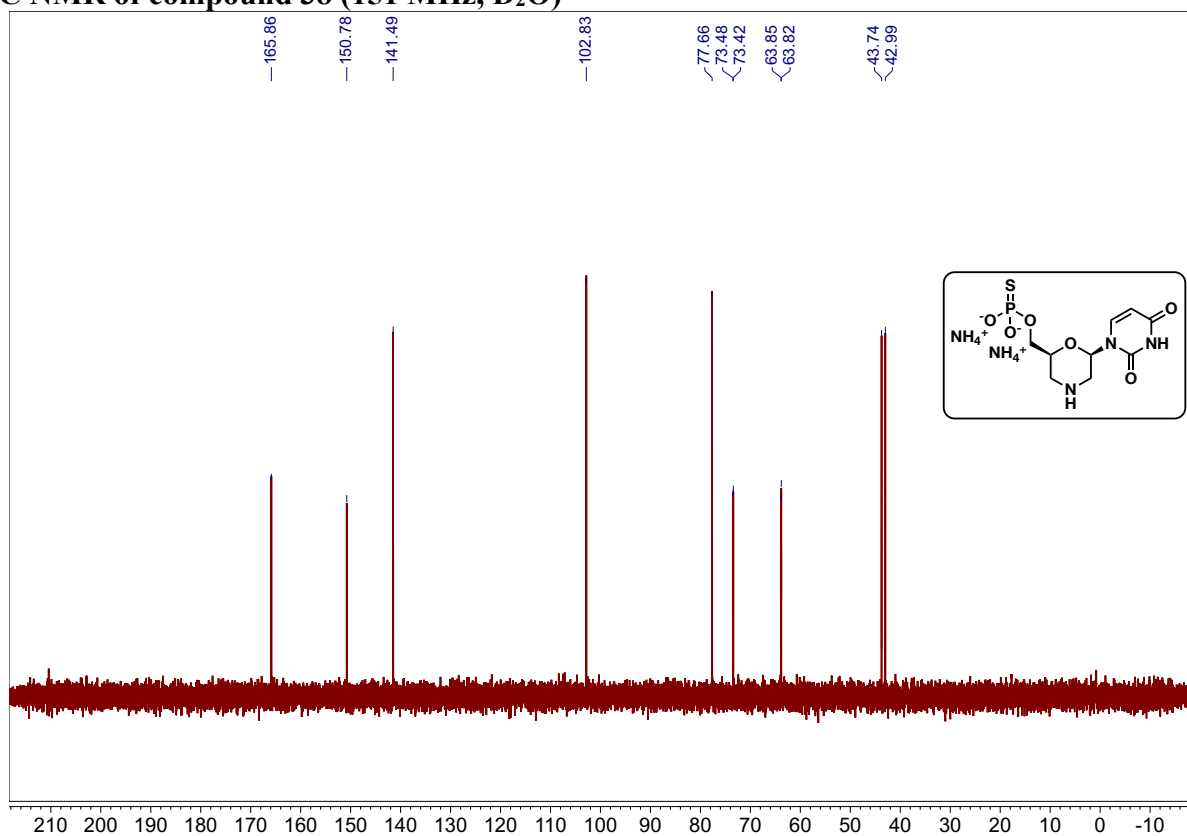
^{19}F NMR of compound 37 (565 MHz, D_2O)



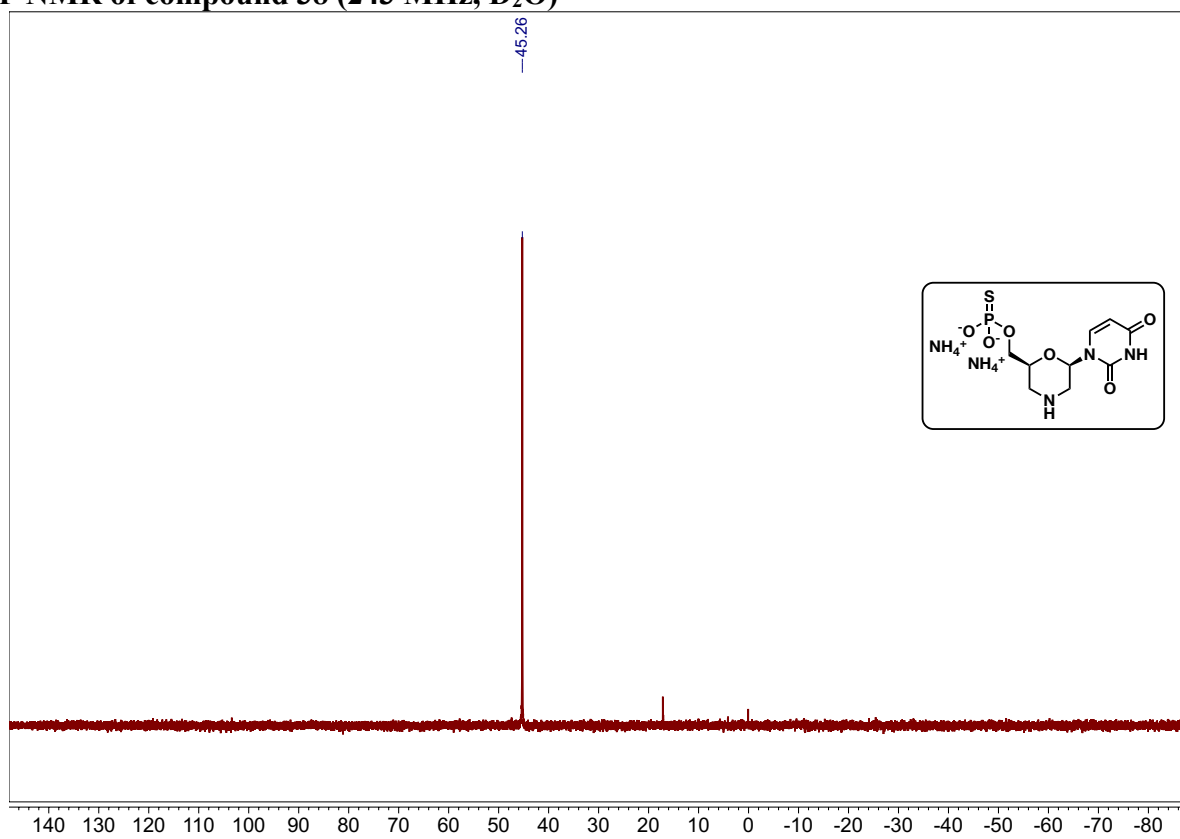
¹H NMR of compound 38 (600 MHz, D₂O)



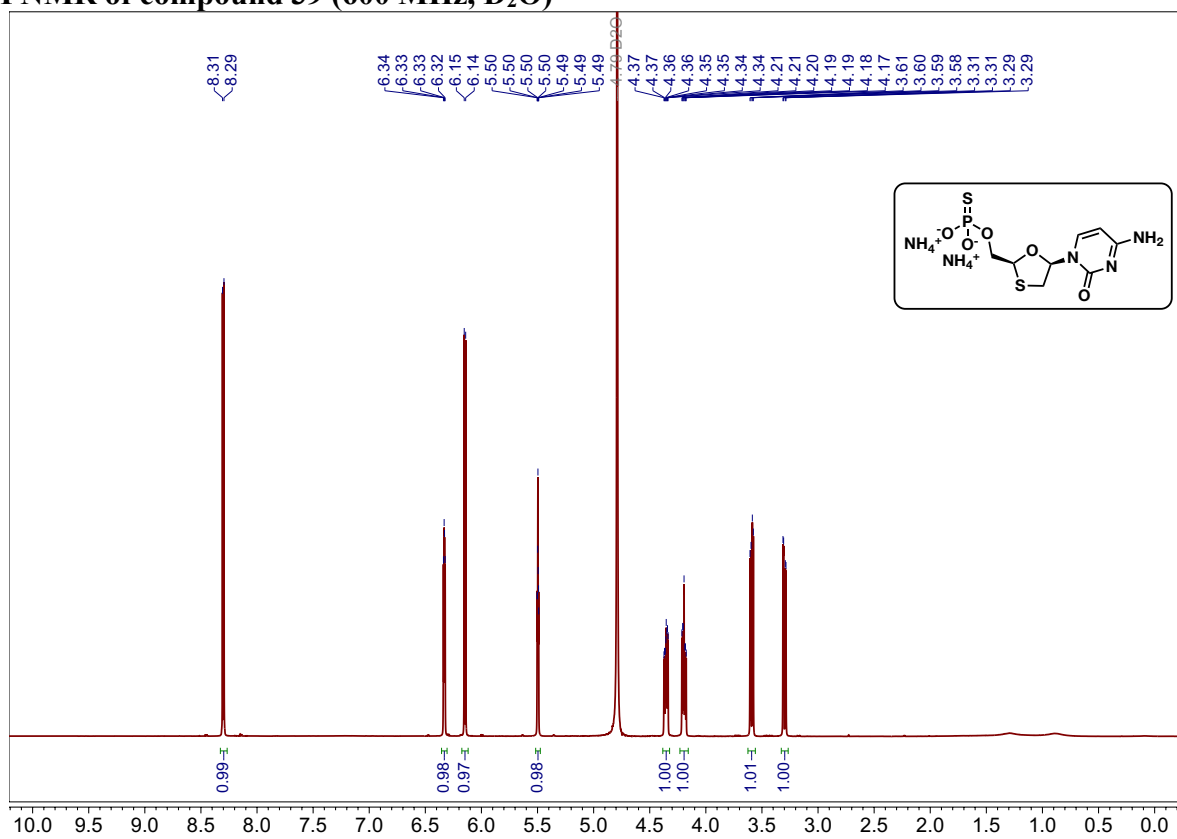
¹³C NMR of compound 38 (151 MHz, D₂O)



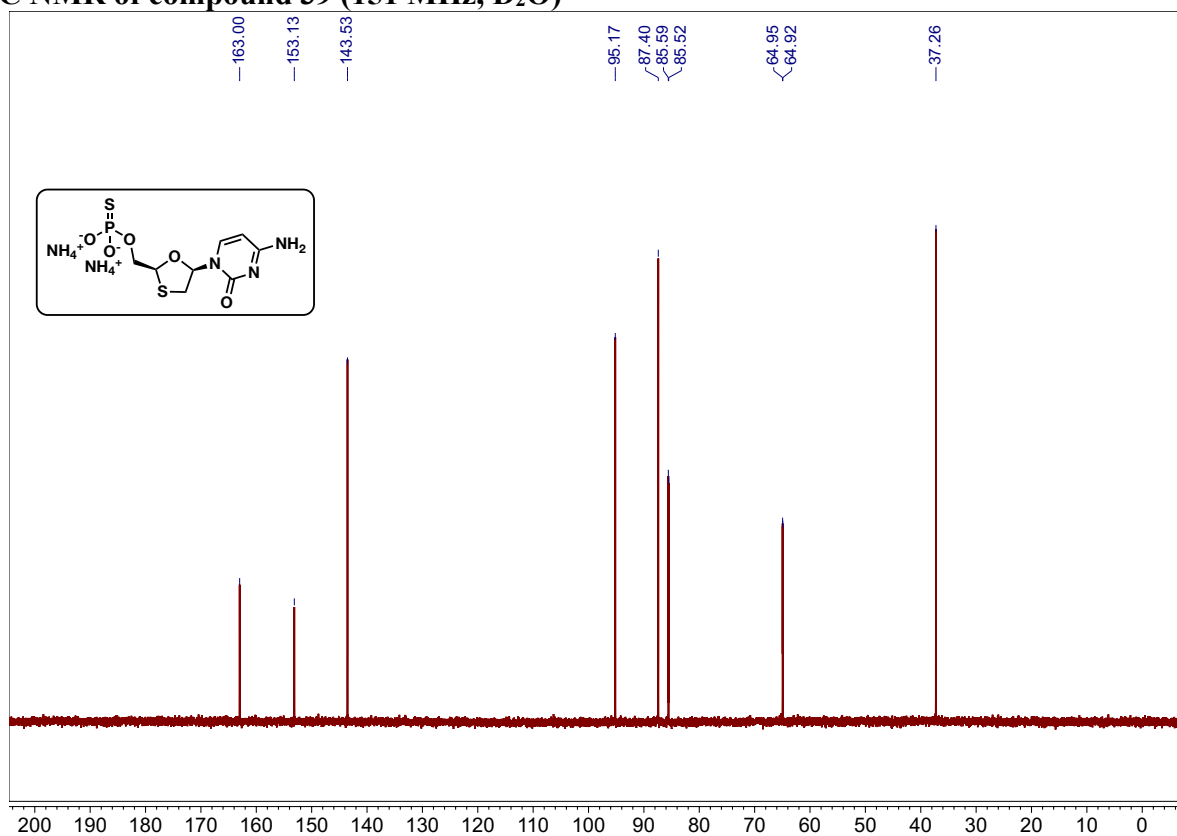
^{31}P NMR of compound 38 (243 MHz, D_2O)



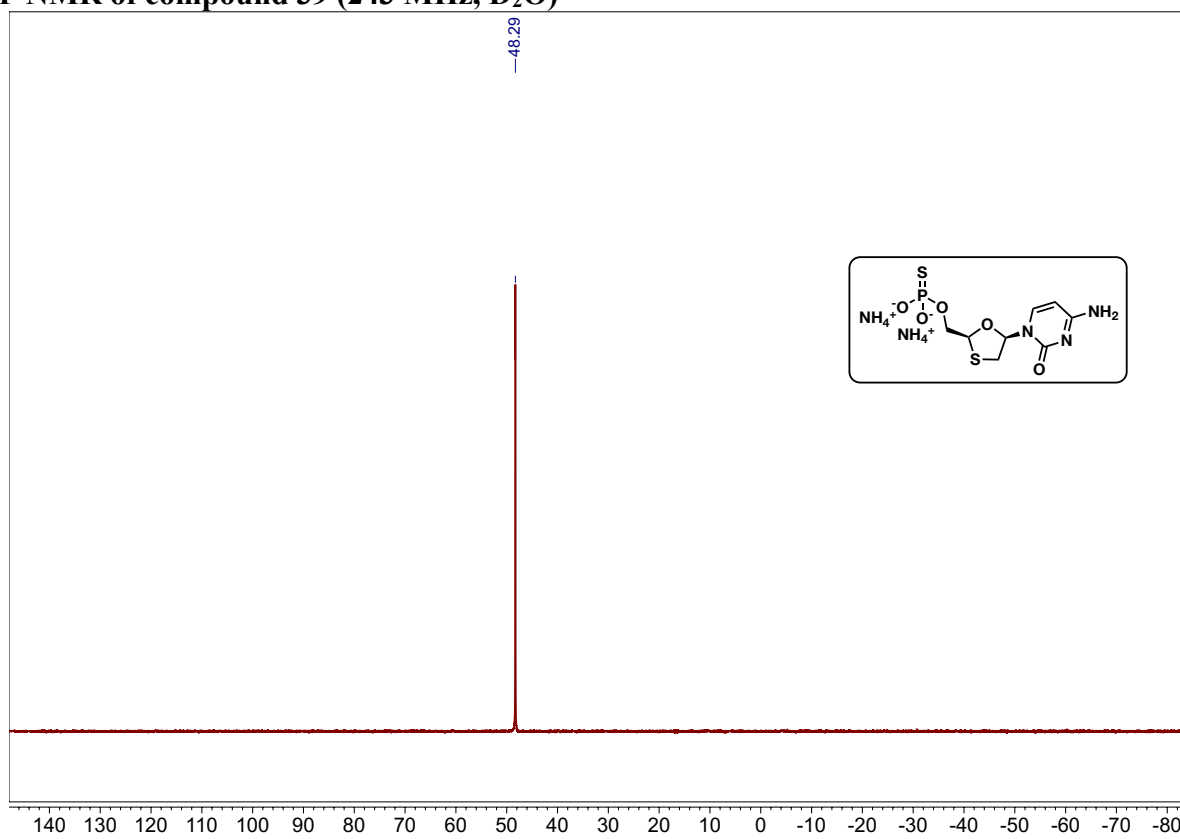
¹H NMR of compound 39 (600 MHz, D₂O)



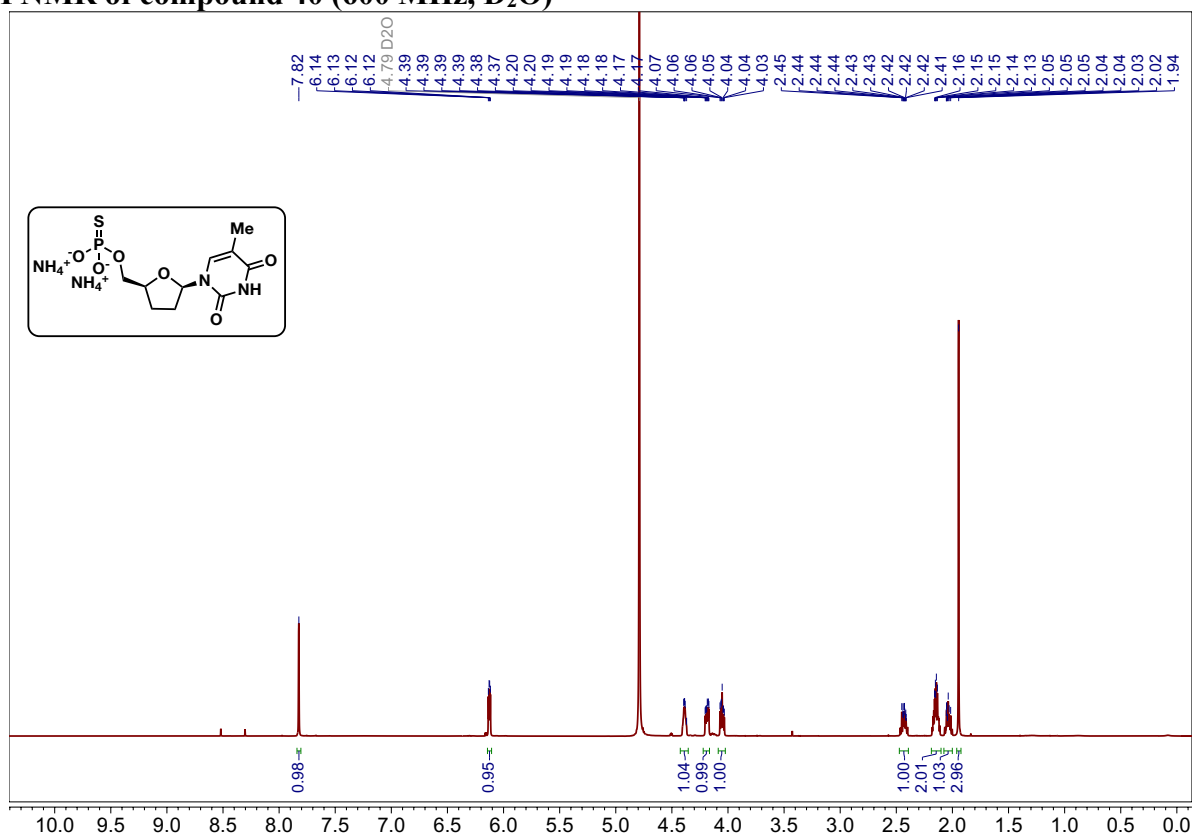
¹³C NMR of compound 39 (151 MHz, D₂O)



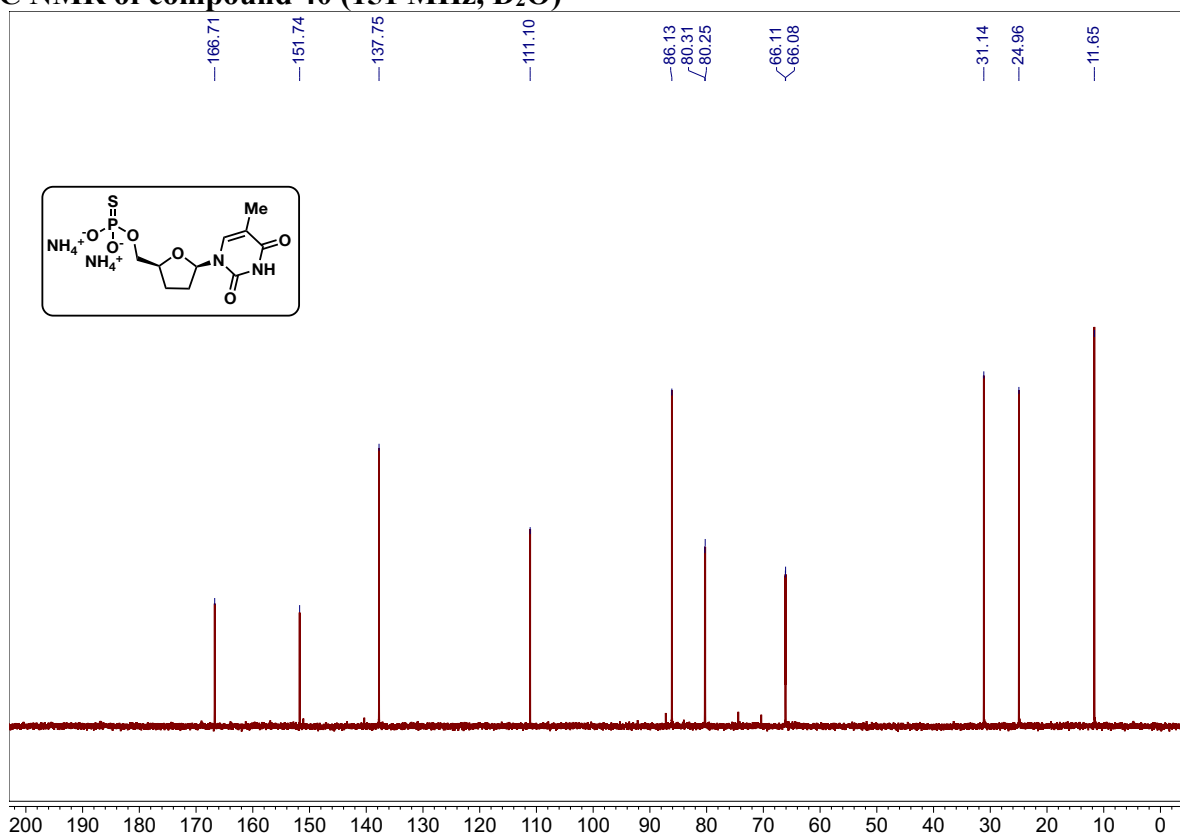
^{31}P NMR of compound 39 (243 MHz, D_2O)



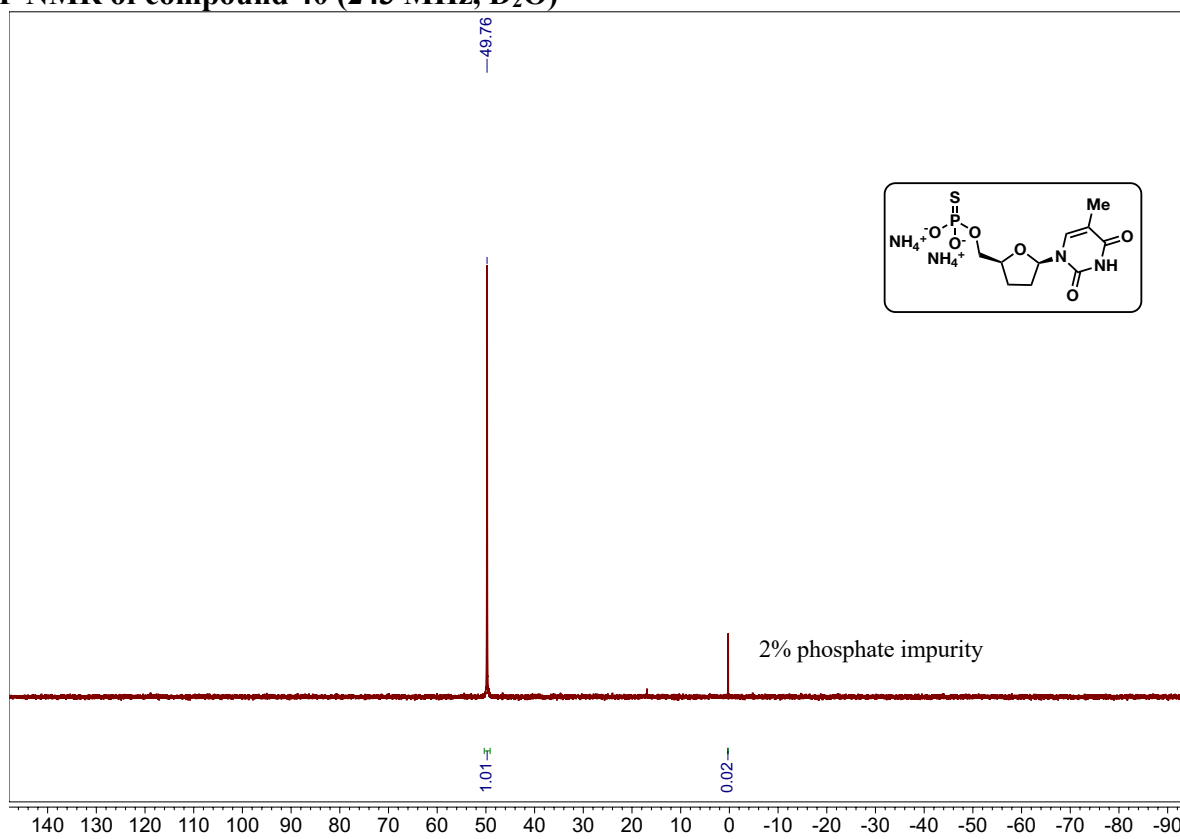
¹H NMR of compound 40 (600 MHz, D₂O)



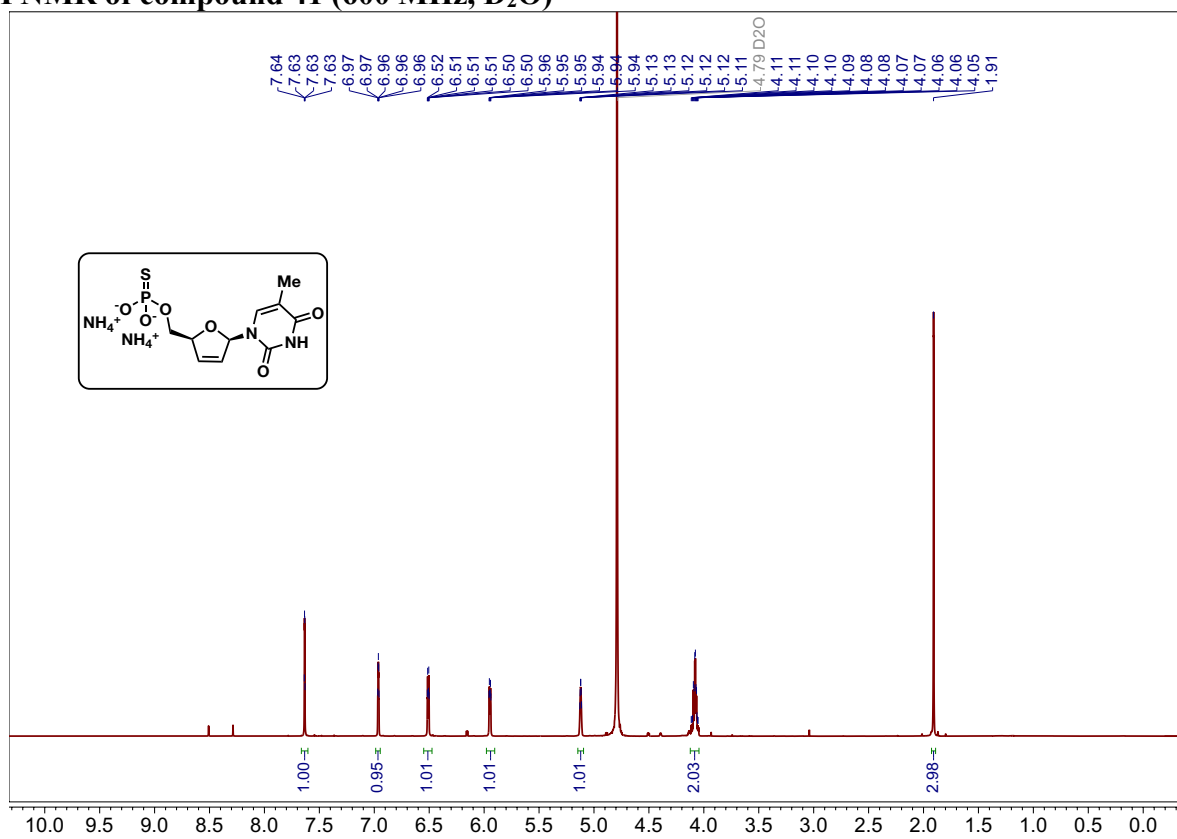
¹³C NMR of compound 40 (151 MHz, D₂O)



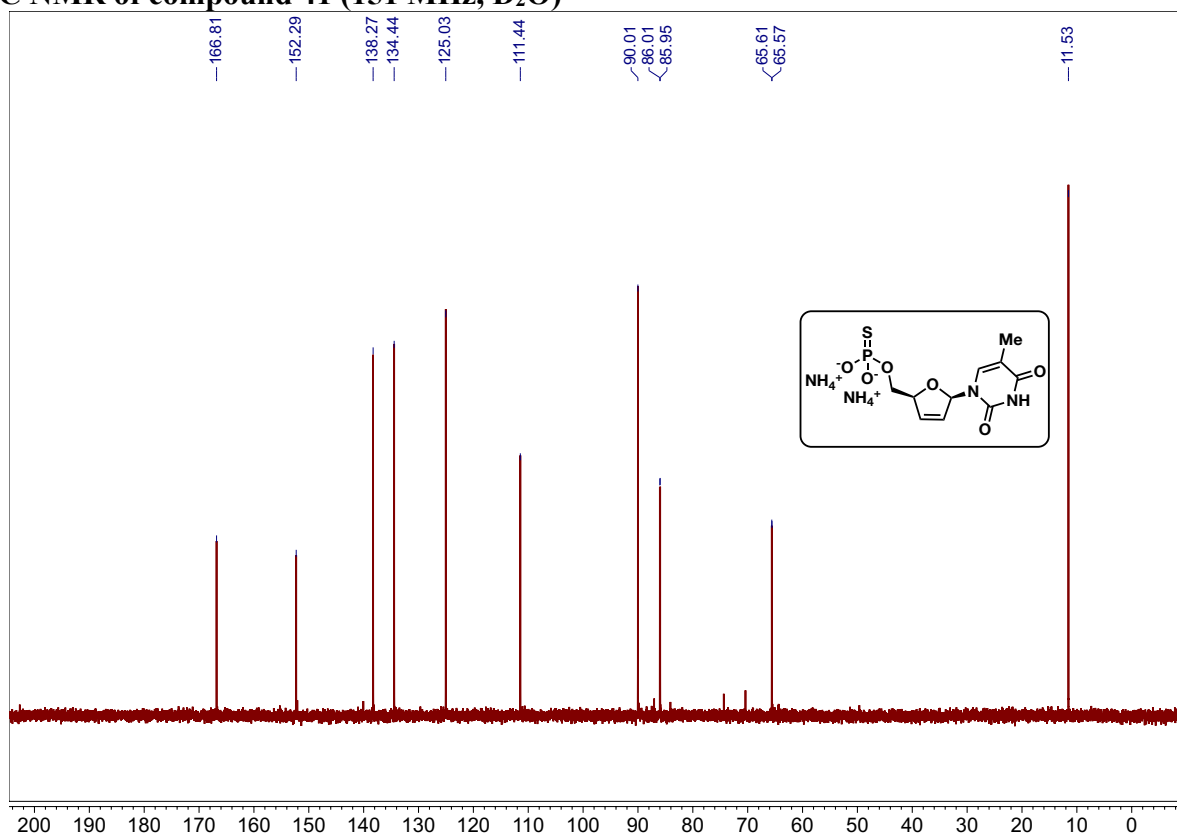
^{31}P NMR of compound 40 (243 MHz, D_2O)



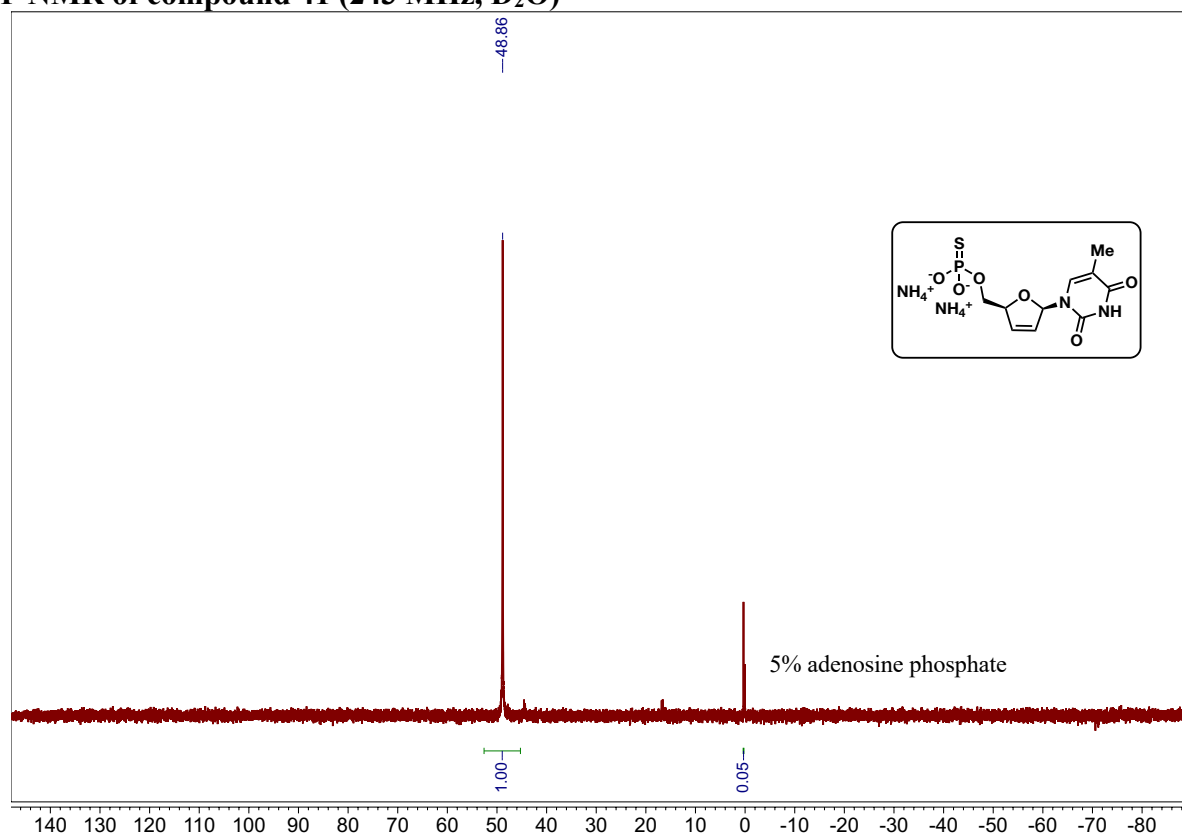
¹H NMR of compound 41 (600 MHz, D₂O)



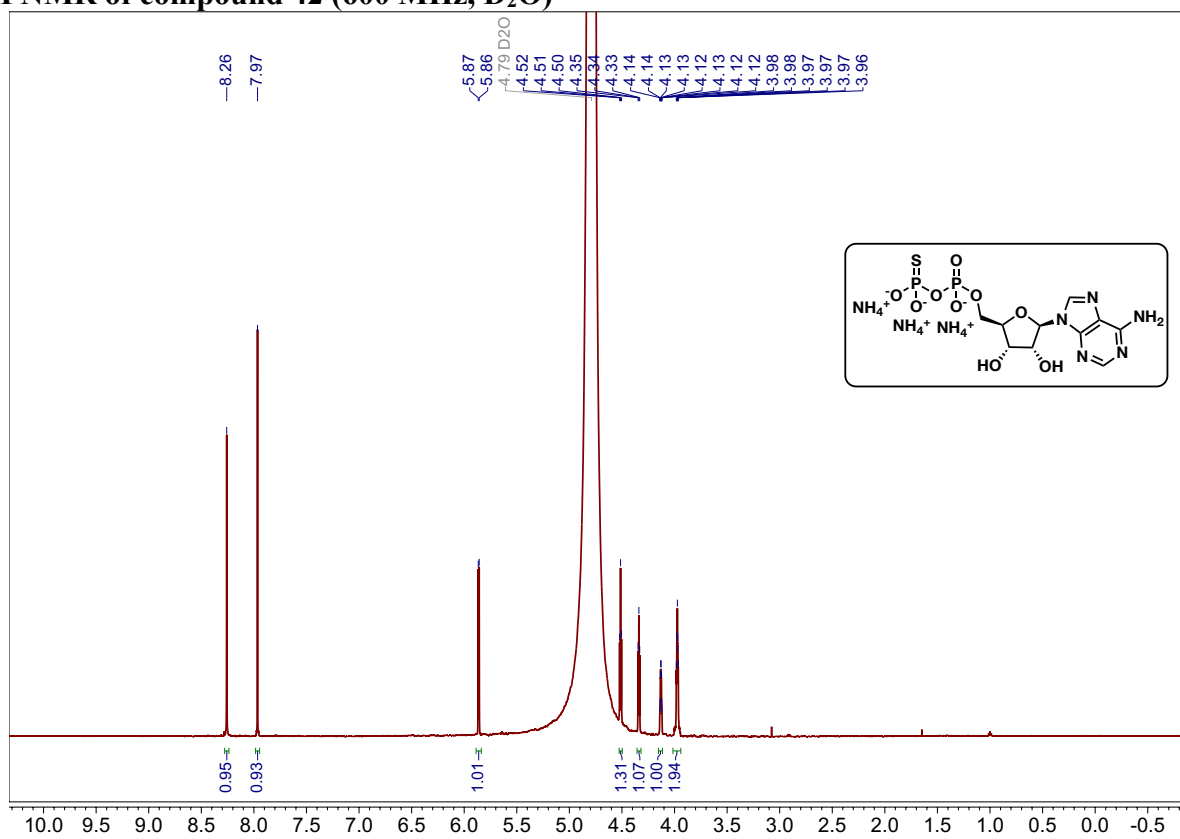
¹³C NMR of compound 41 (151 MHz, D₂O)



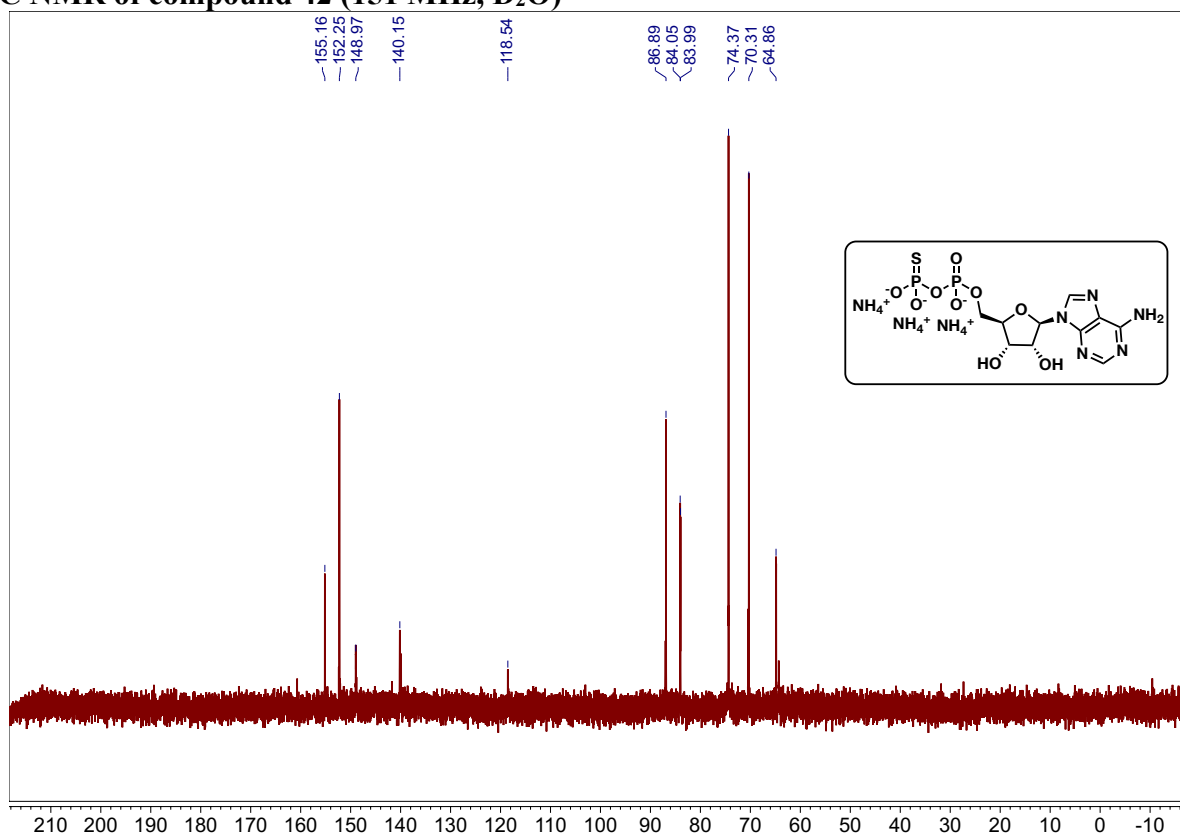
^{31}P NMR of compound 41 (243 MHz, D_2O)



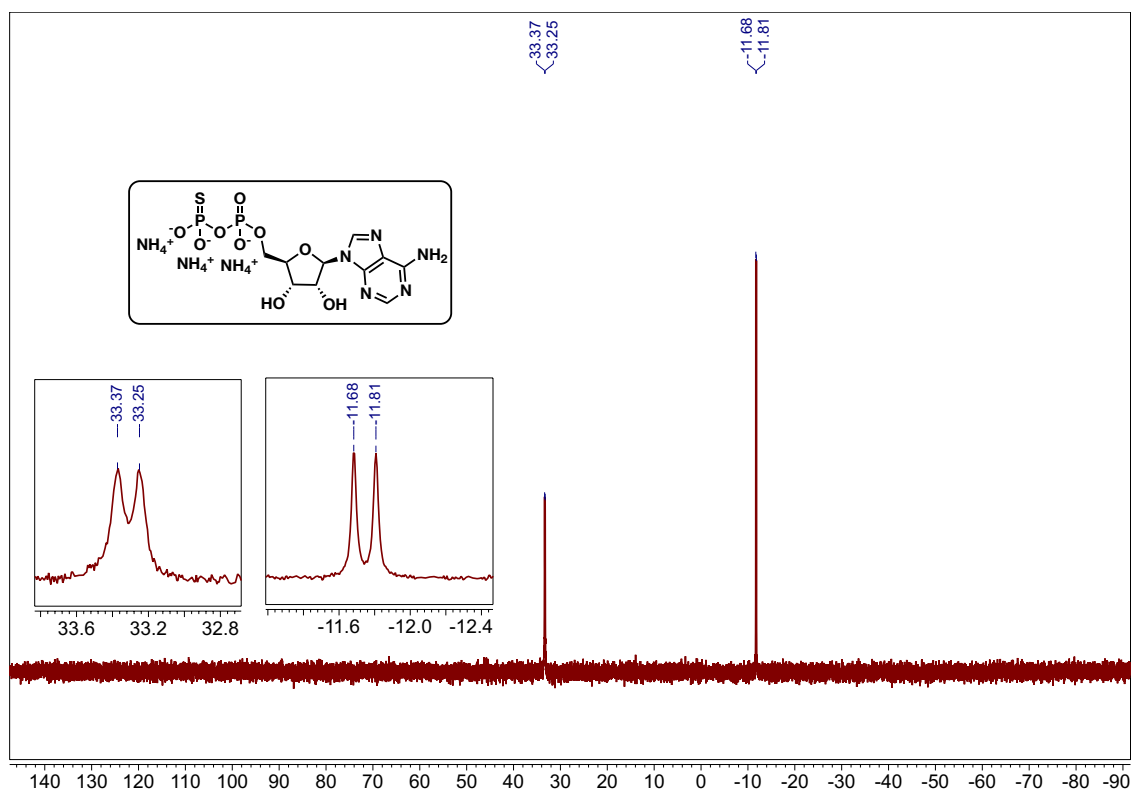
^1H NMR of compound 42 (600 MHz, D_2O)



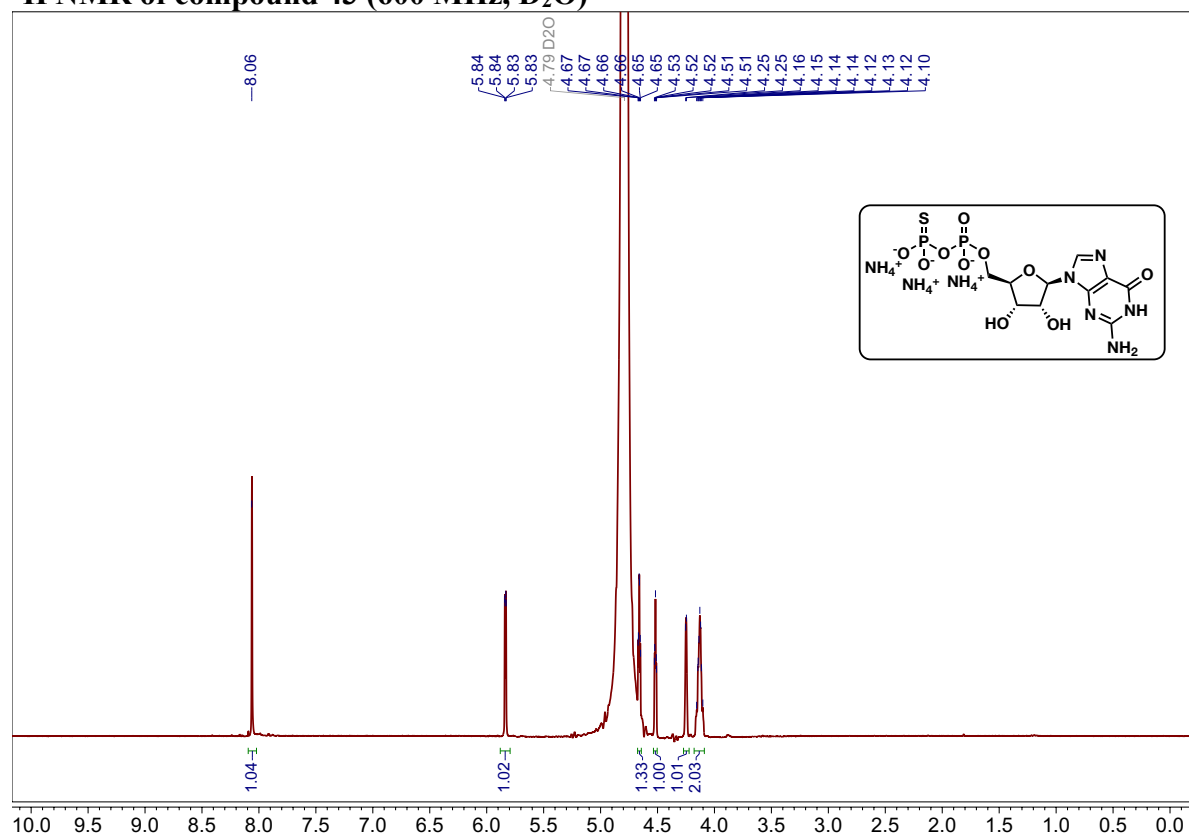
^{13}C NMR of compound 42 (151 MHz, D_2O)



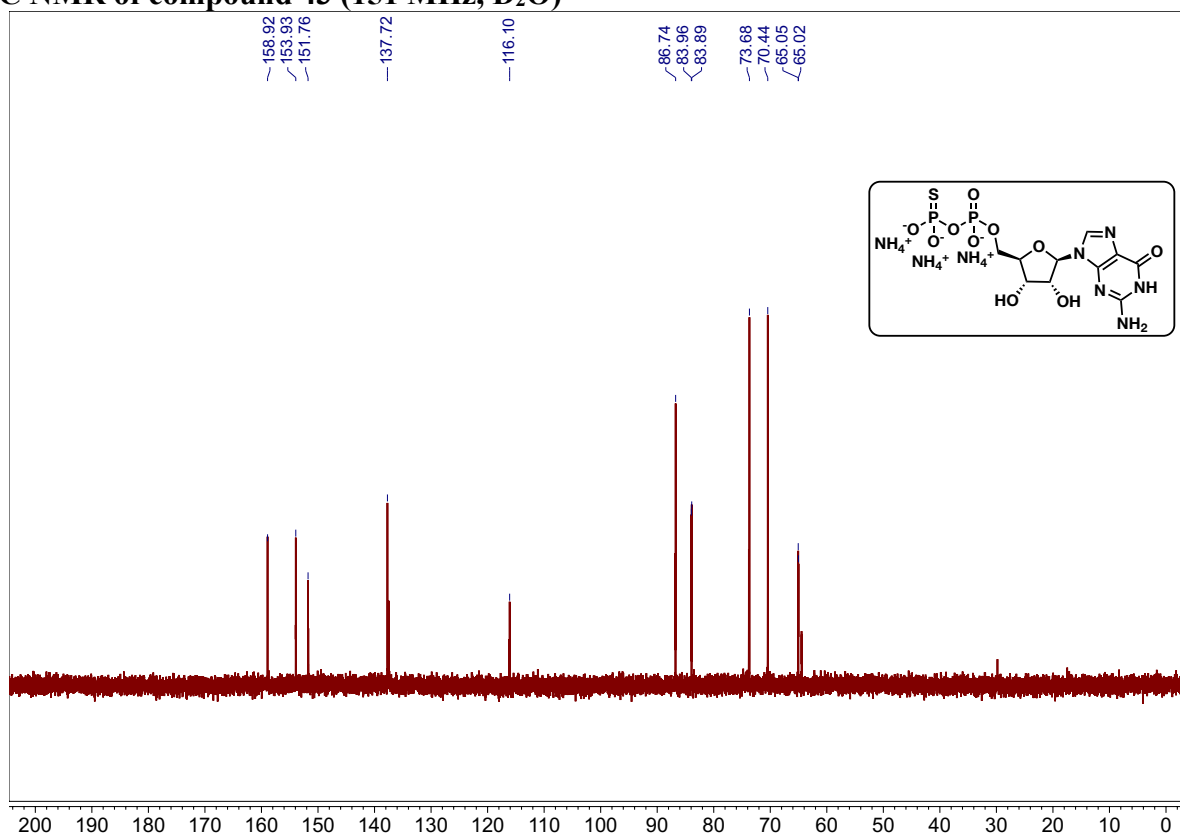
^{31}P NMR of compound 42 (243 MHz, D_2O)



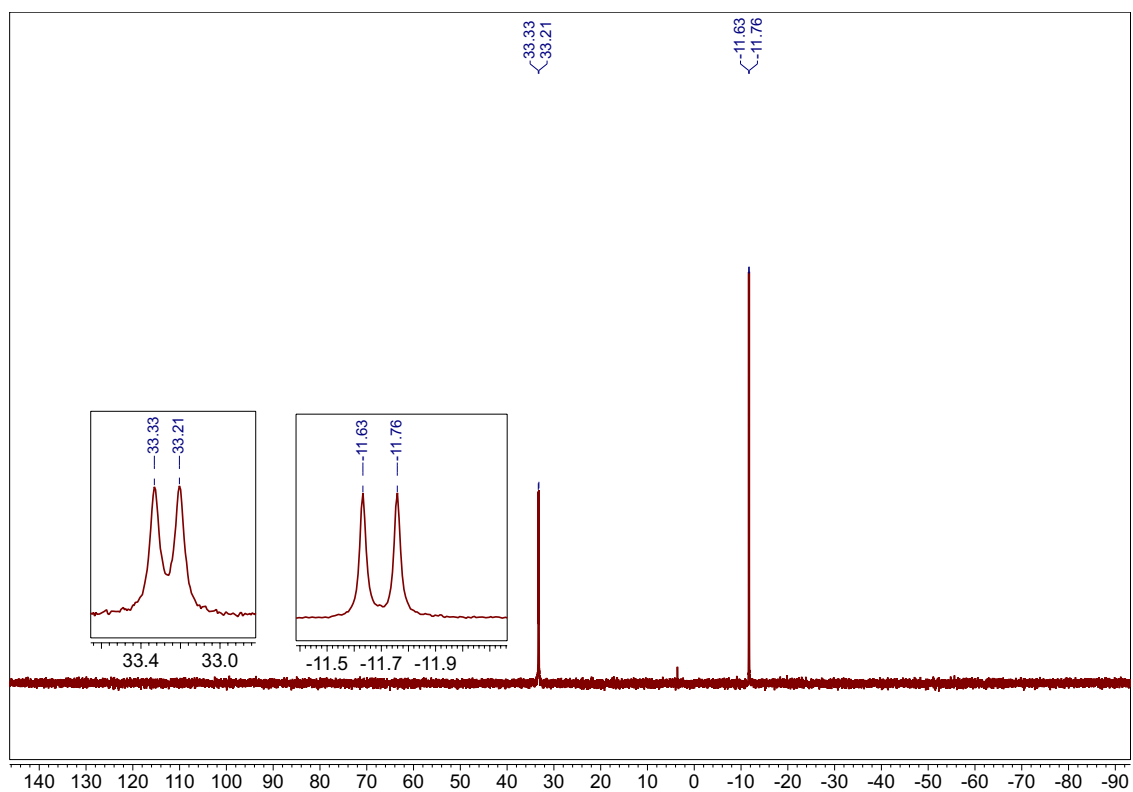
¹H NMR of compound 43 (600 MHz, D₂O)



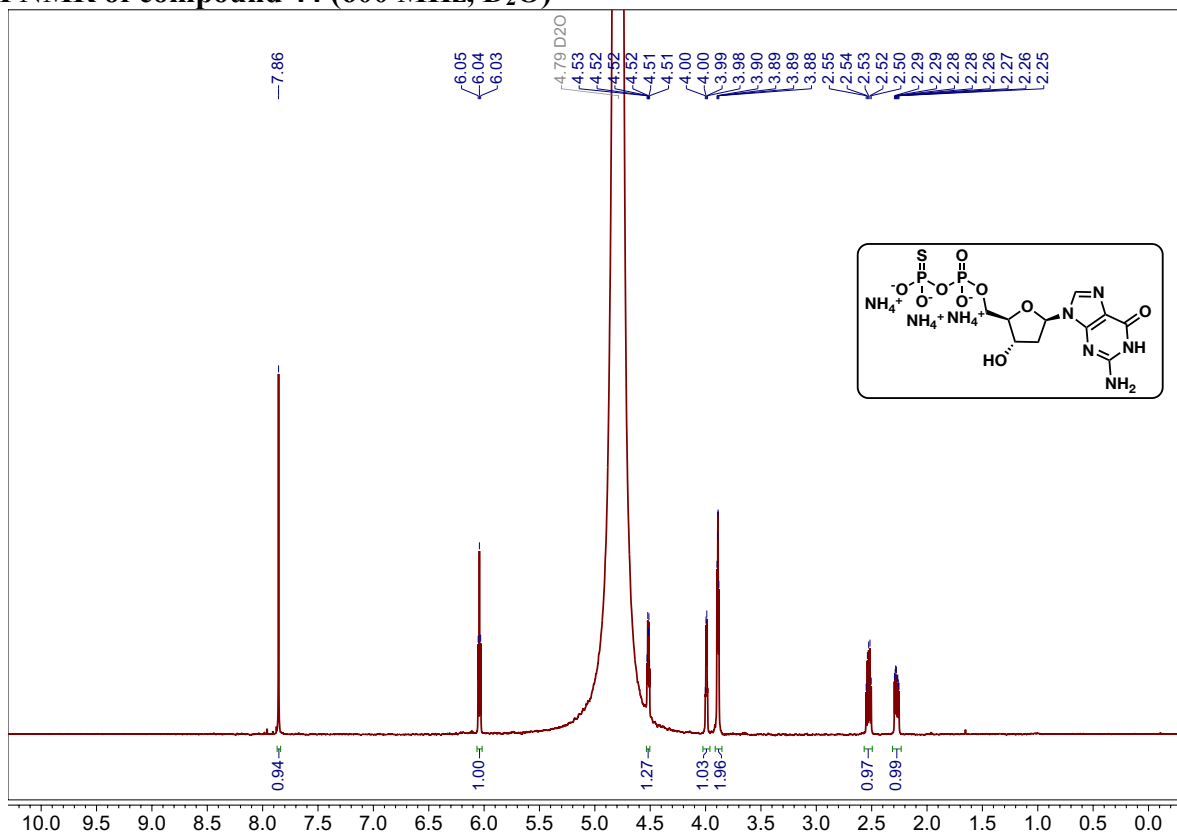
¹³C NMR of compound 43 (151 MHz, D₂O)



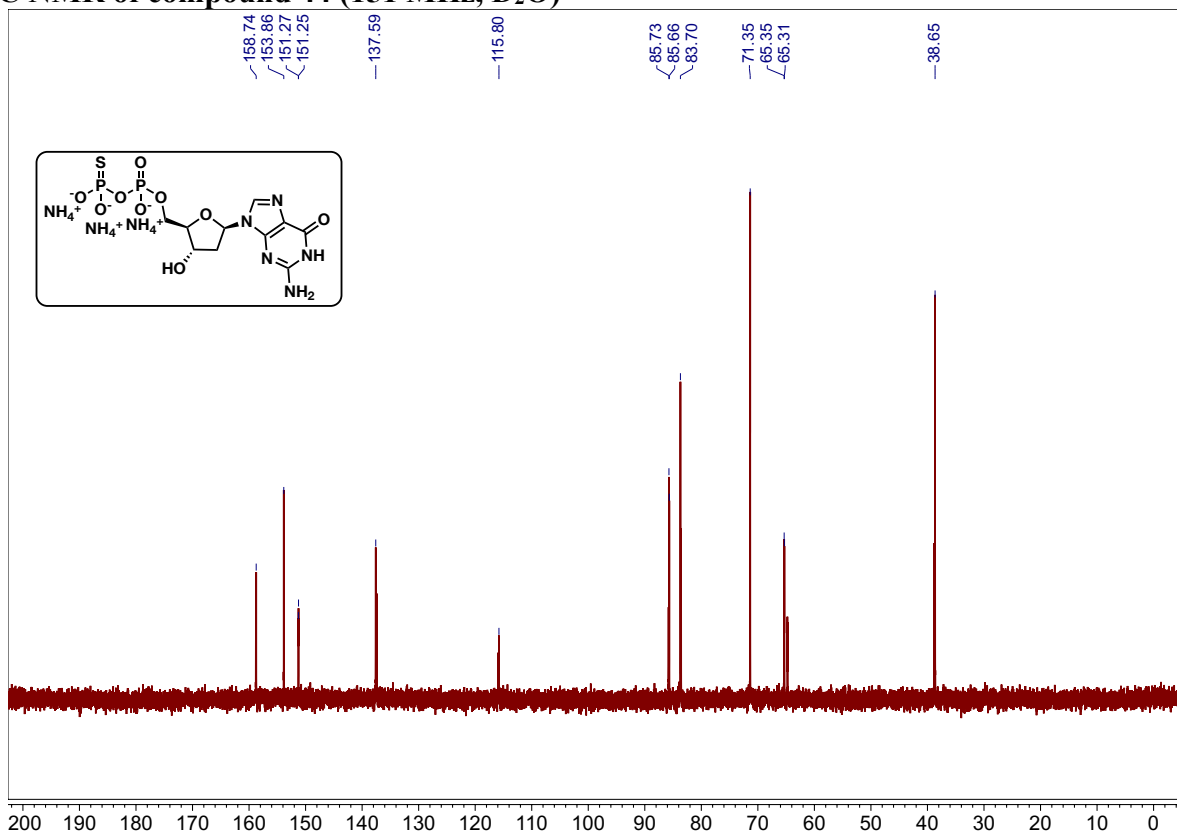
^{31}P NMR of compound 43 (243 MHz, D_2O)



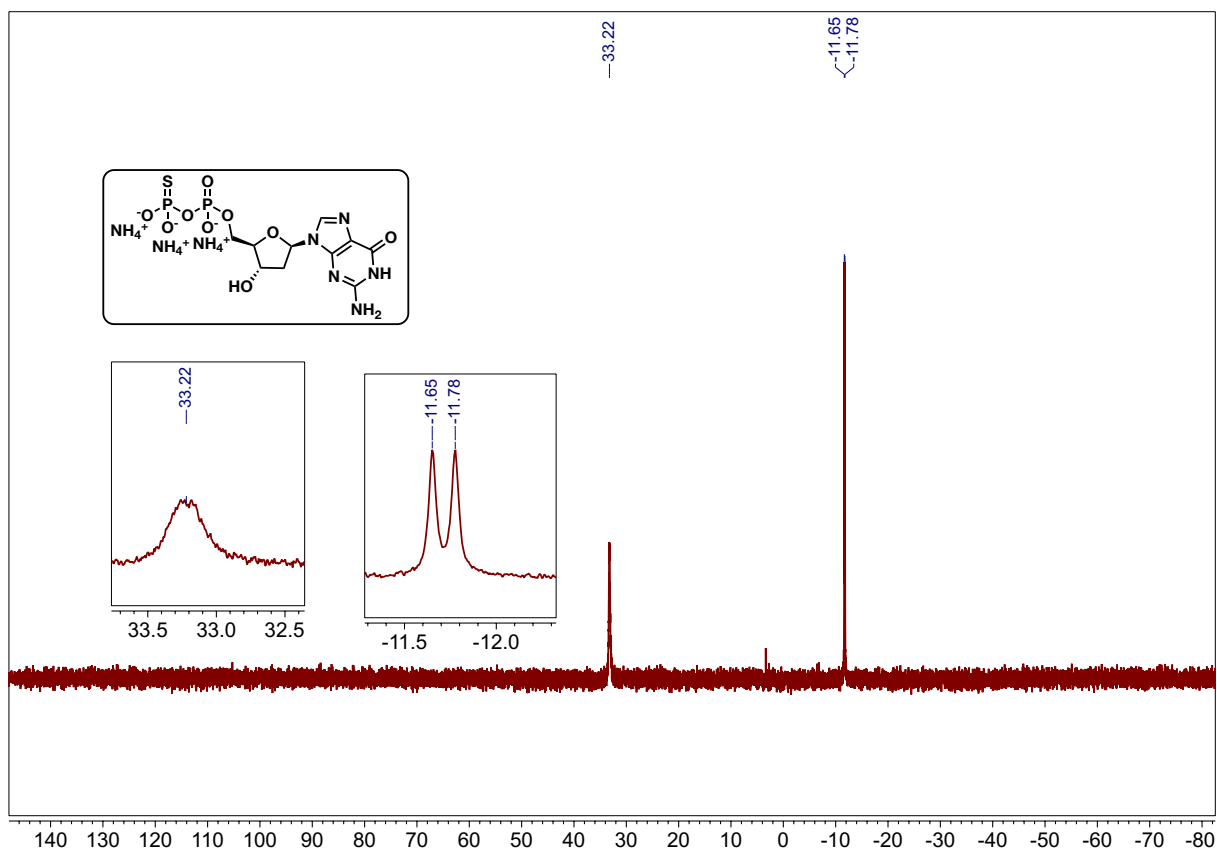
¹H NMR of compound 44 (600 MHz, D₂O)



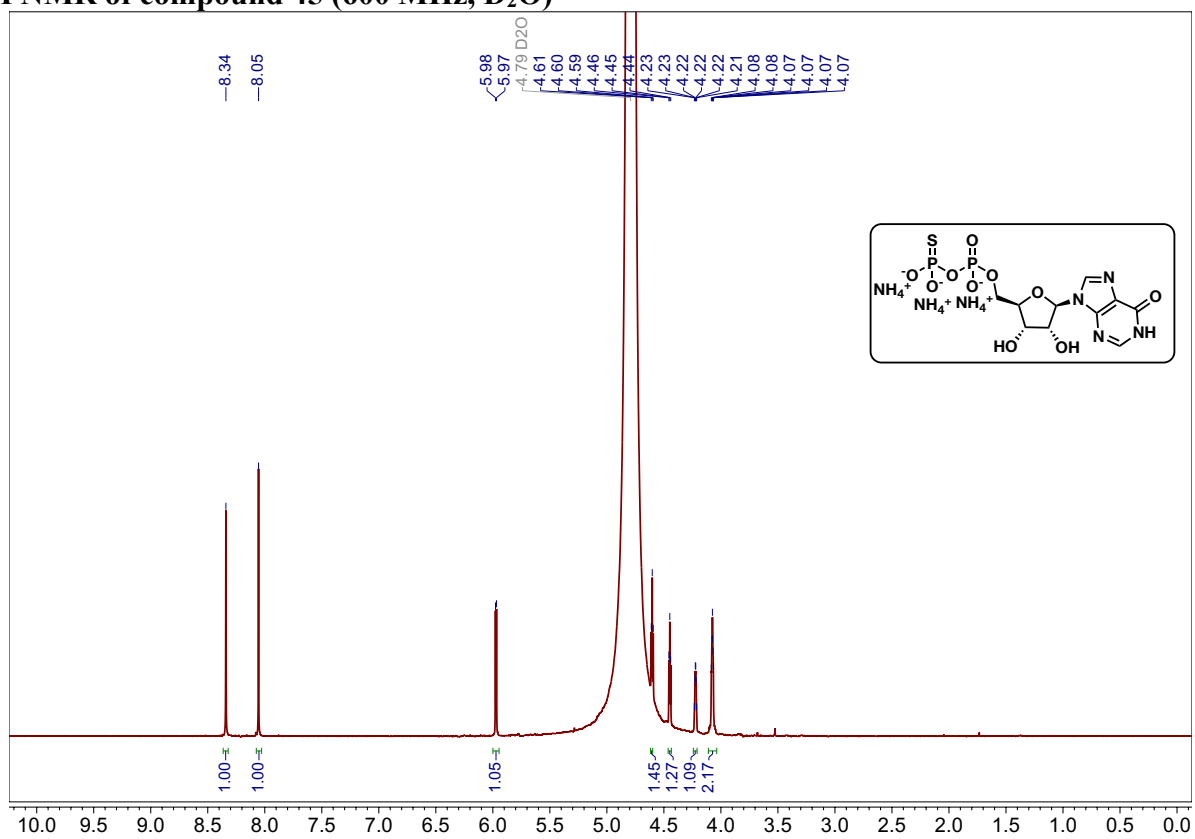
¹³C NMR of compound 44 (151 MHz, D₂O)



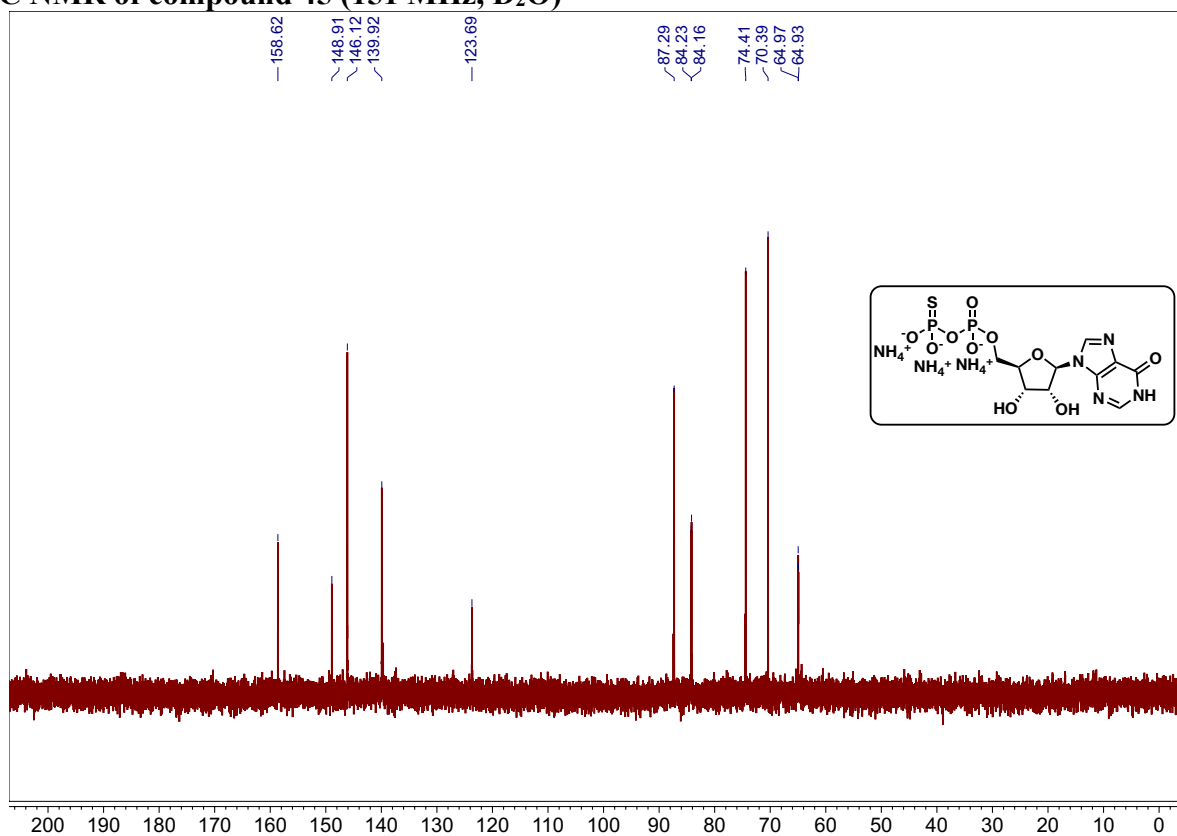
^{31}P NMR of compound 44 (243 MHz, D_2O)



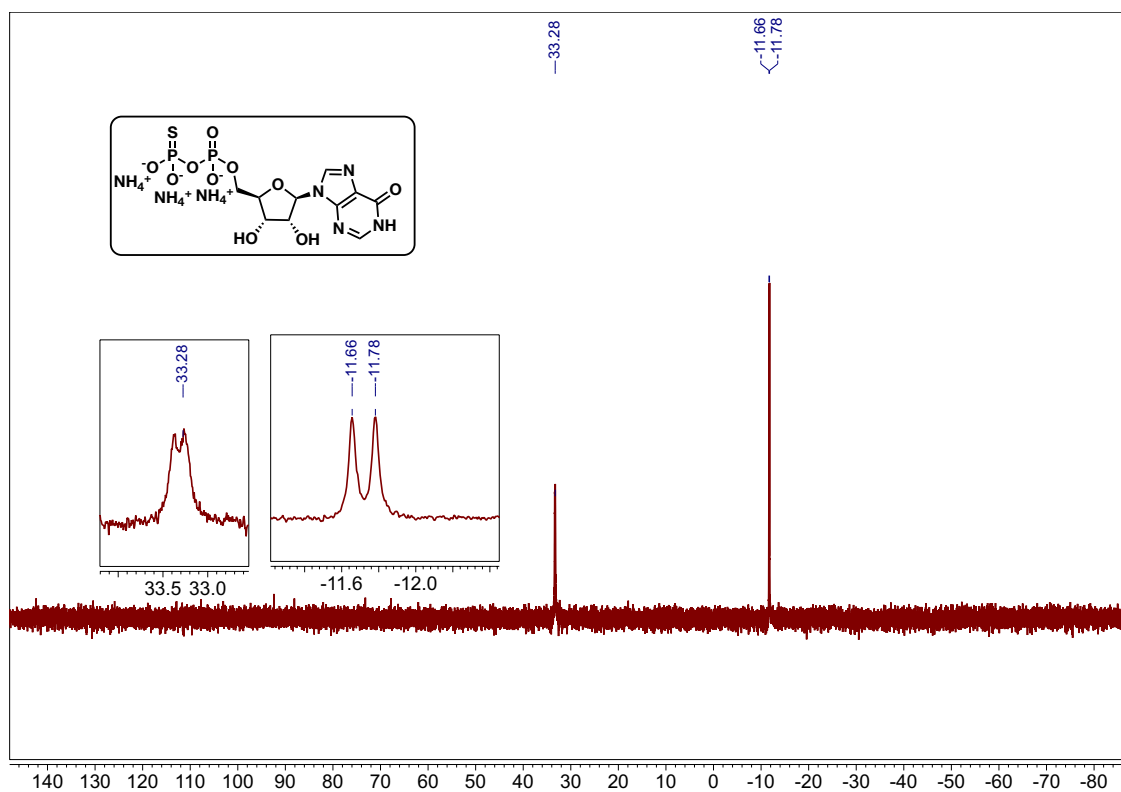
¹H NMR of compound 45 (600 MHz, D₂O)



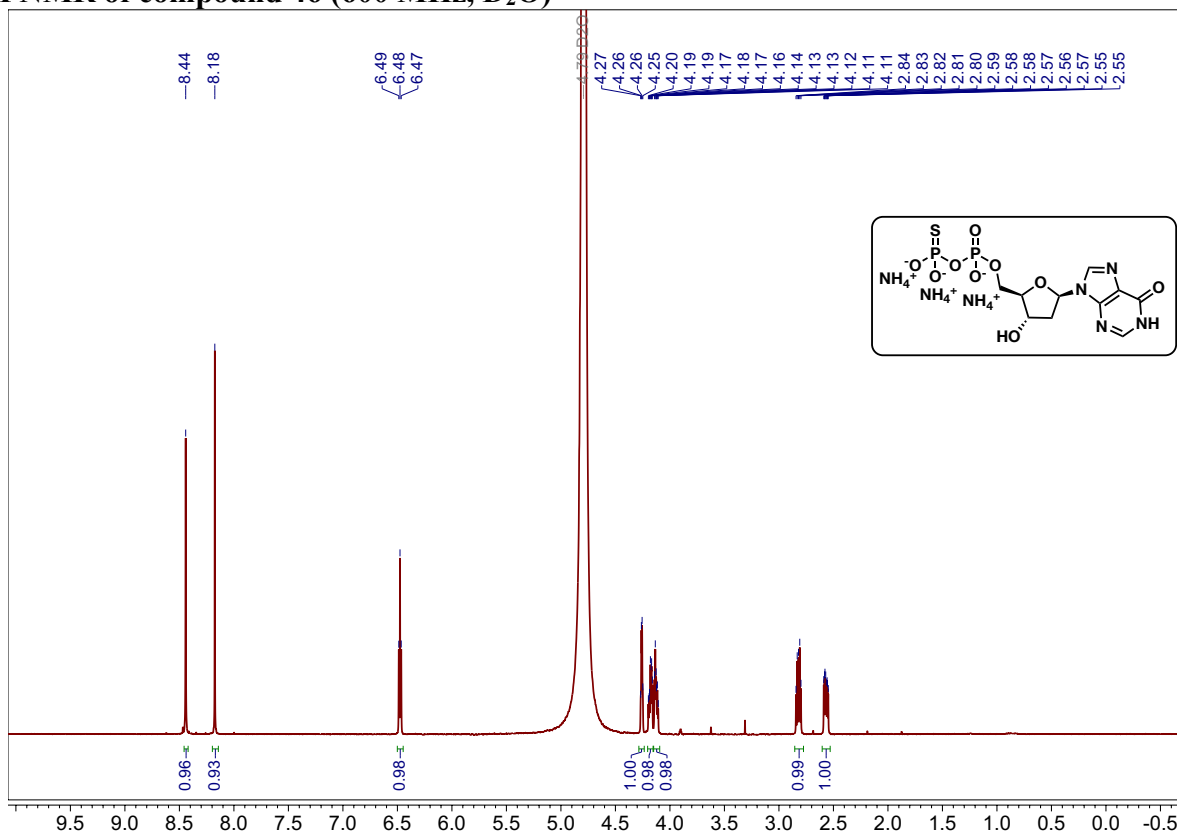
¹³C NMR of compound 45 (151 MHz, D₂O)



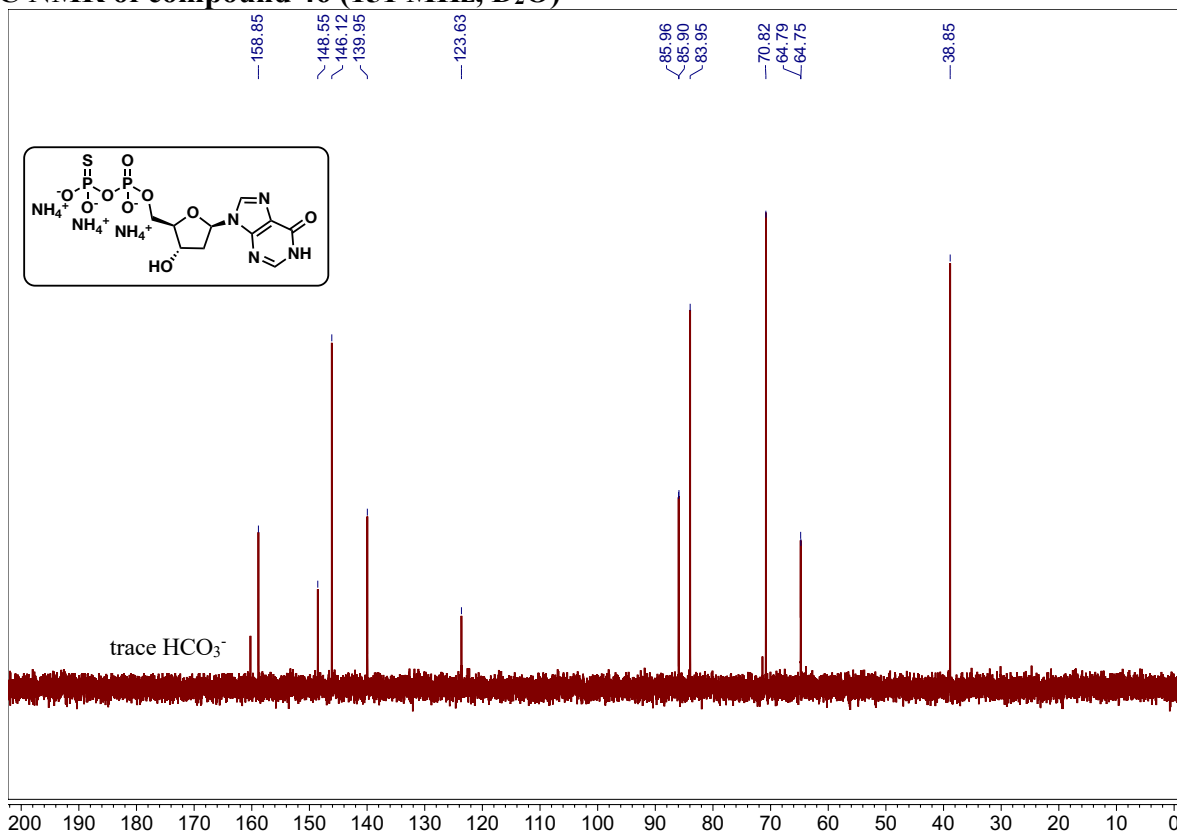
^{31}P NMR of compound 45 (243 MHz, D_2O)



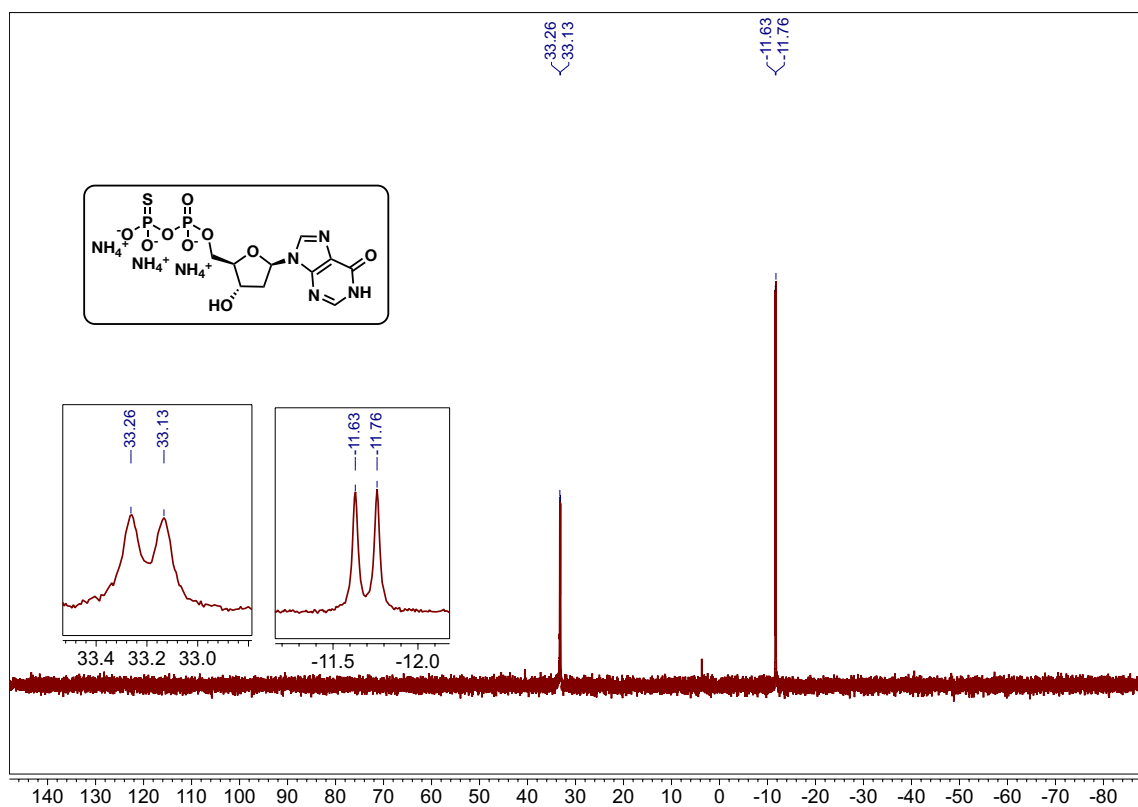
¹H NMR of compound 46 (600 MHz, D₂O)



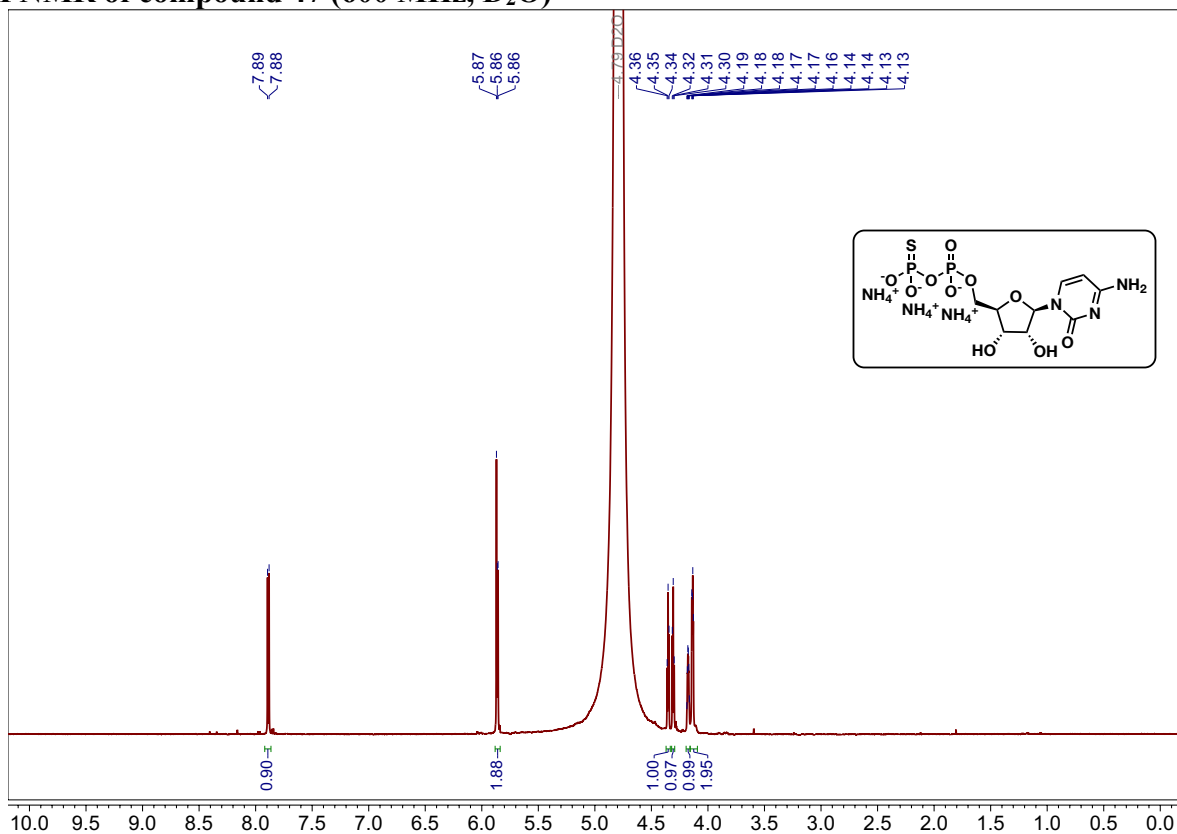
¹³C NMR of compound 46 (151 MHz, D₂O)



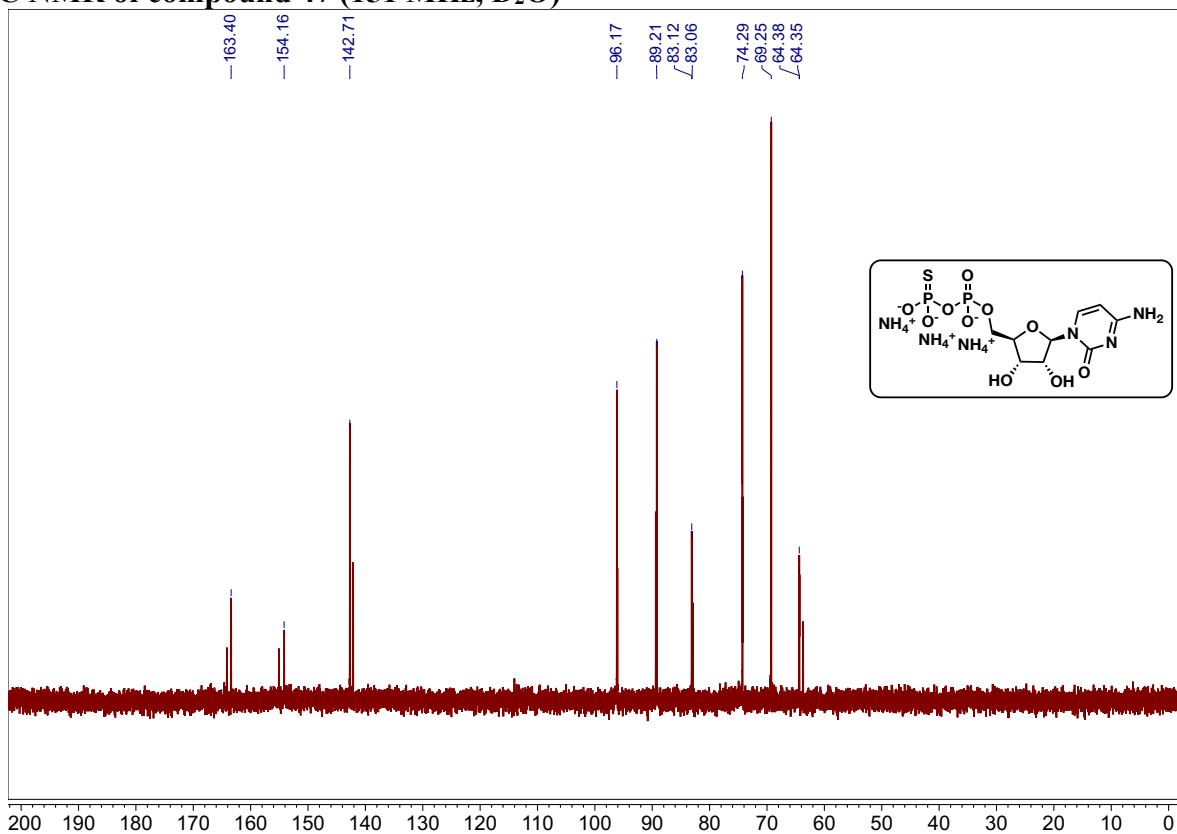
^{31}P NMR of compound 46 (243 MHz, D_2O)



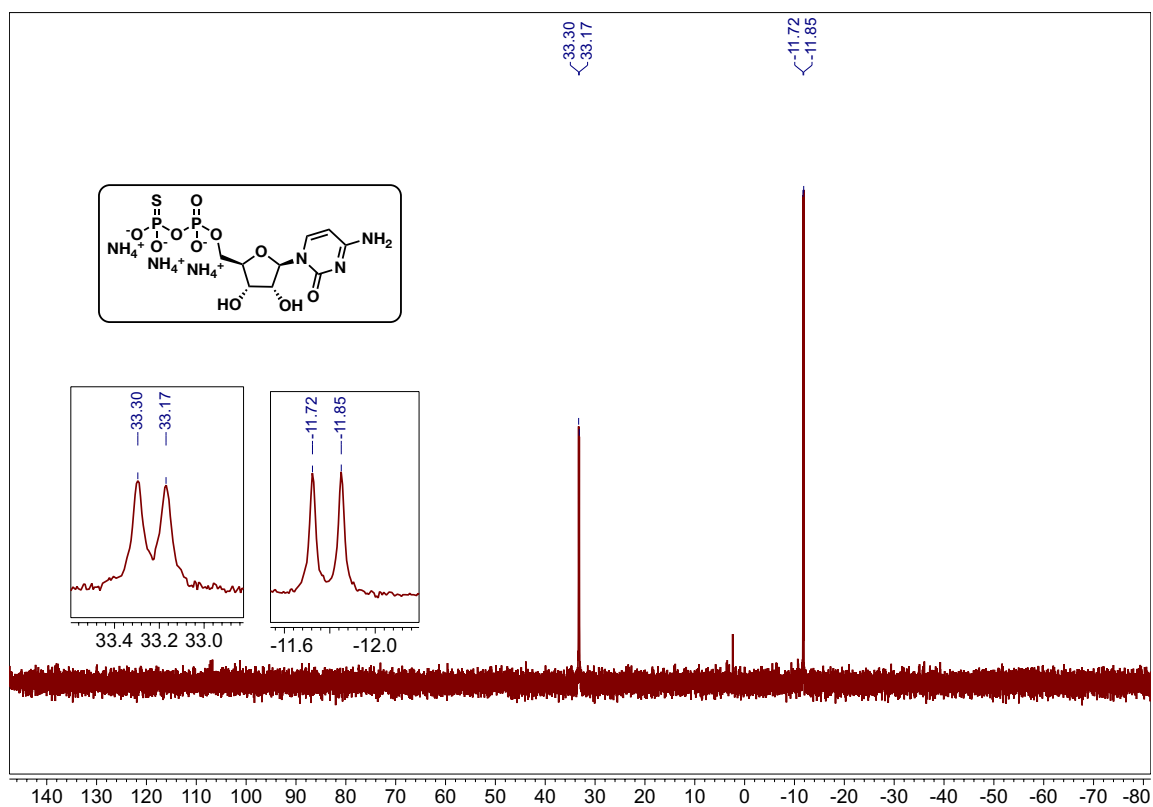
¹H NMR of compound 47 (600 MHz, D₂O)



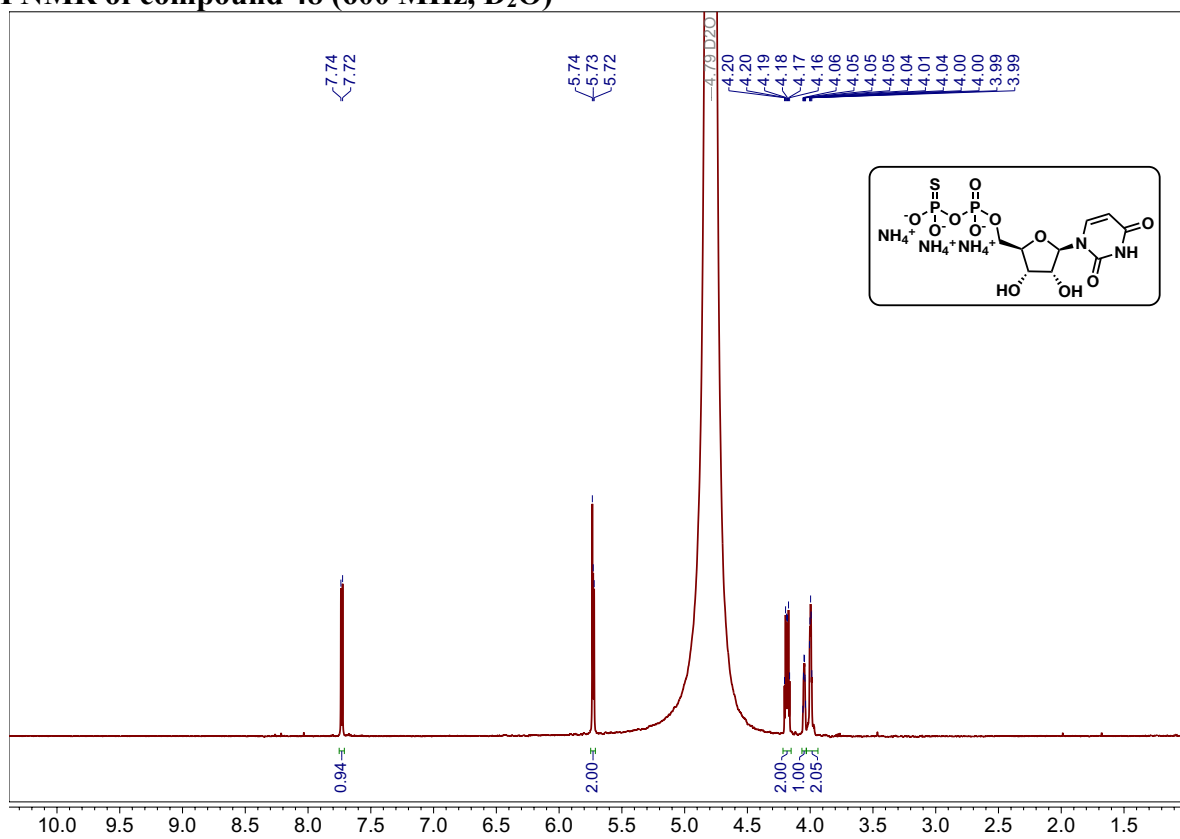
¹³C NMR of compound 47 (151 MHz, D₂O)



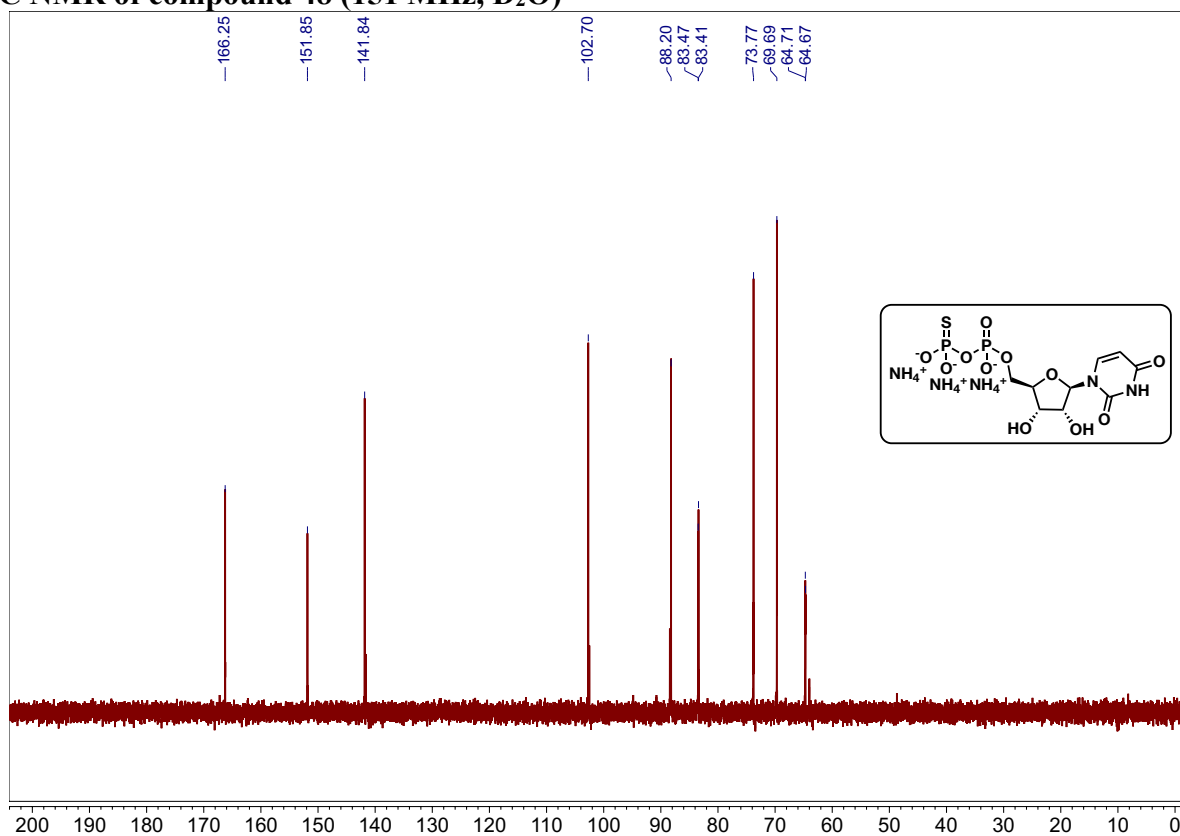
^{31}P NMR of compound 47 (243 MHz, D_2O)



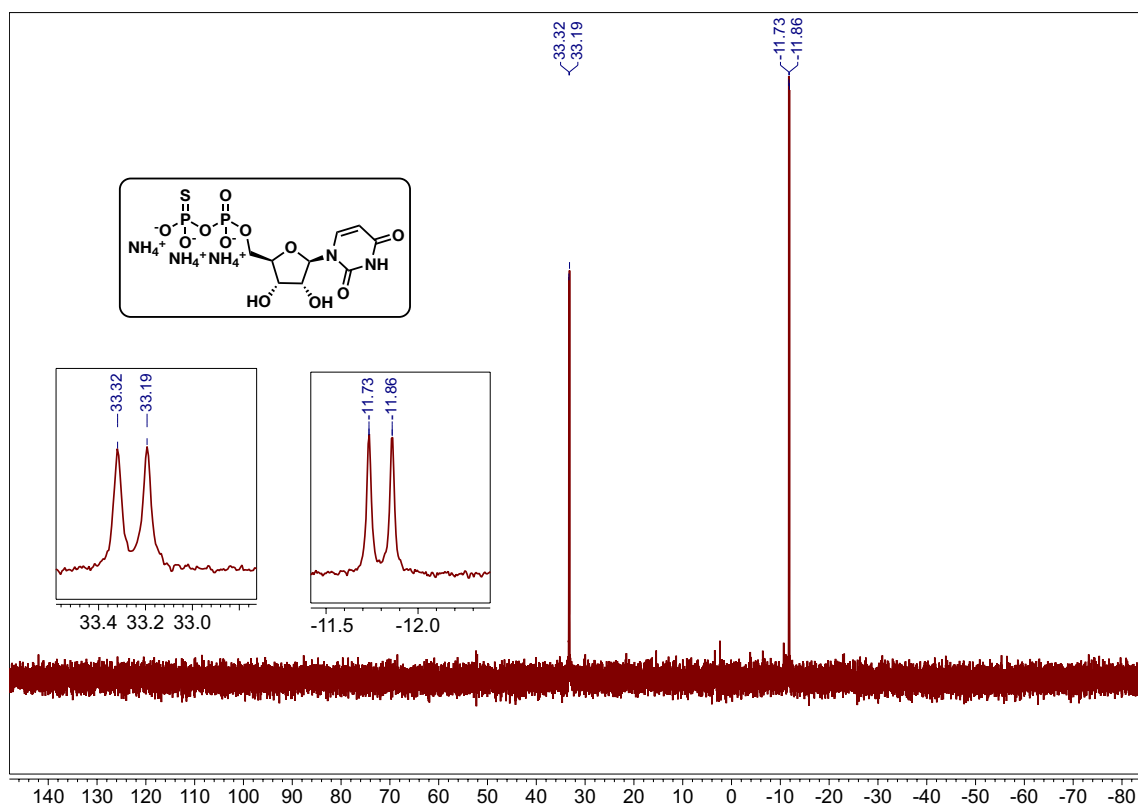
¹H NMR of compound 48 (600 MHz, D₂O)



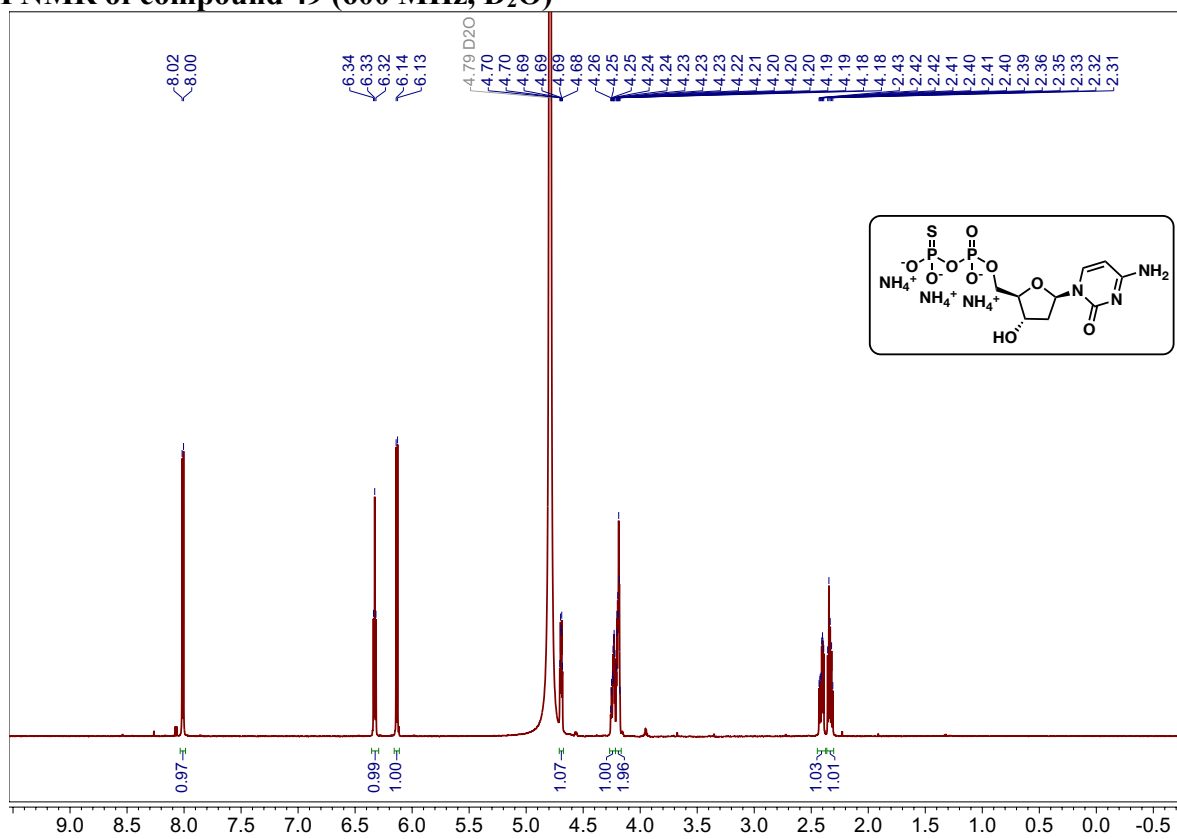
¹³C NMR of compound 48 (151 MHz, D₂O)



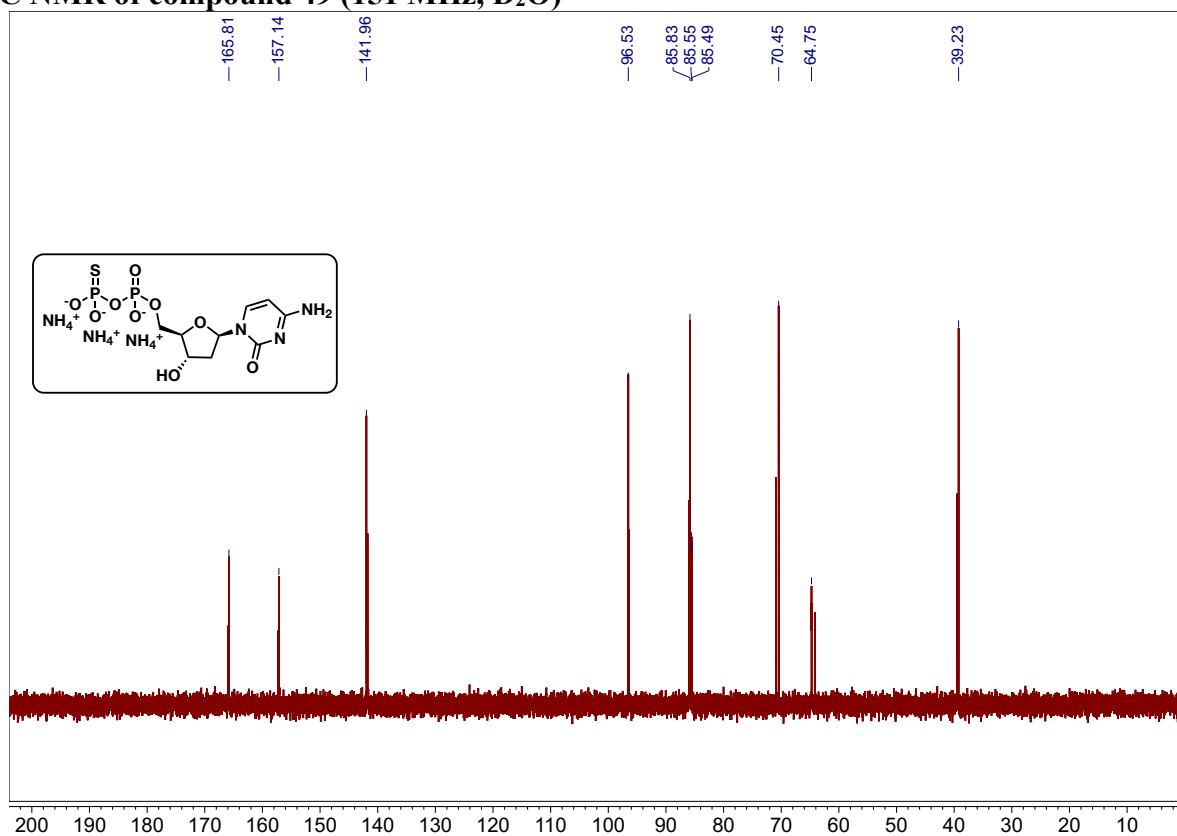
³¹P NMR of compound 48 (243 MHz, D₂O)



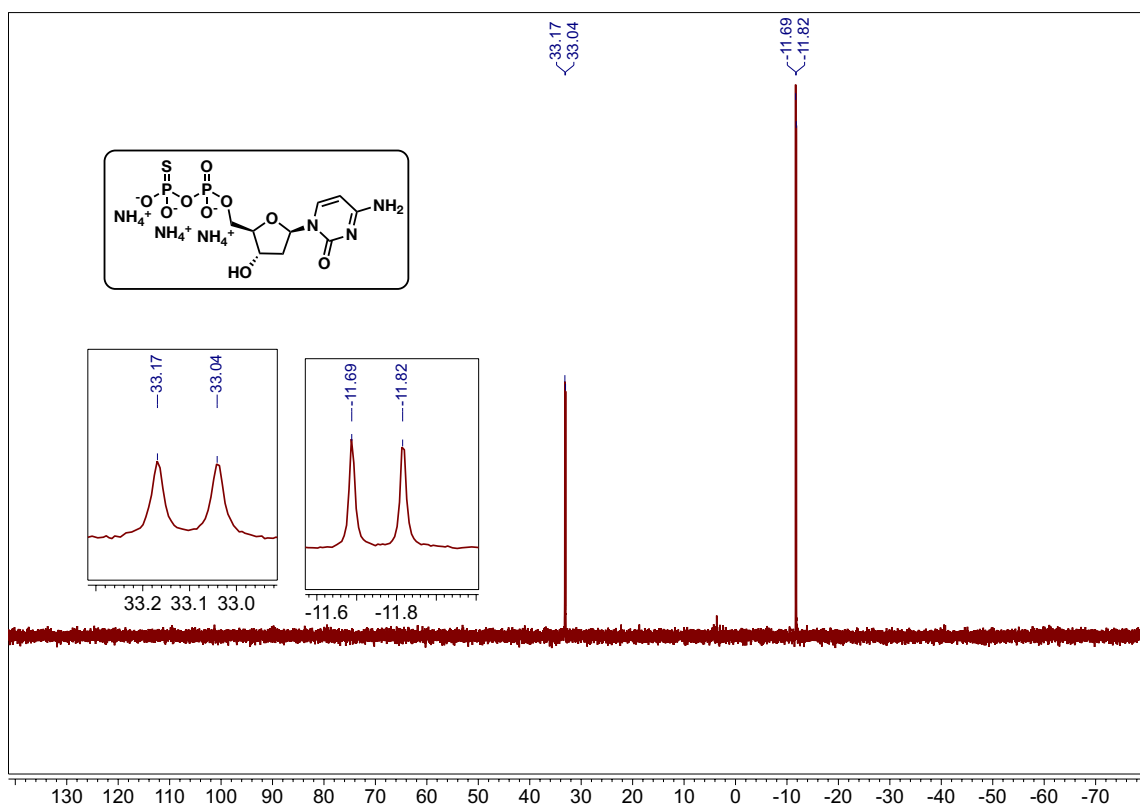
¹H NMR of compound 49 (600 MHz, D₂O)



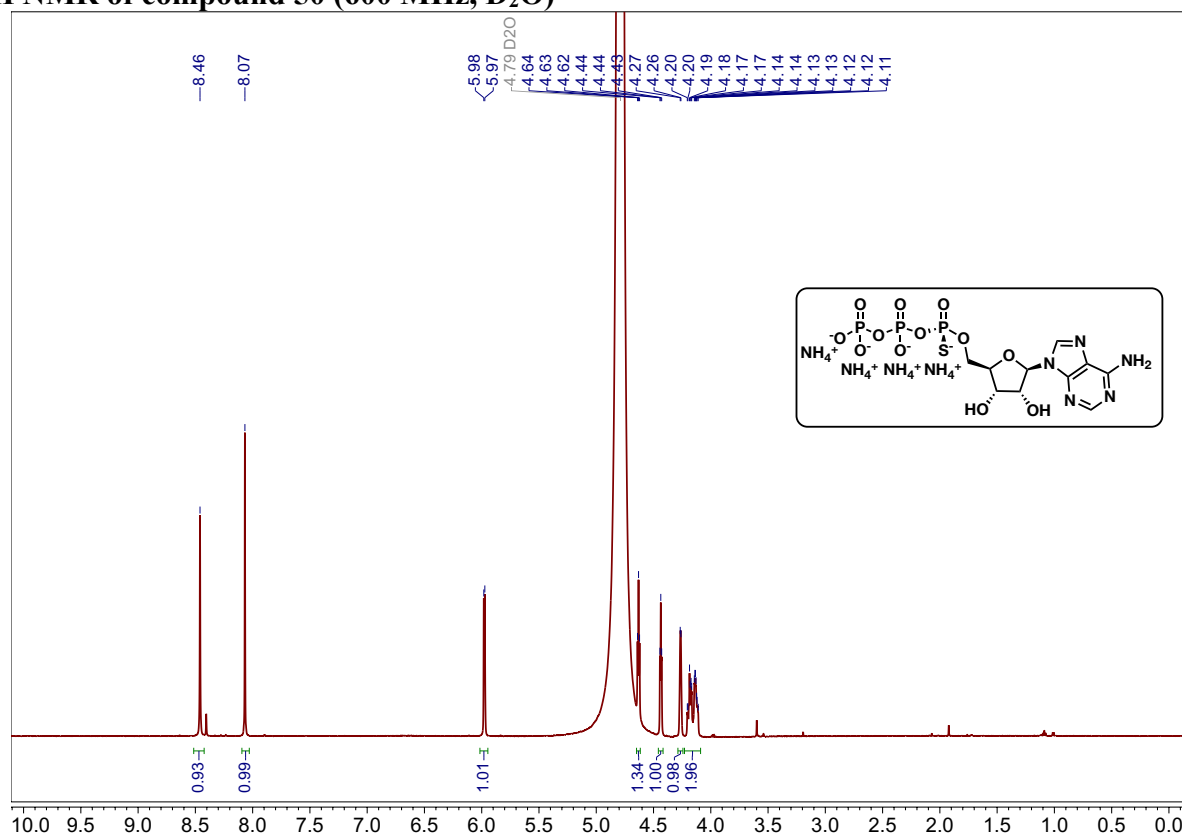
¹³C NMR of compound 49 (151 MHz, D₂O)



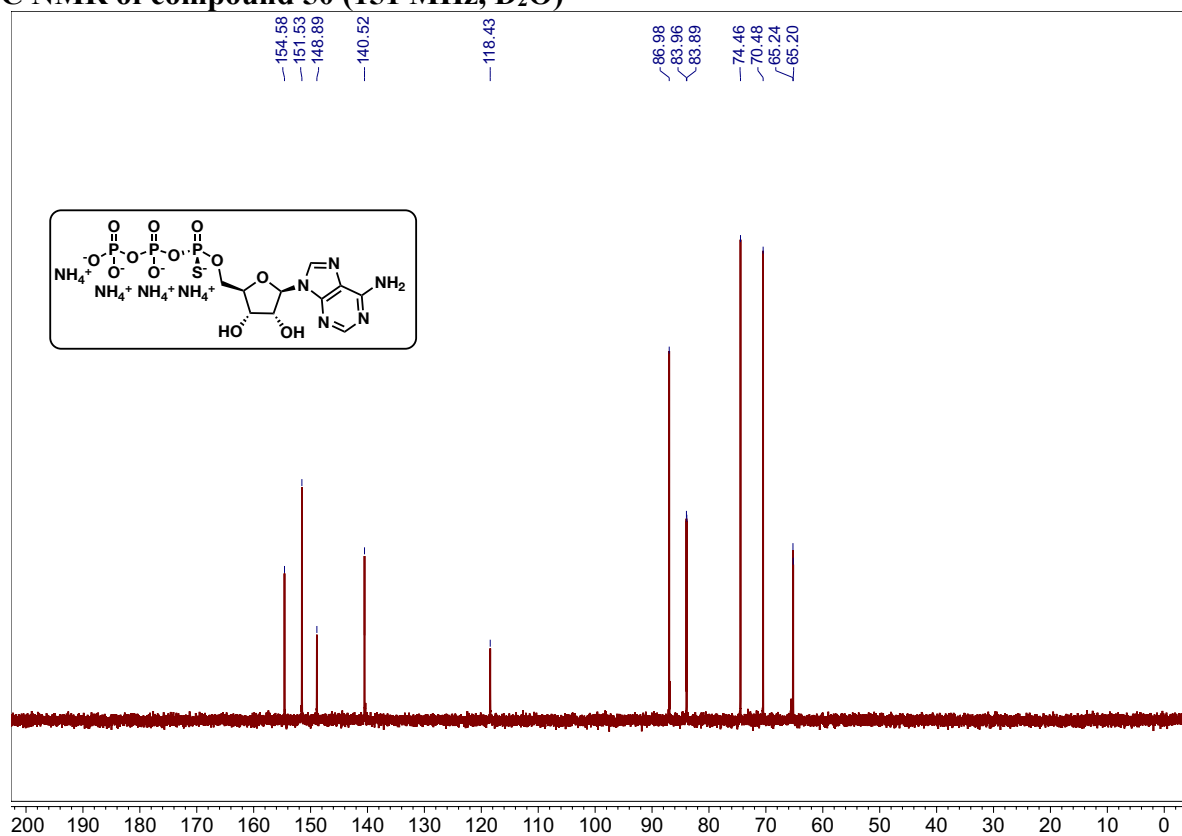
^{31}P NMR of compound 49 (243 MHz, D_2O)



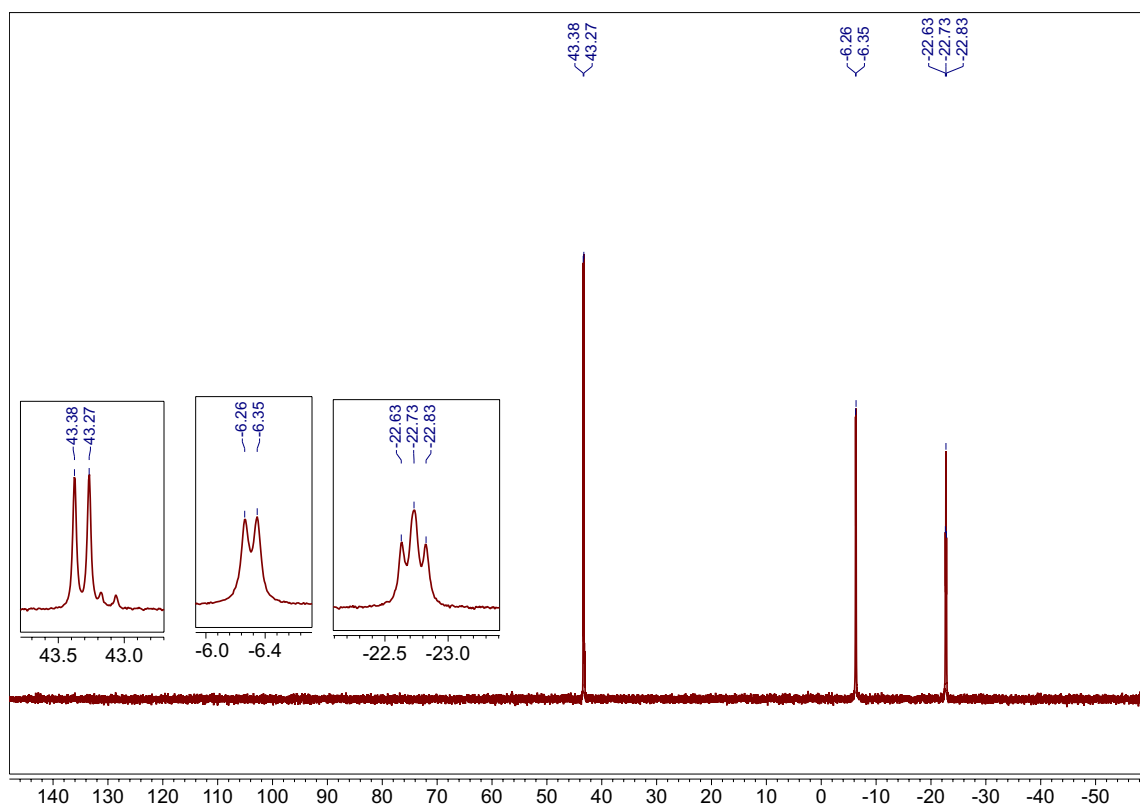
¹H NMR of compound 50 (600 MHz, D₂O)



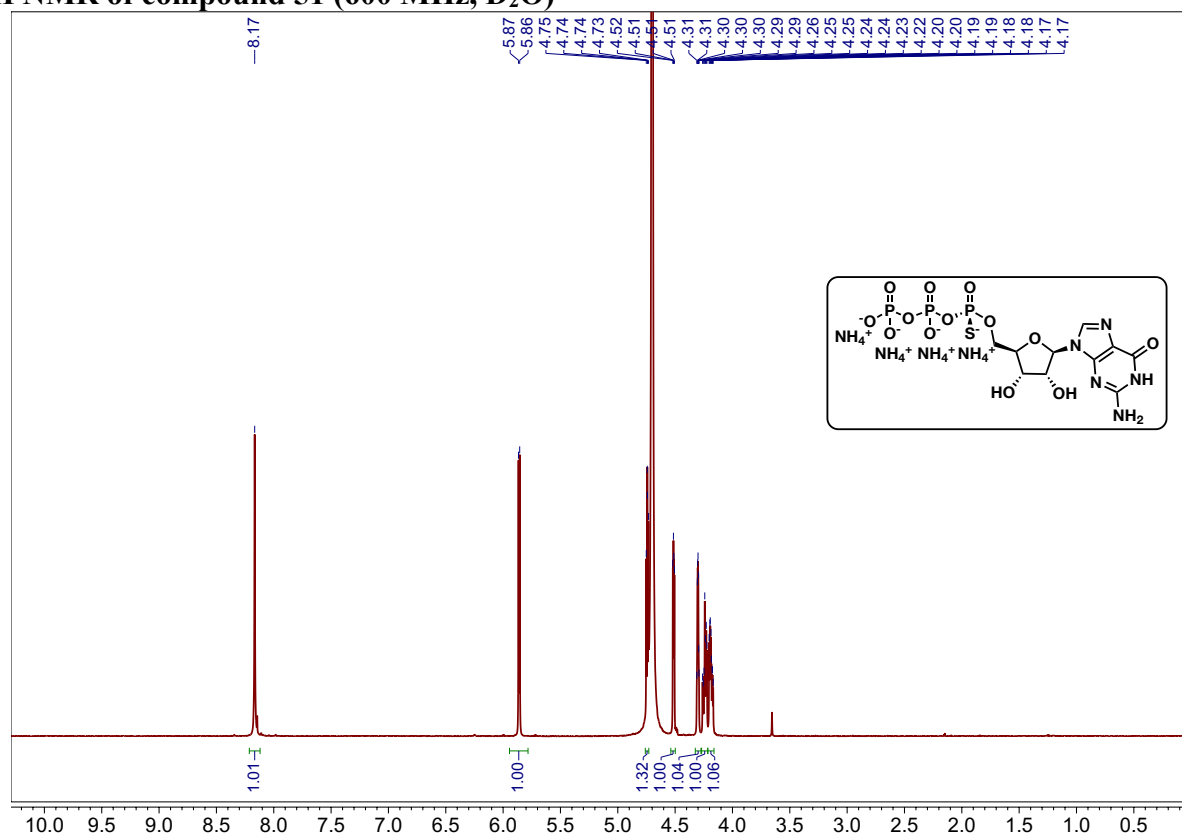
¹³C NMR of compound 50 (151 MHz, D₂O)



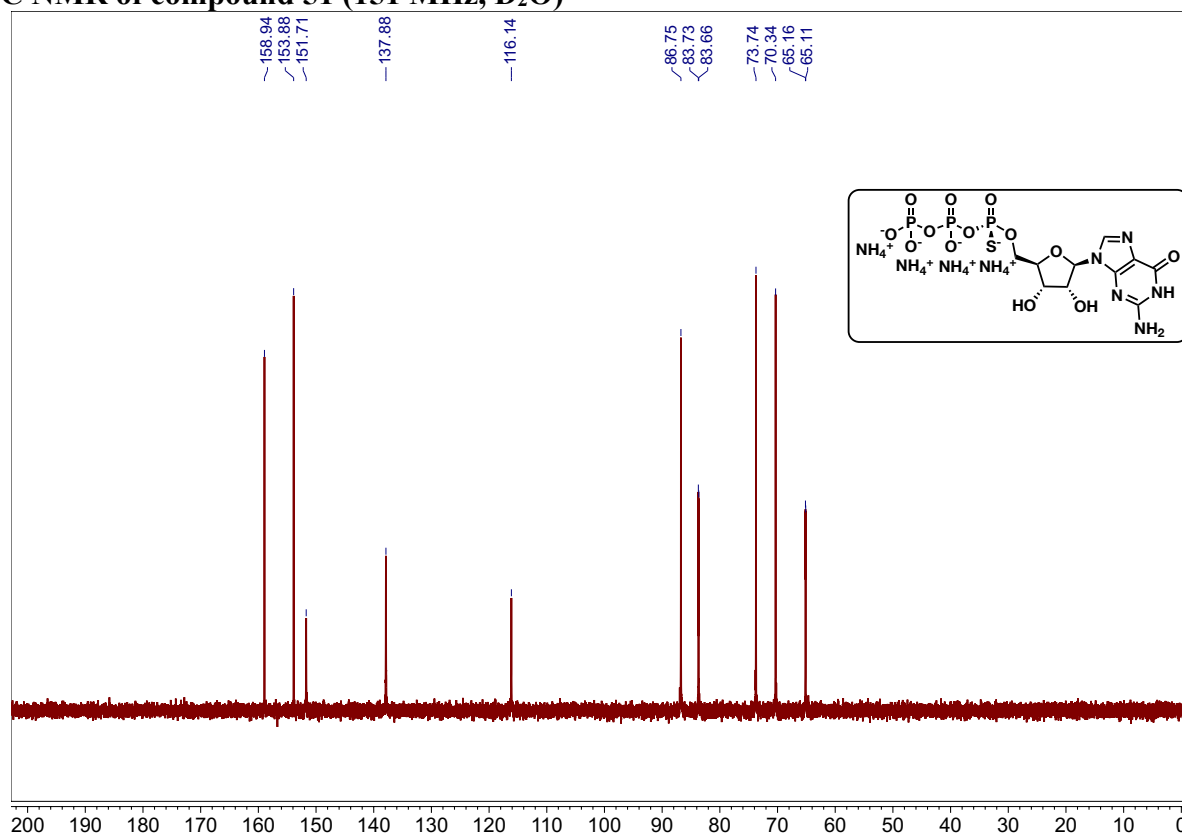
^{31}P NMR of compound 50 (243 MHz, D_2O)



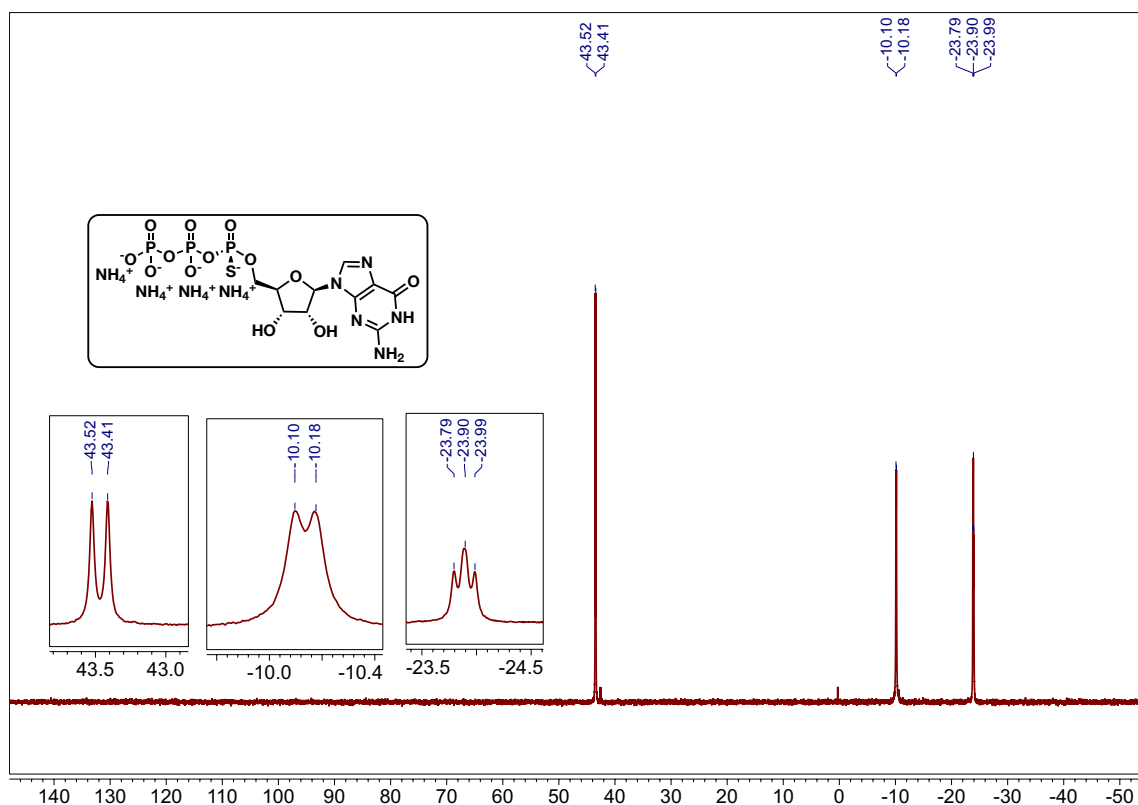
^1H NMR of compound 51 (600 MHz, D_2O)



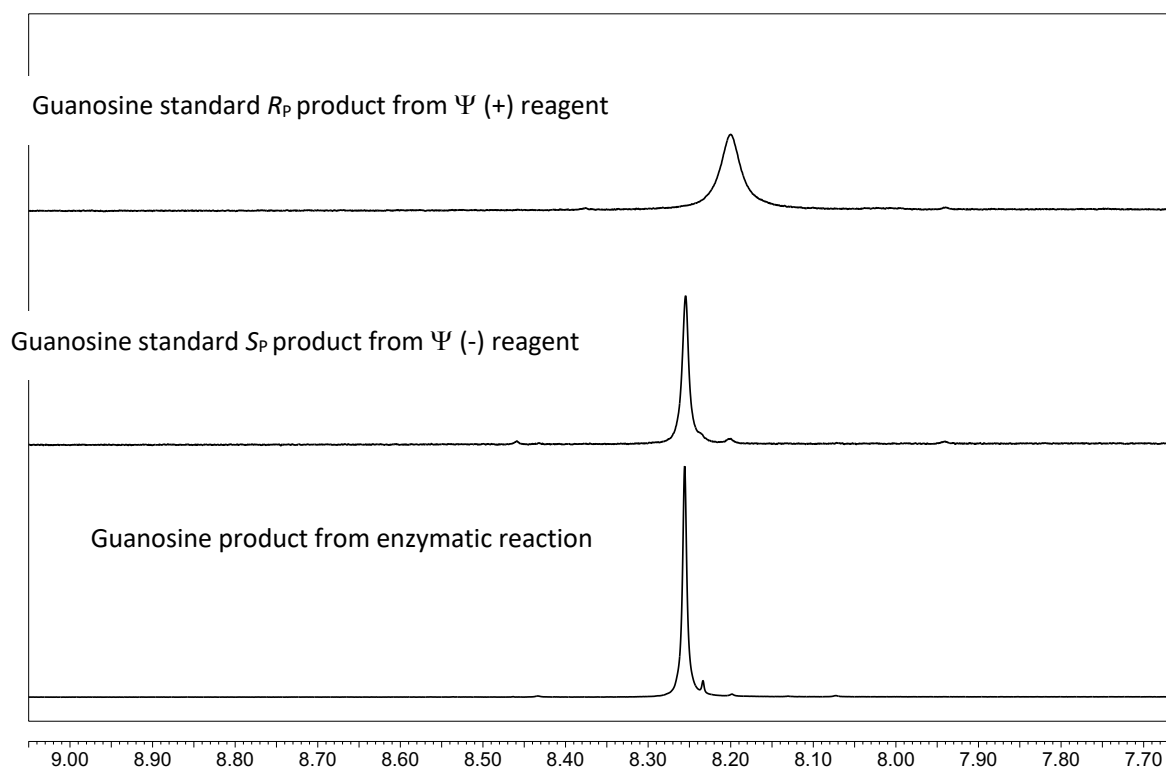
^{13}C NMR of compound 51 (151 MHz, D_2O)



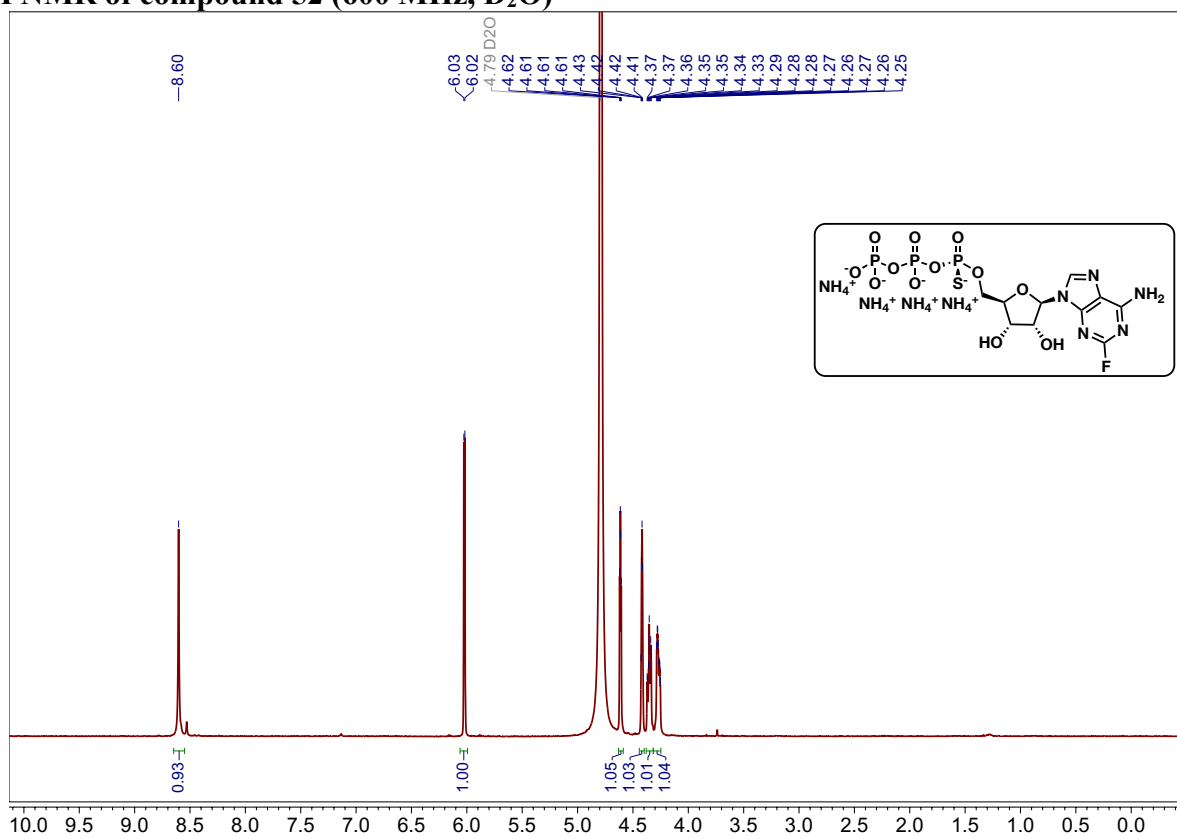
³¹P NMR of compound 51 (243 MHz, D₂O)



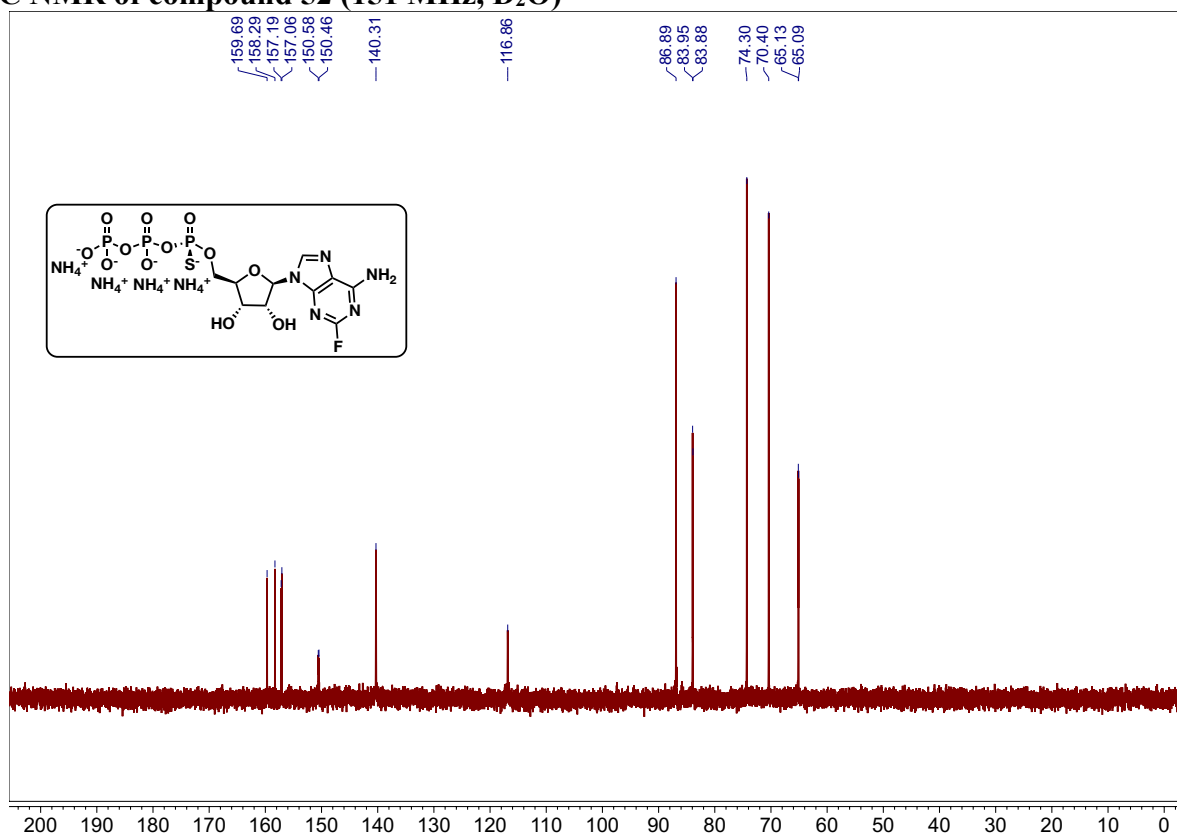
Comparison of enzymatic product and standard samples



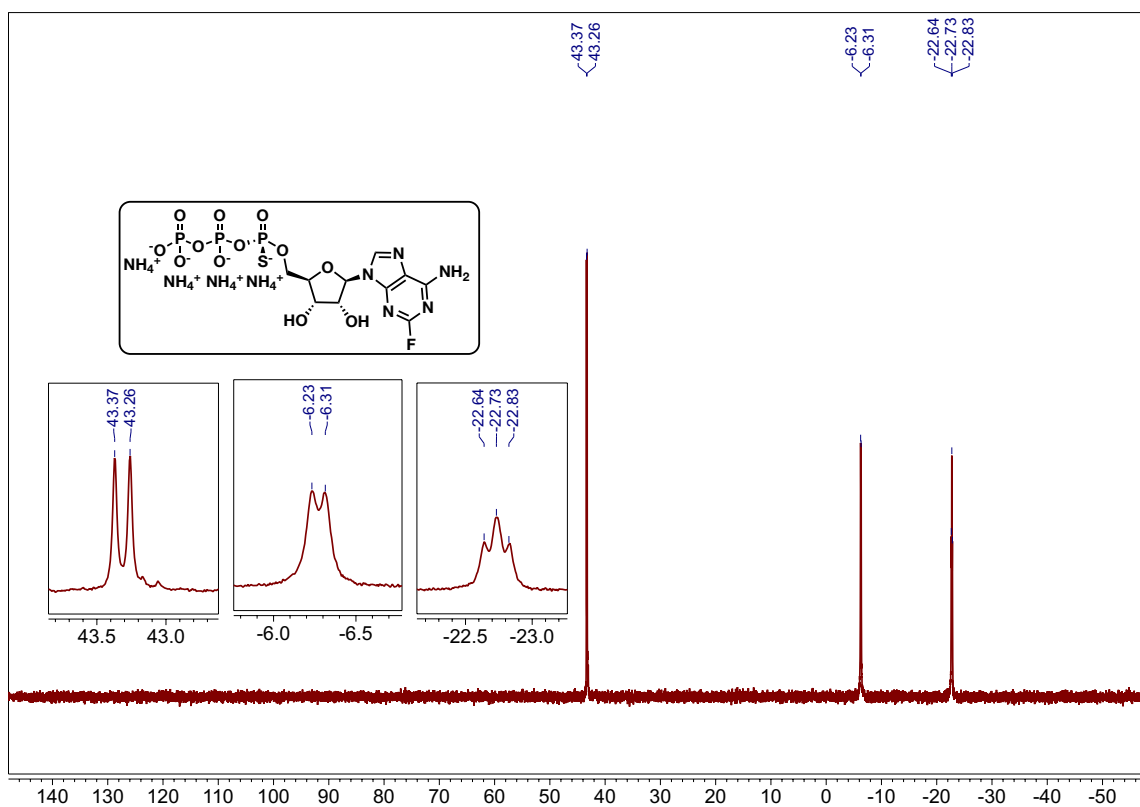
¹H NMR of compound 52 (600 MHz, D₂O)



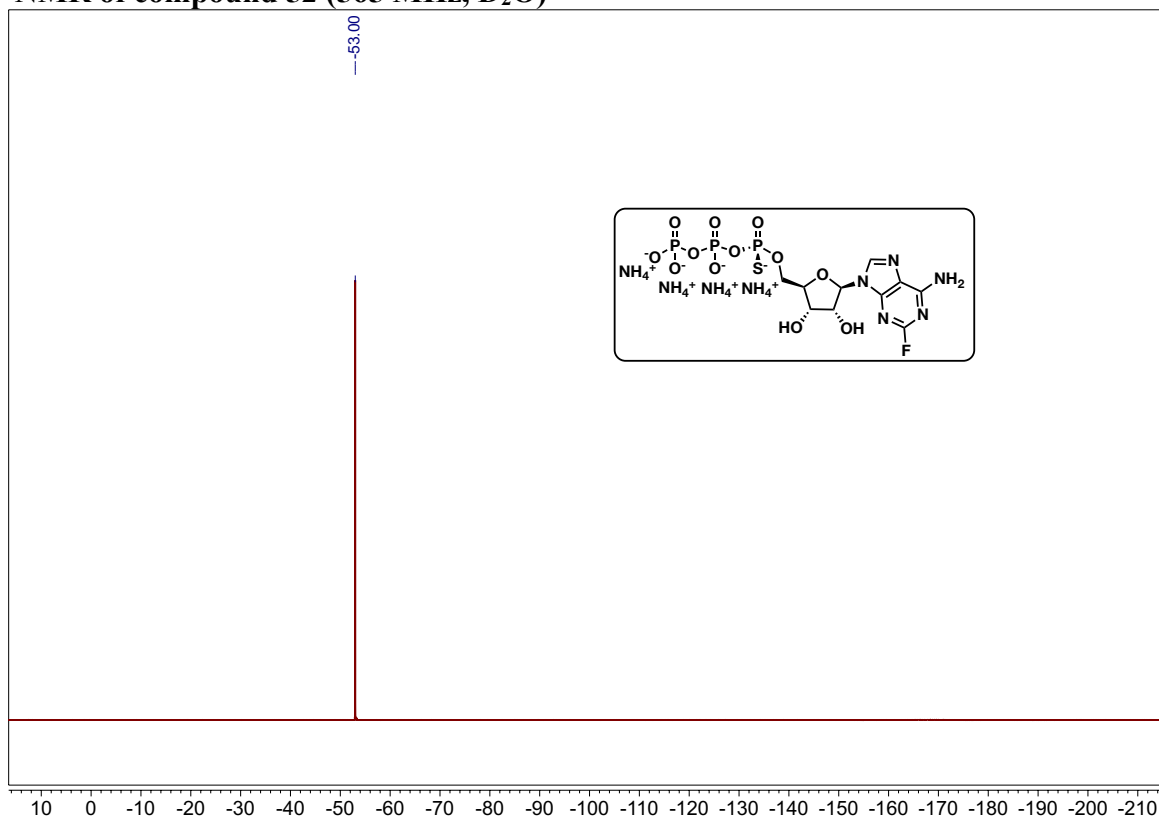
¹³C NMR of compound 52 (151 MHz, D₂O)



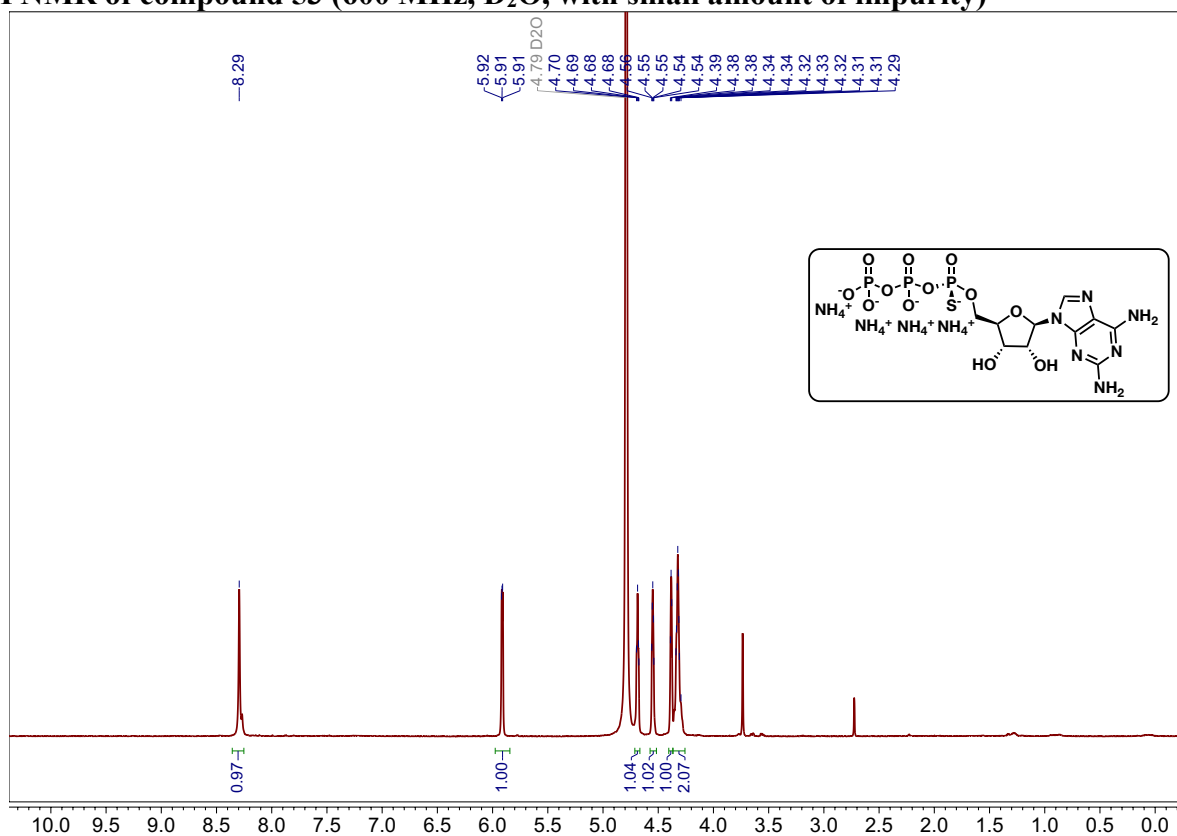
^{31}P NMR of compound 52 (243 MHz, D_2O)



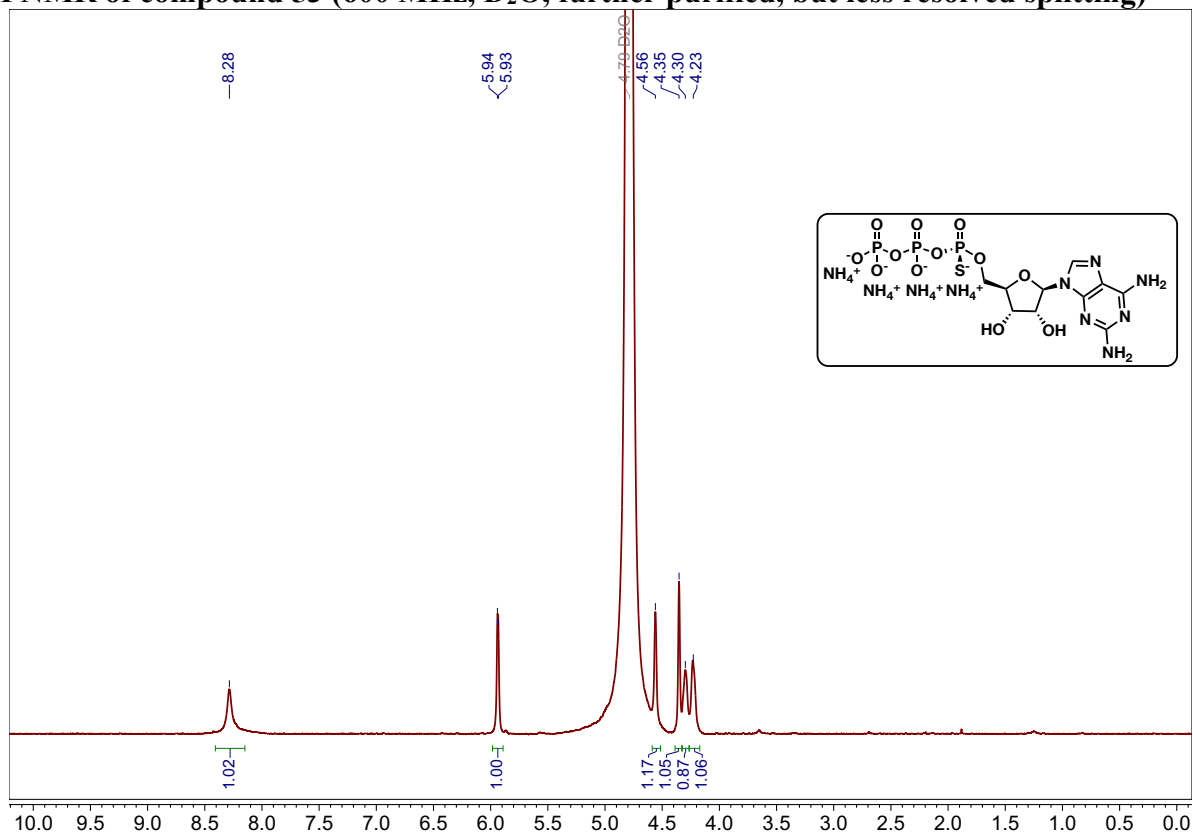
^{19}F NMR of compound 52 (565 MHz, D_2O)



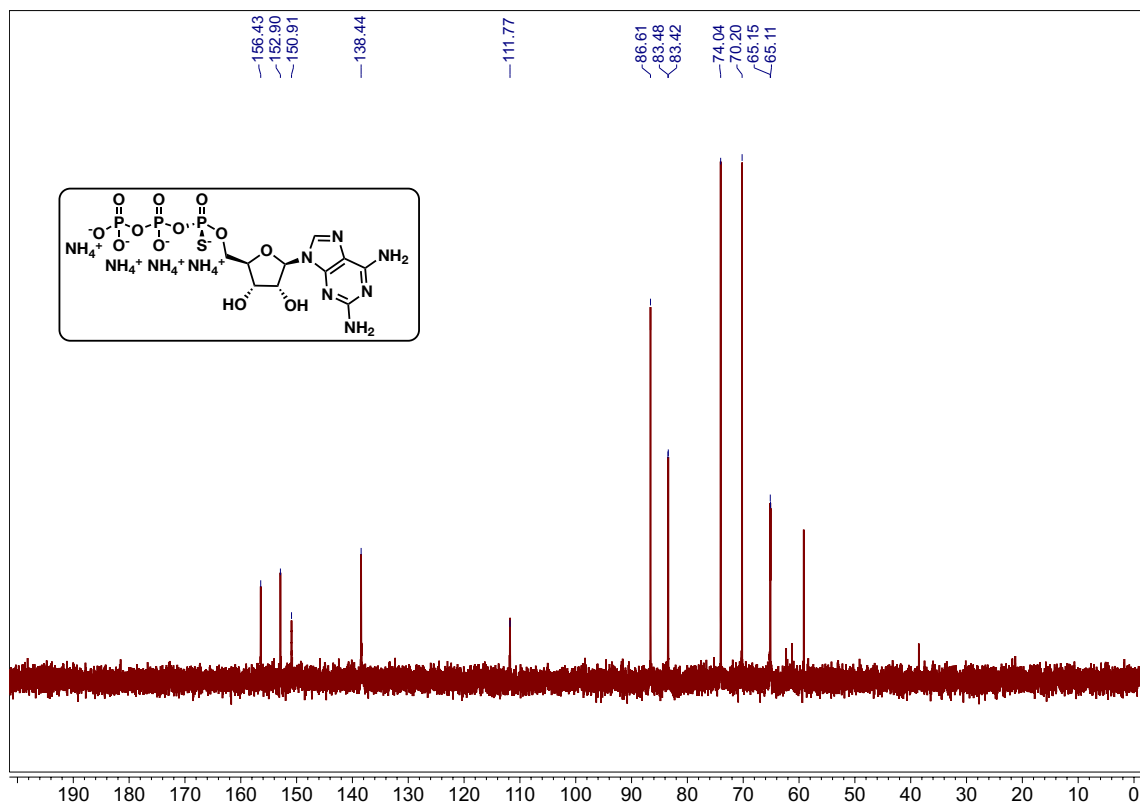
^1H NMR of compound 53 (600 MHz, D_2O , with small amount of impurity)



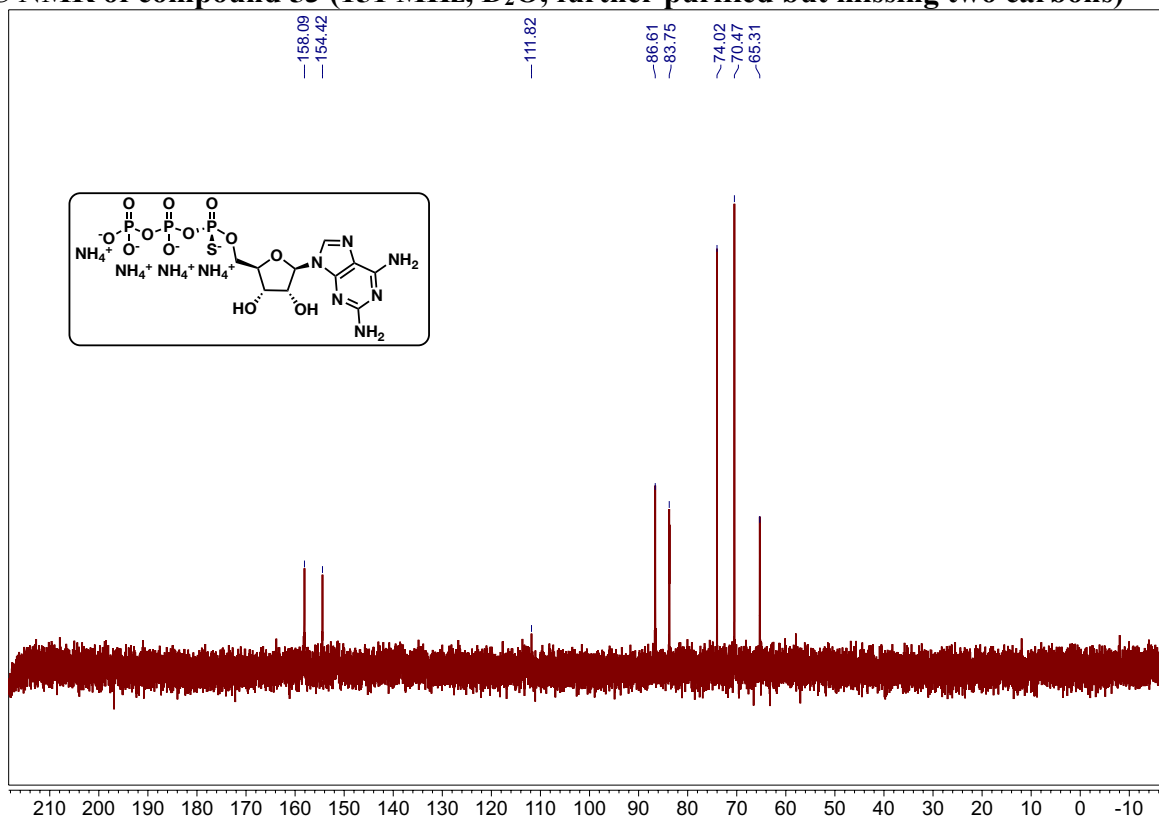
^1H NMR of compound 53 (600 MHz, D_2O , further purified, but less resolved splitting)



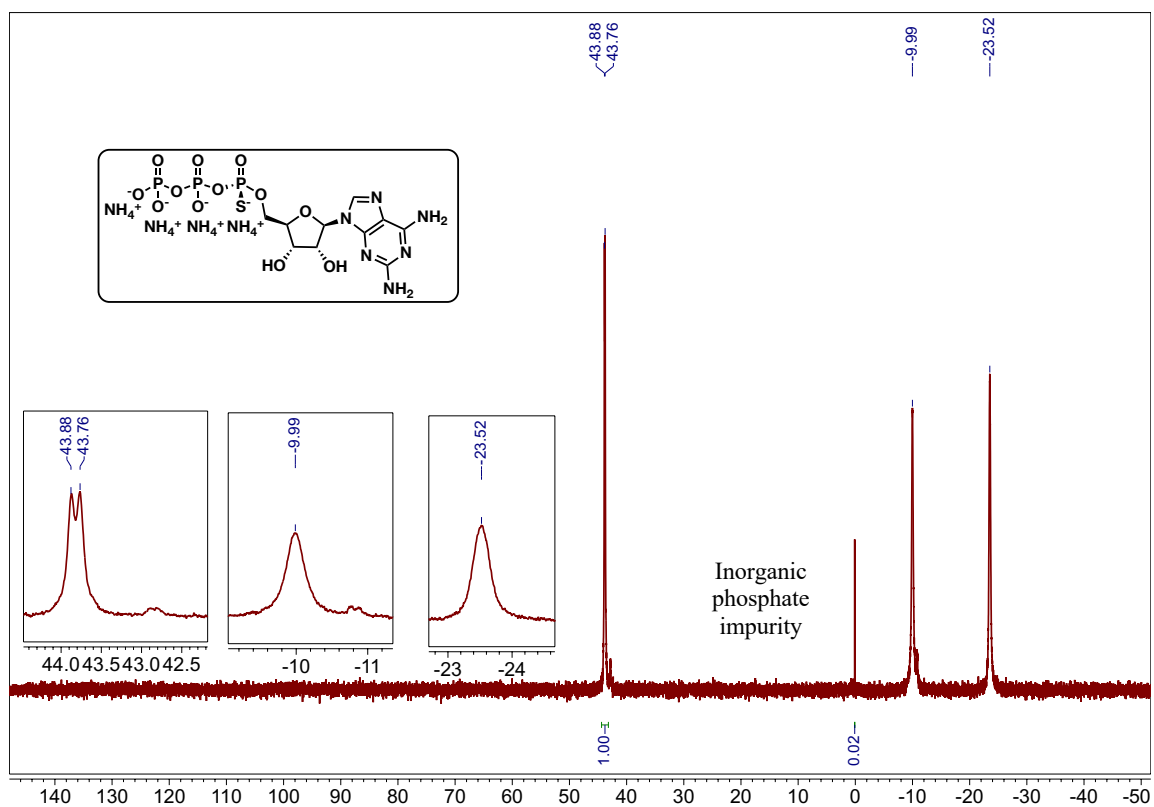
^{13}C NMR of compound 53 (151 MHz, D_2O , with small amount of impurity)



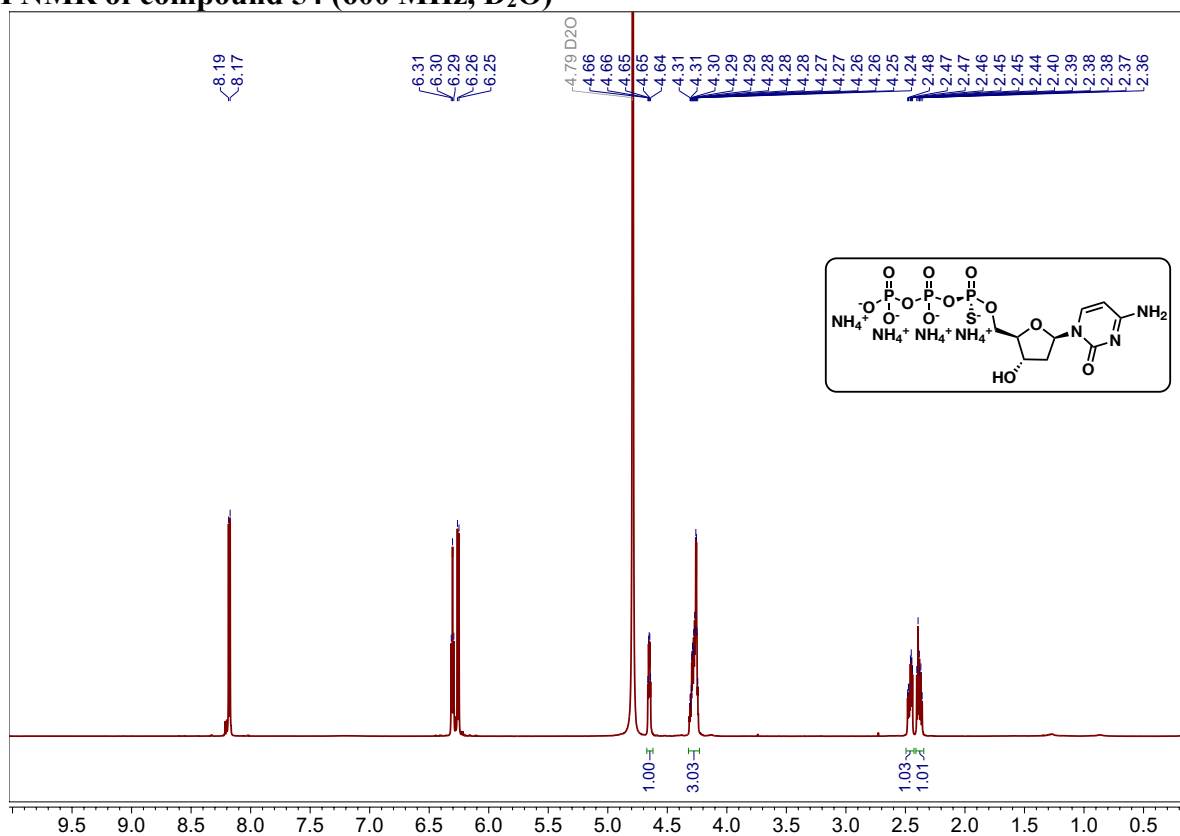
^{13}C NMR of compound 53 (151 MHz, D_2O , further purified but missing two carbons)



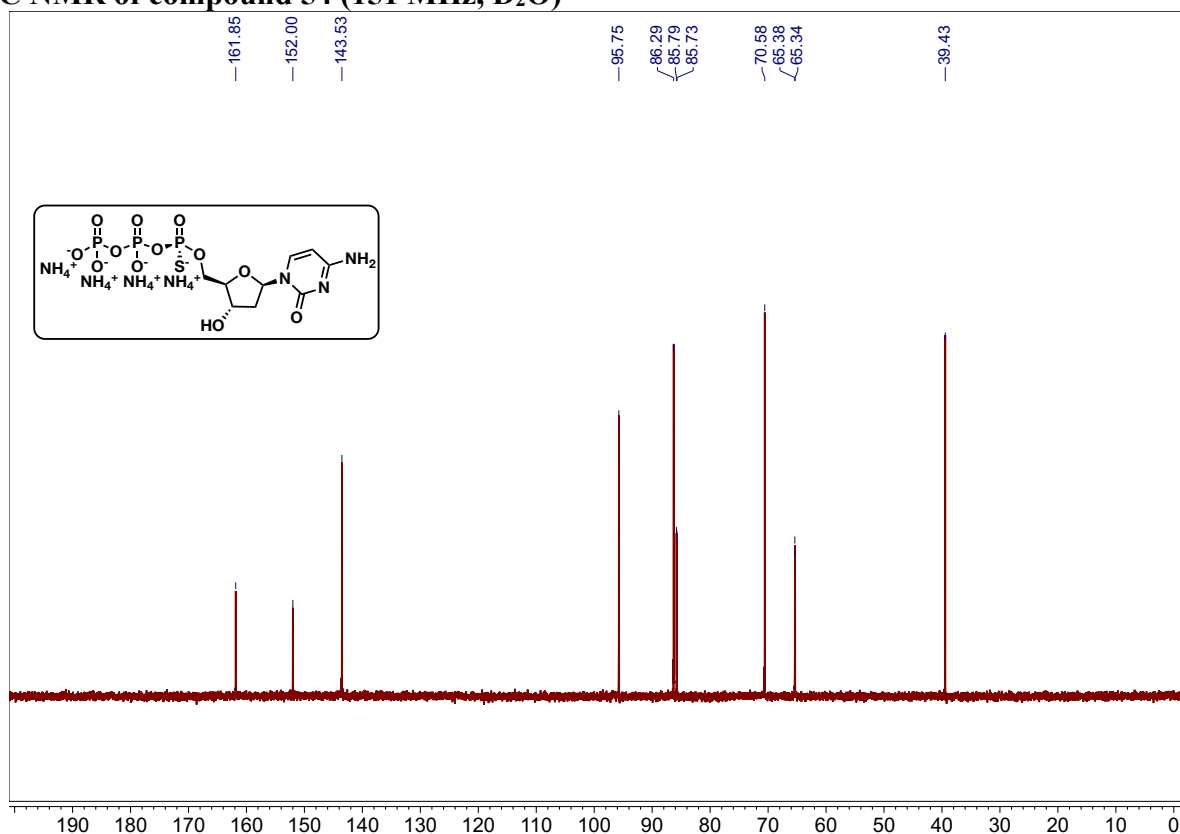
^{31}P NMR of compound 53 (243 MHz, D_2O)



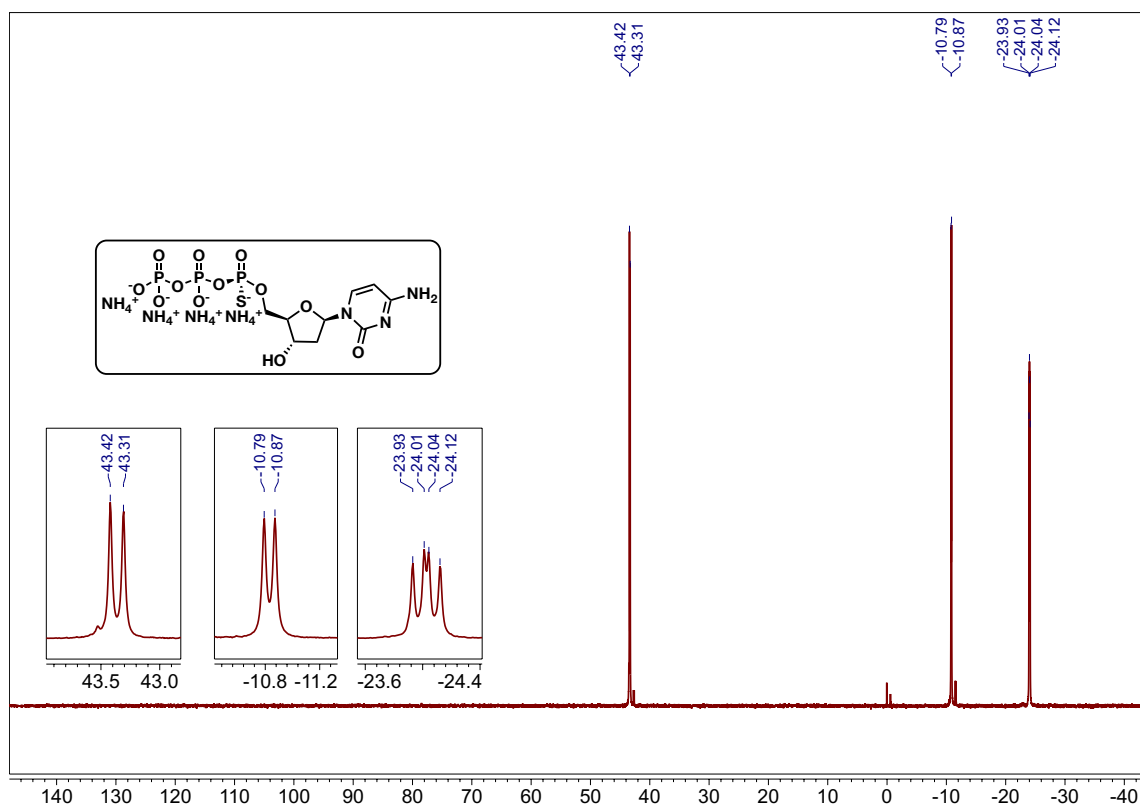
¹H NMR of compound 54 (600 MHz, D₂O)



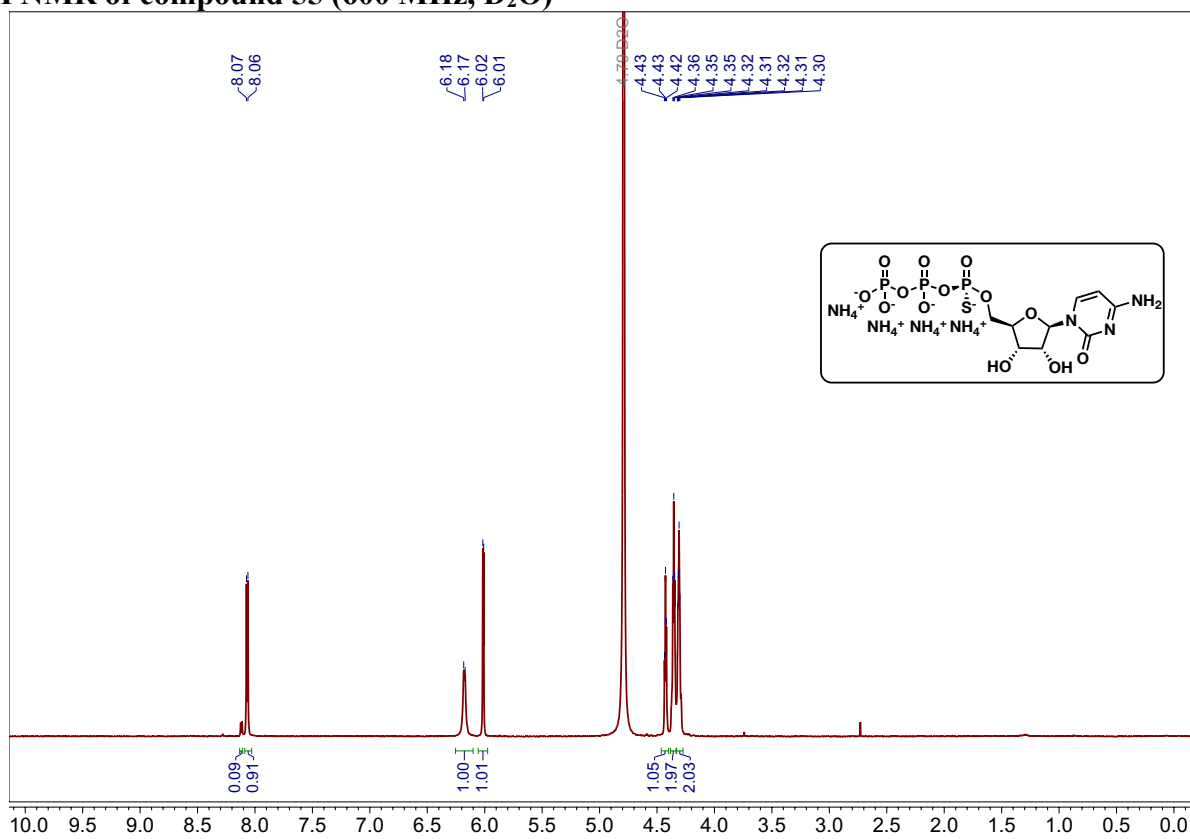
¹³C NMR of compound 54 (151 MHz, D₂O)



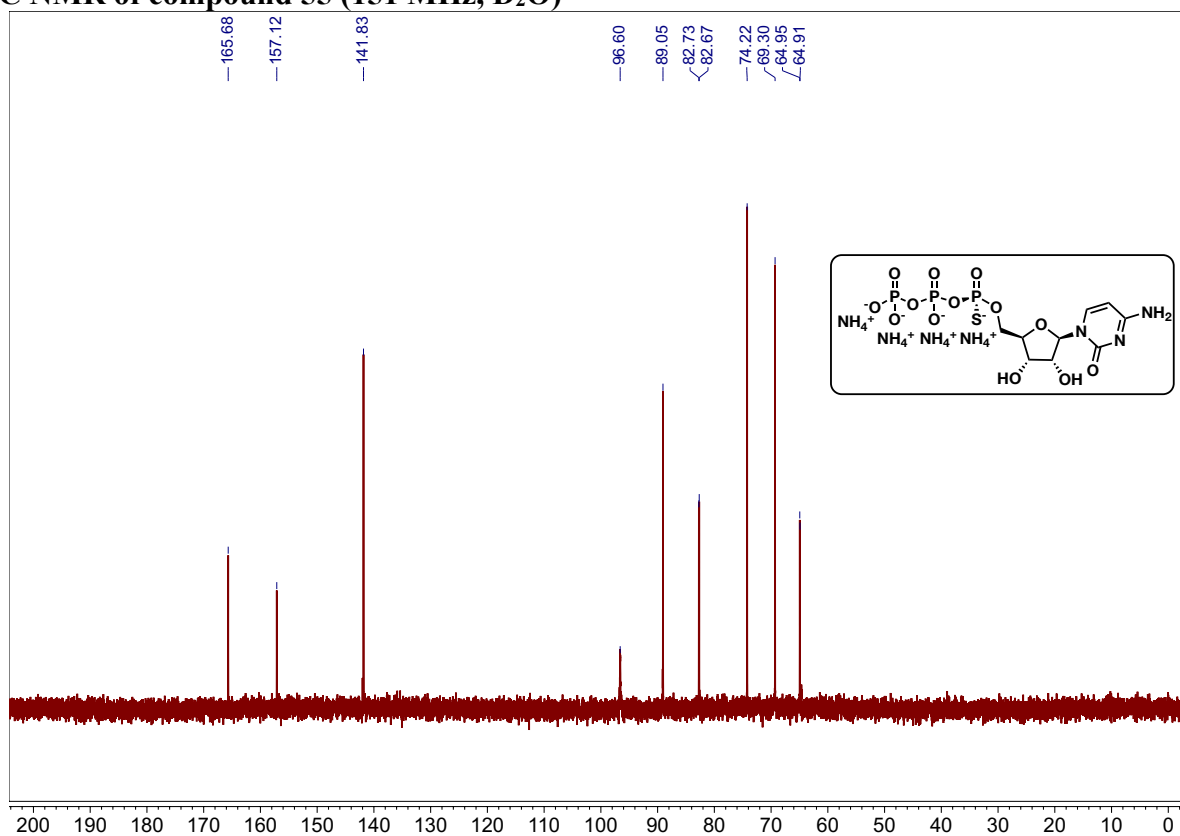
^{31}P NMR of compound 54 (243 MHz, D_2O)



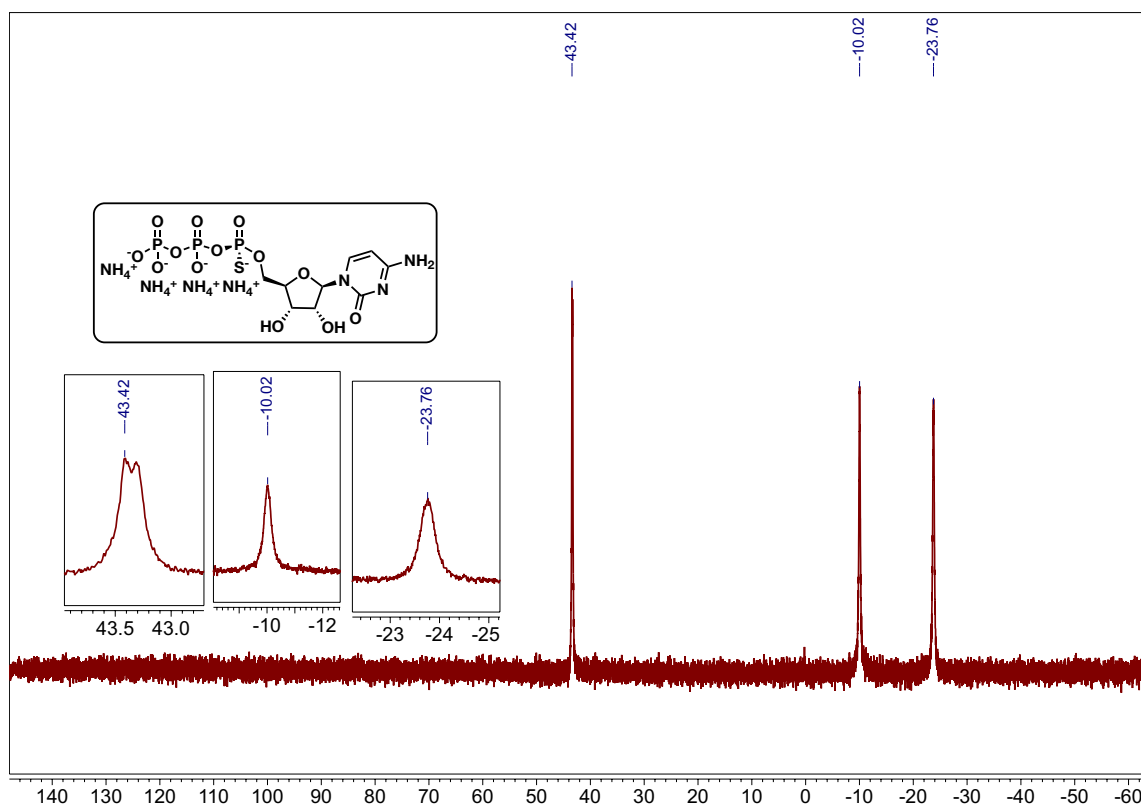
¹H NMR of compound 55 (600 MHz, D₂O)



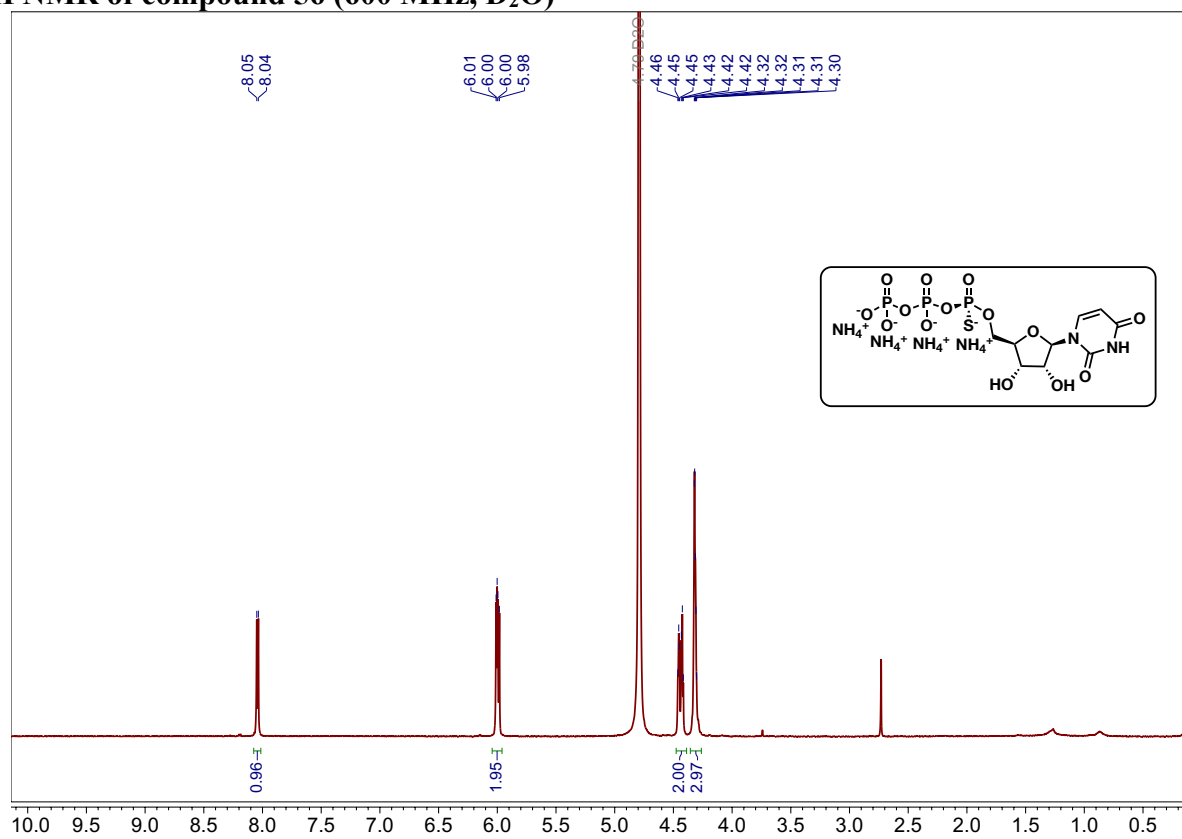
¹³C NMR of compound 55 (151 MHz, D₂O)



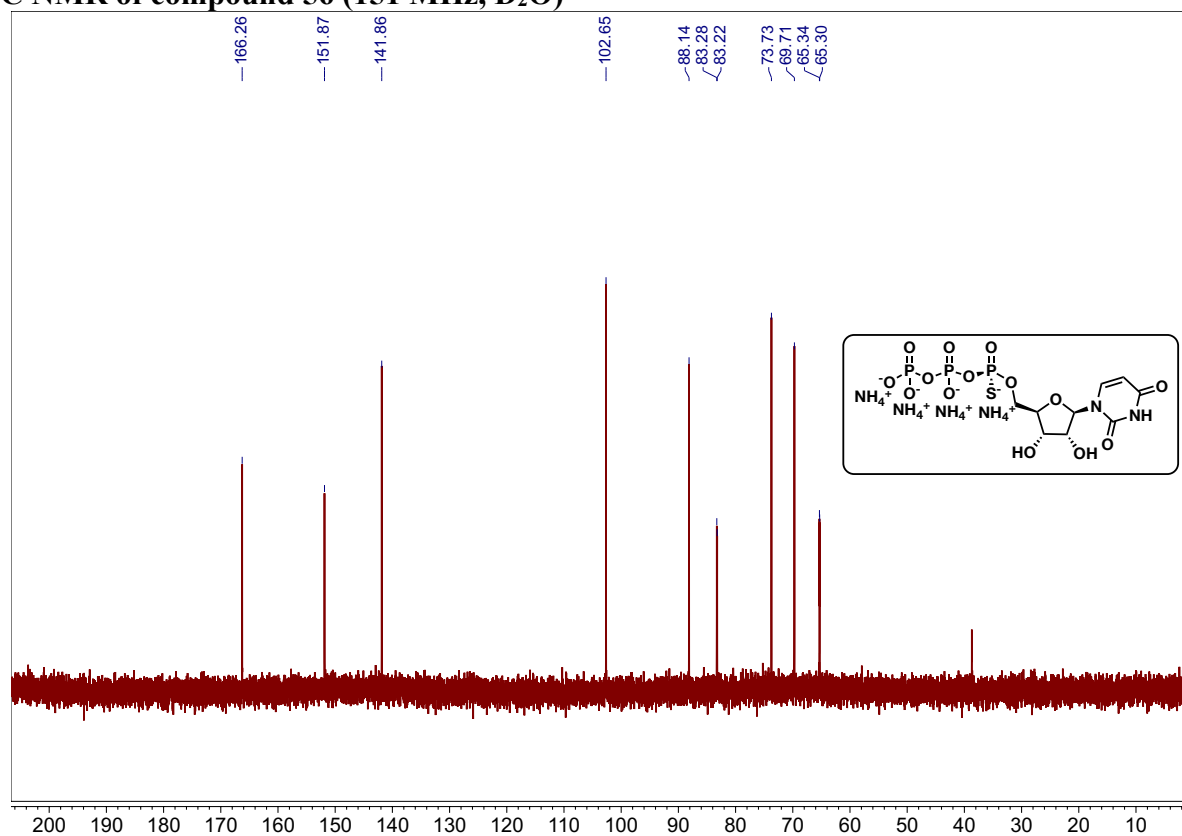
^{31}P NMR of compound 55 (243 MHz, D_2O)



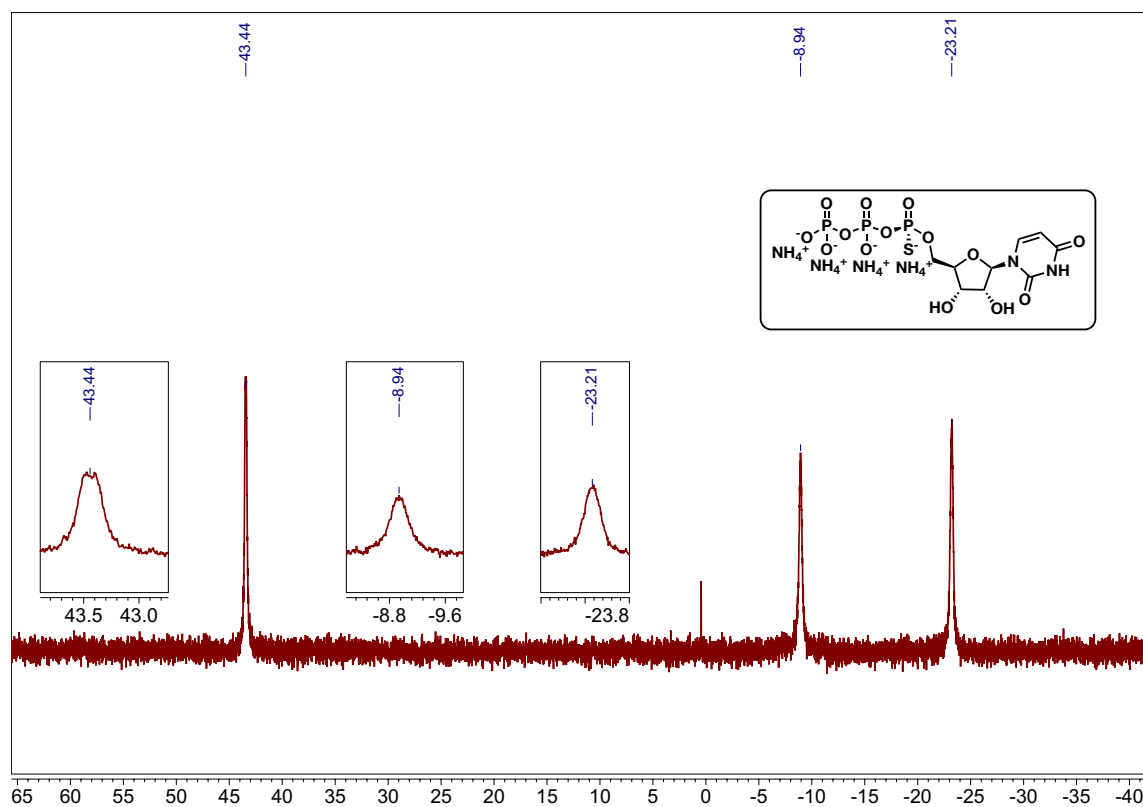
^1H NMR of compound 56 (600 MHz, D_2O)



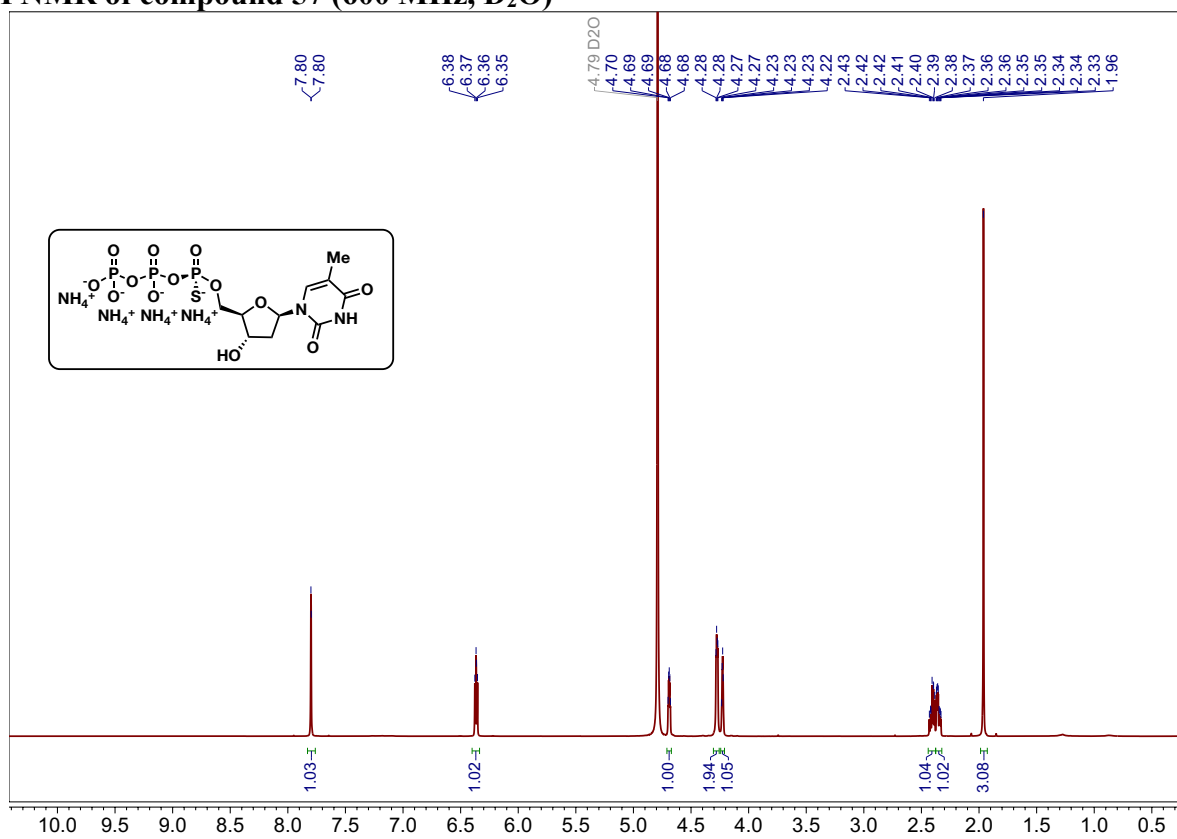
^{13}C NMR of compound 56 (151 MHz, D_2O)



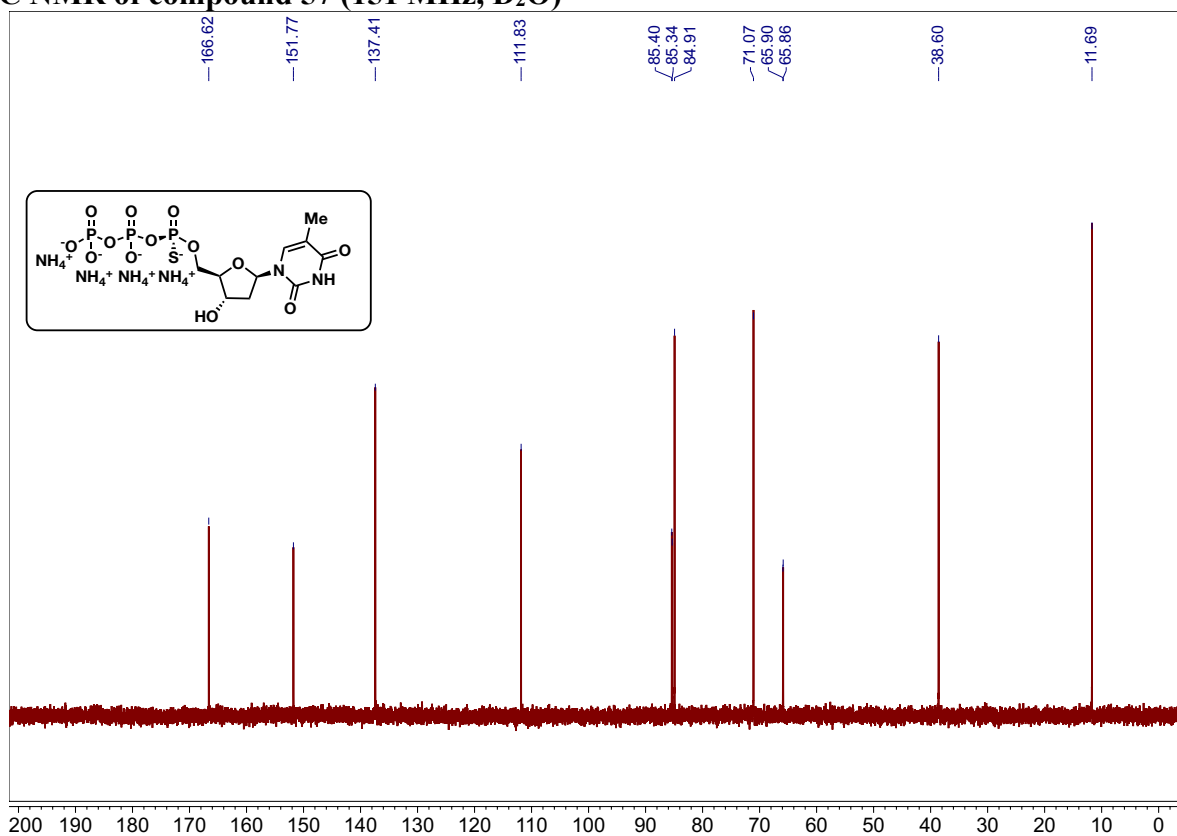
^{31}P NMR of compound 56 (243 MHz, D_2O)



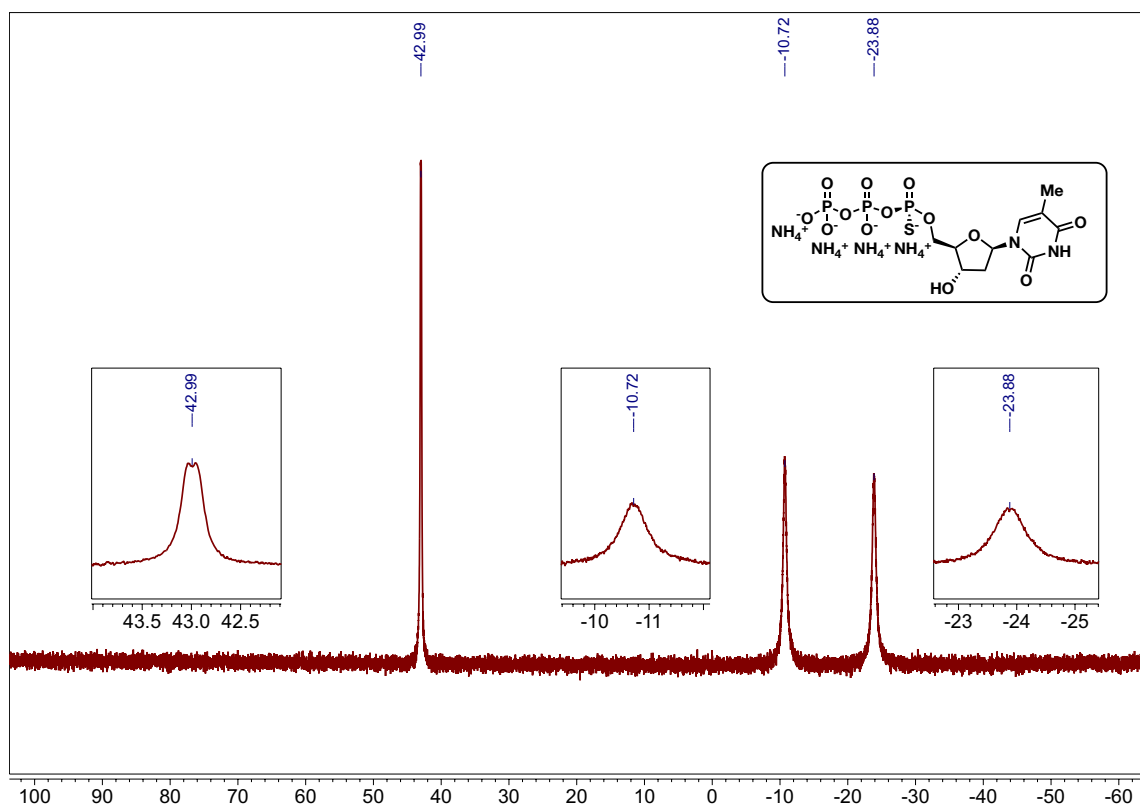
^1H NMR of compound 57 (600 MHz, D_2O)



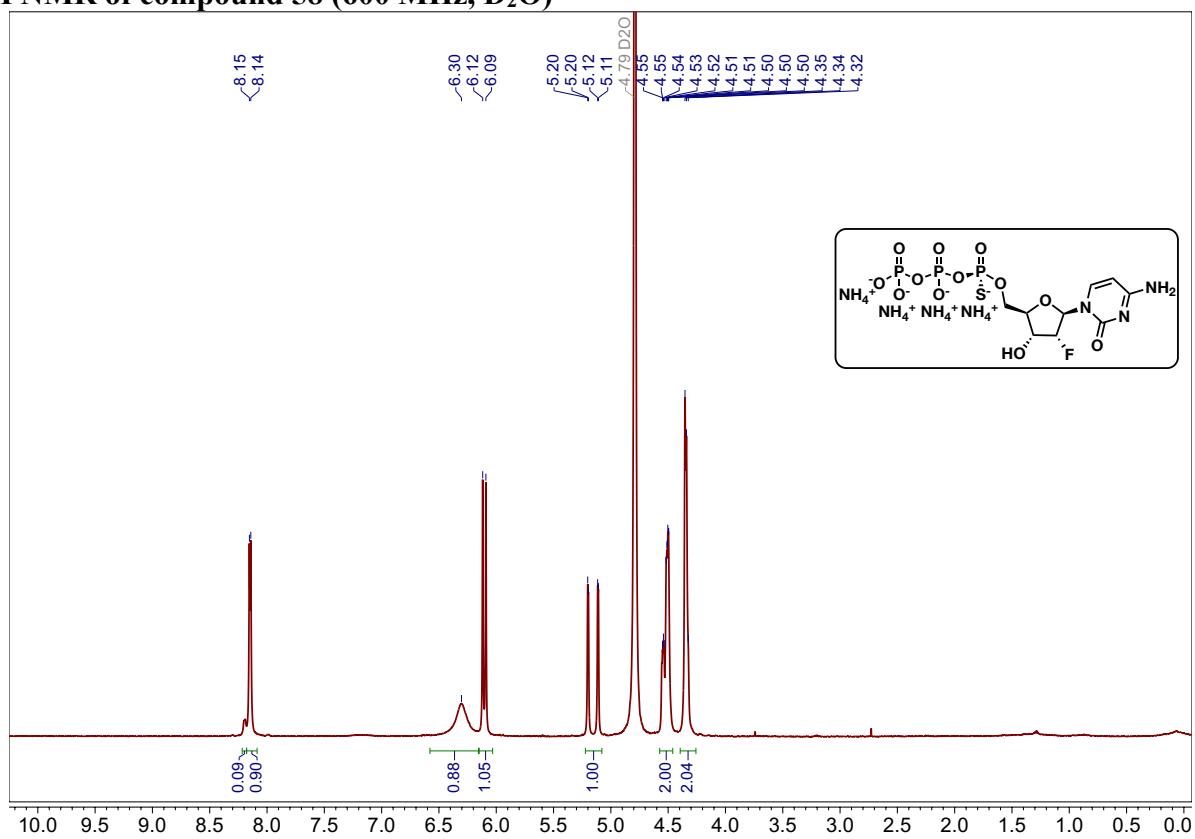
^{13}C NMR of compound 57 (151 MHz, D_2O)



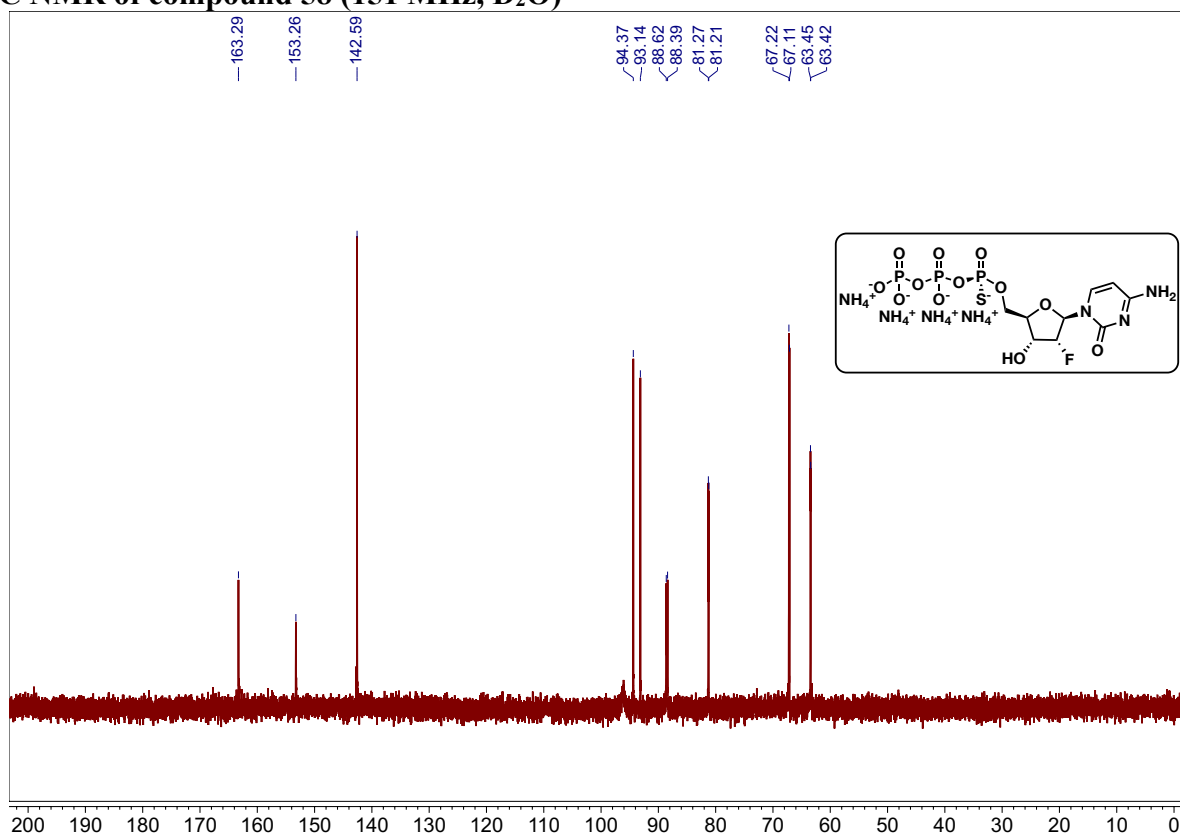
^{31}P NMR of compound 57 (243 MHz, D_2O)



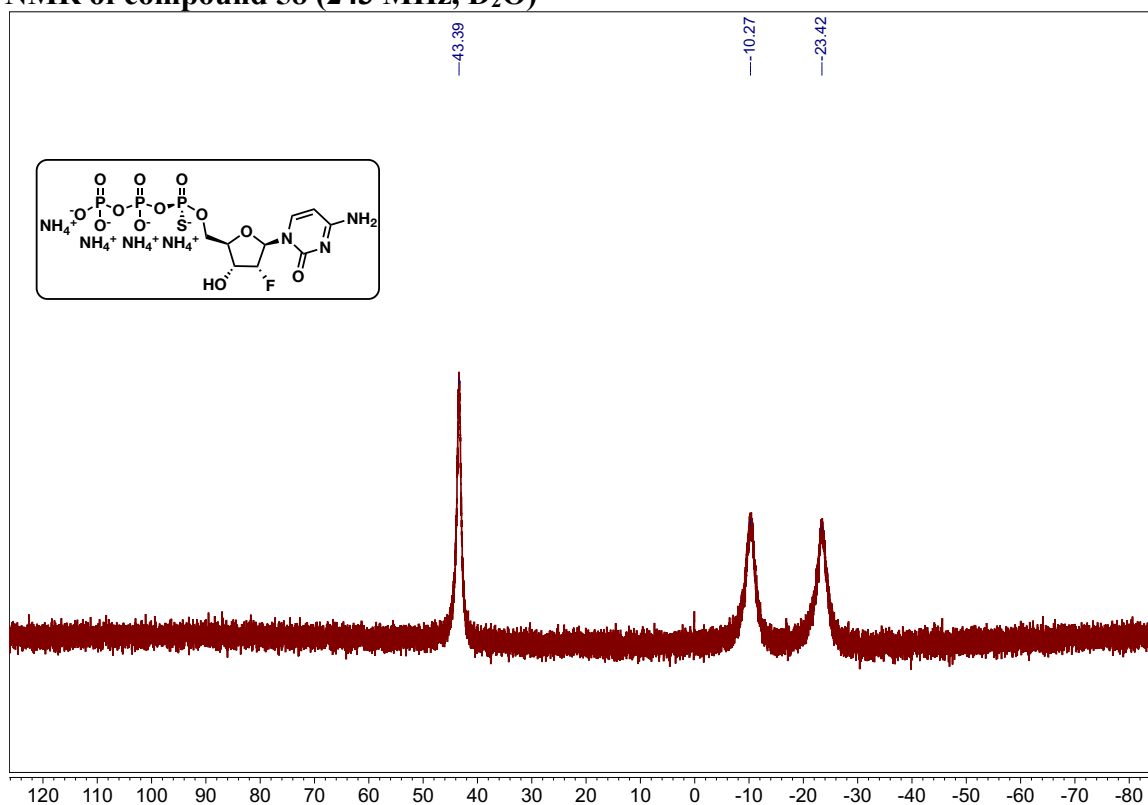
^1H NMR of compound 58 (600 MHz, D_2O)



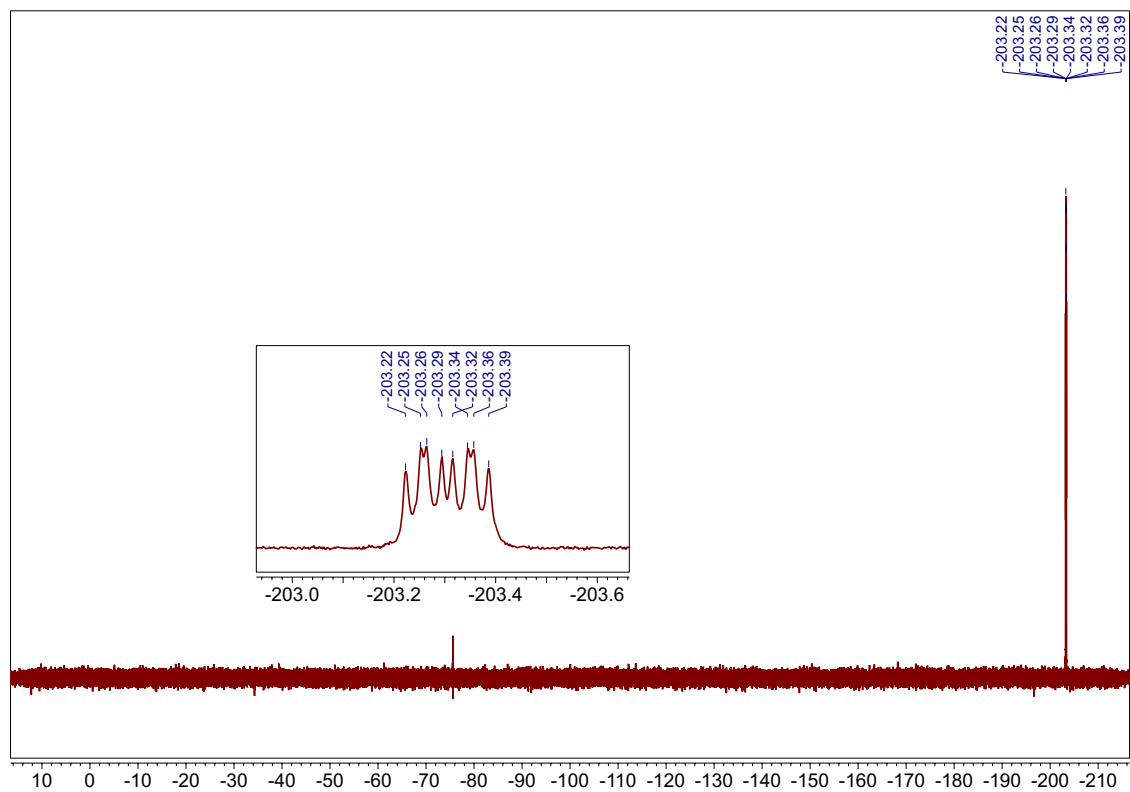
^{13}C NMR of compound 58 (151 MHz, D_2O)



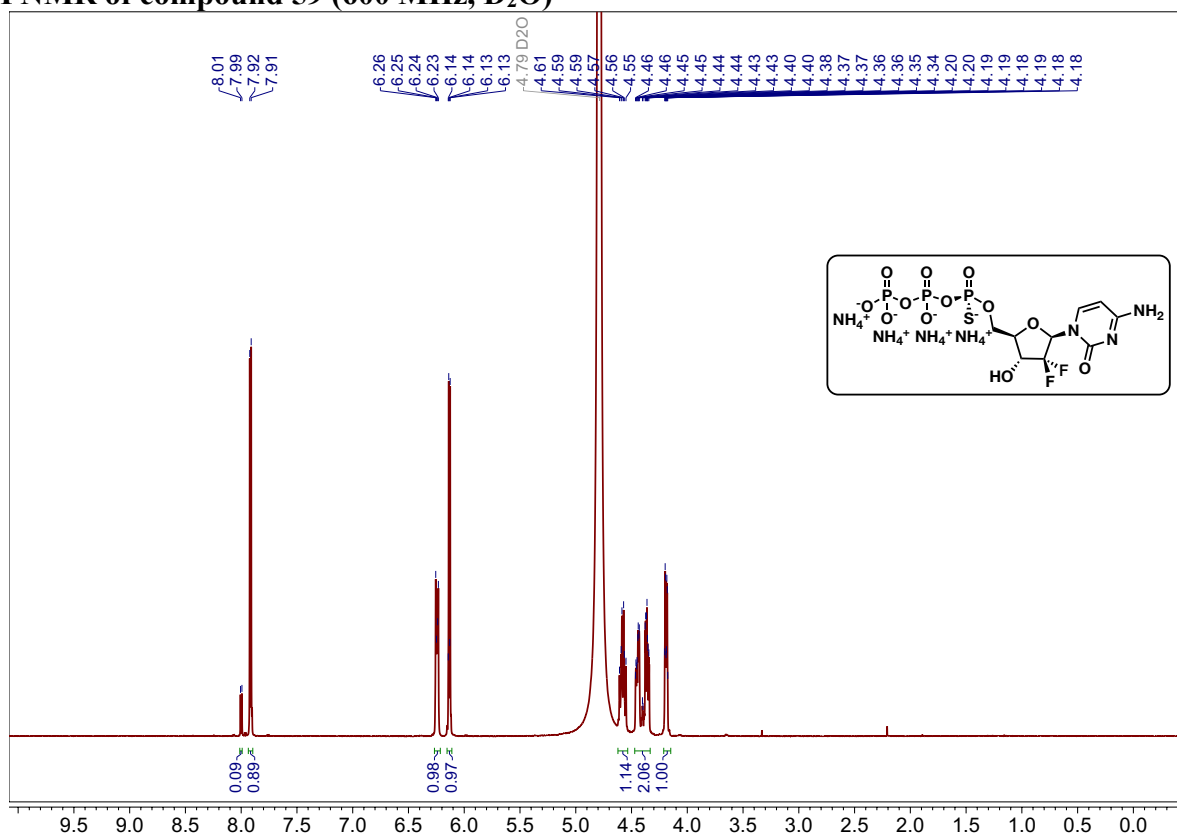
^{31}P NMR of compound 58 (243 MHz, D_2O)



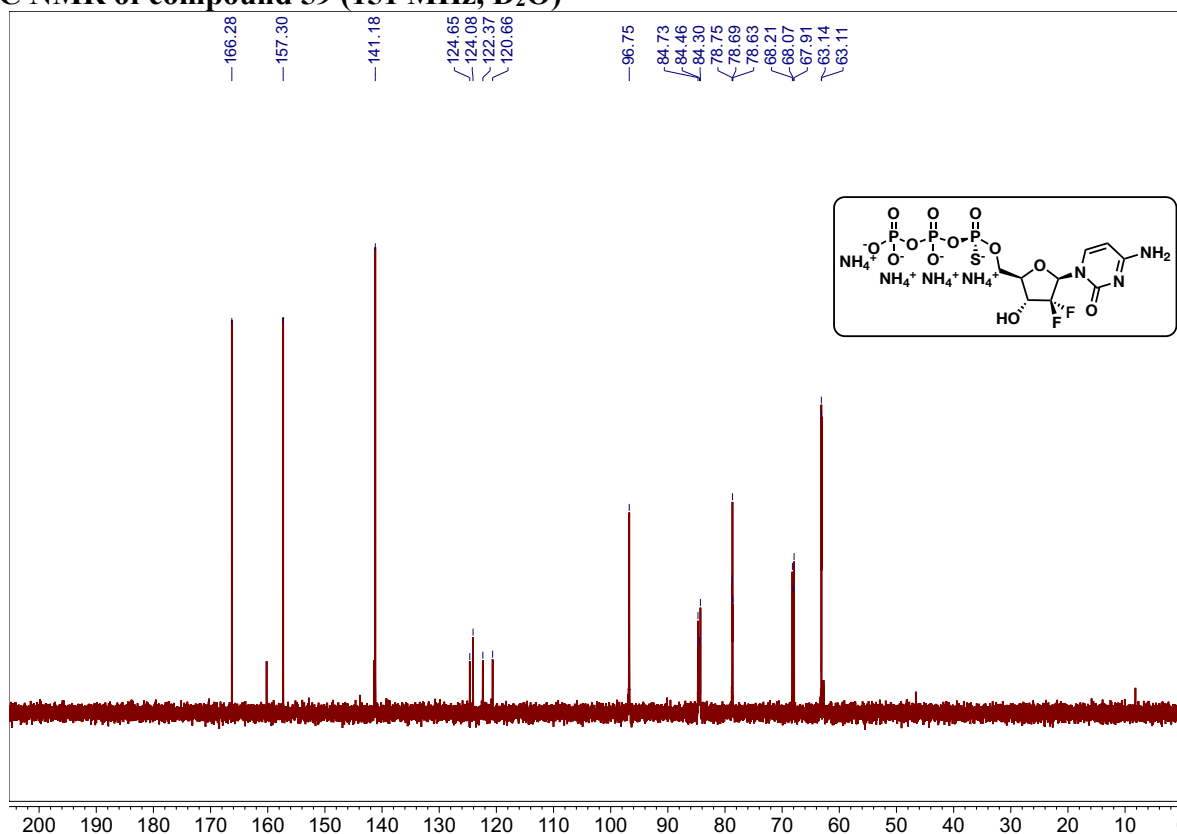
^{19}F NMR of compound 58 (565 MHz, D_2O)



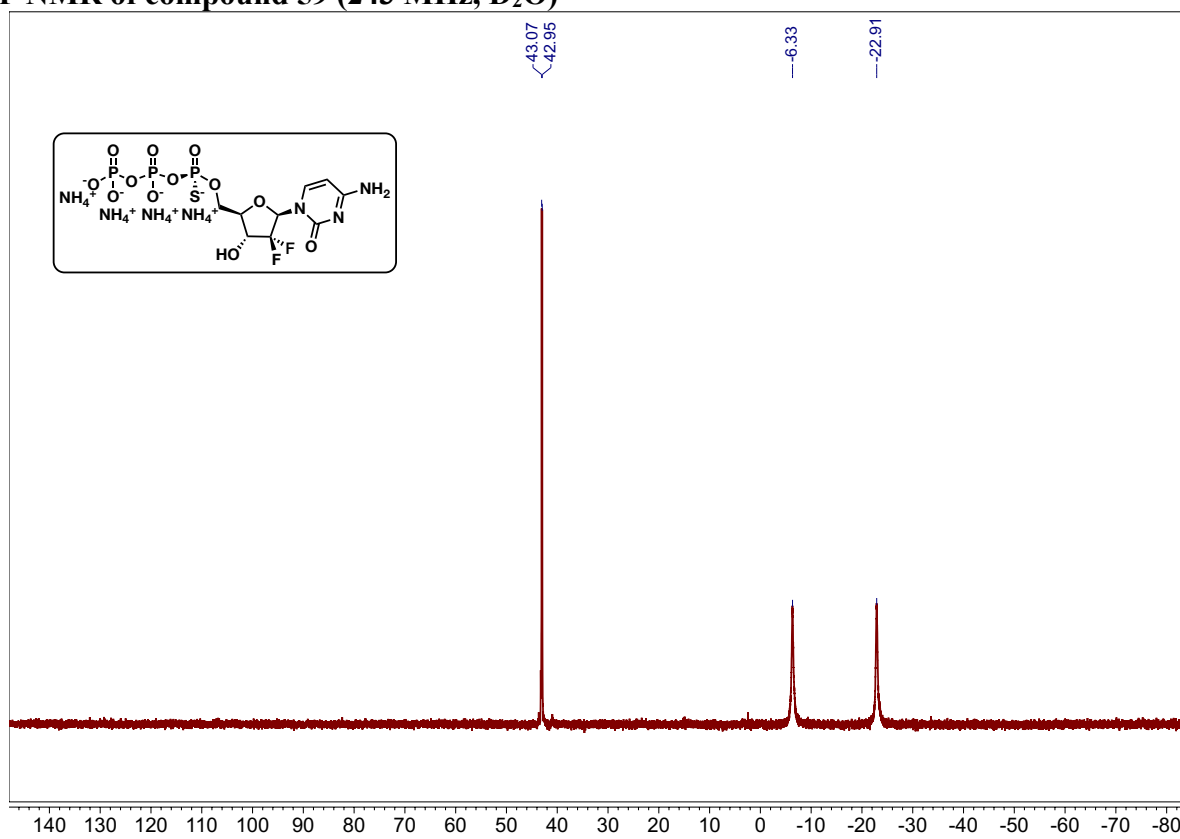
^1H NMR of compound 59 (600 MHz, D_2O)



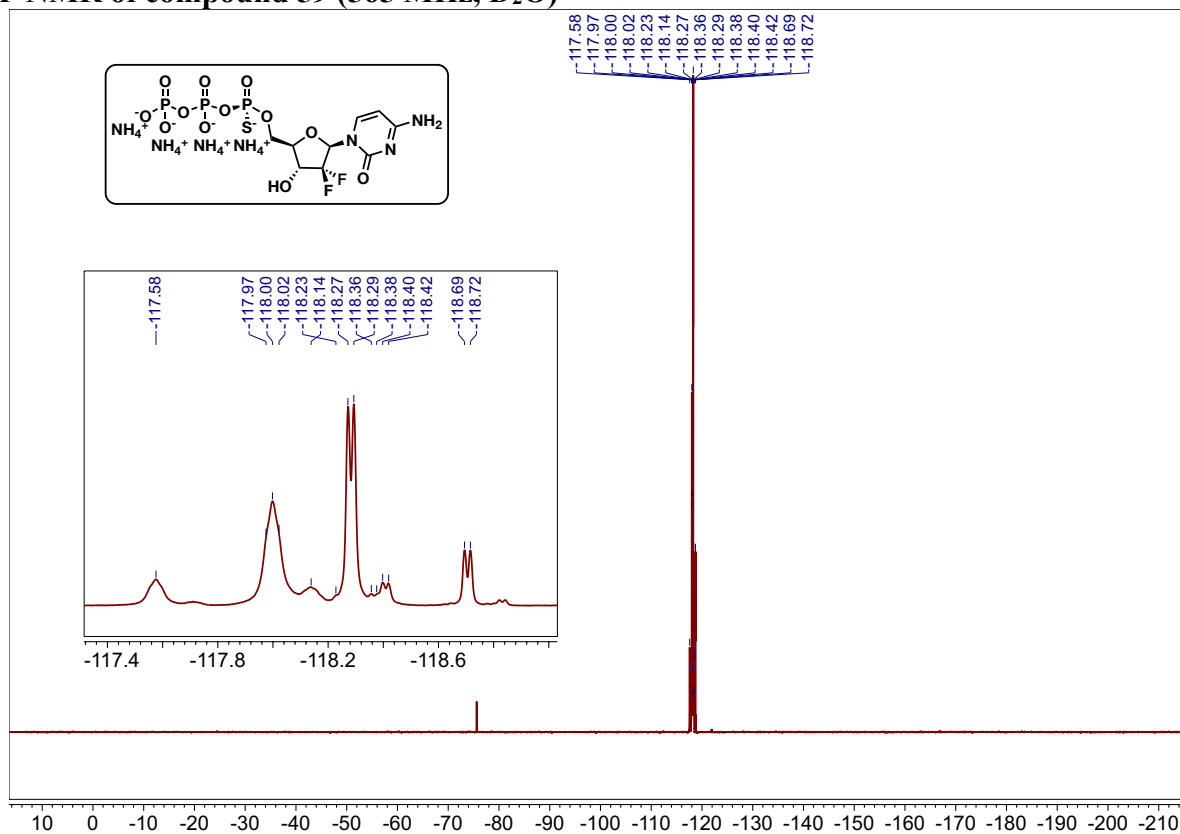
^{13}C NMR of compound 59 (151 MHz, D_2O)



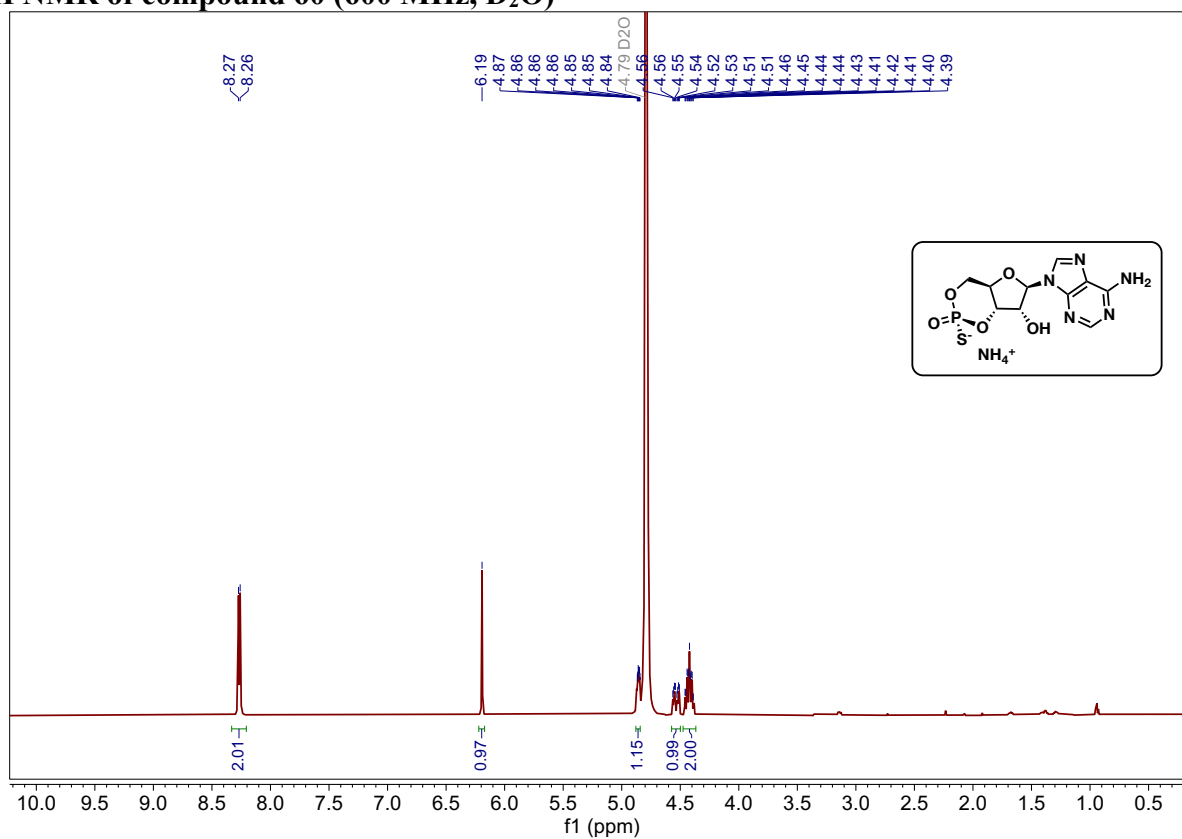
^{31}P NMR of compound 59 (243 MHz, D_2O)



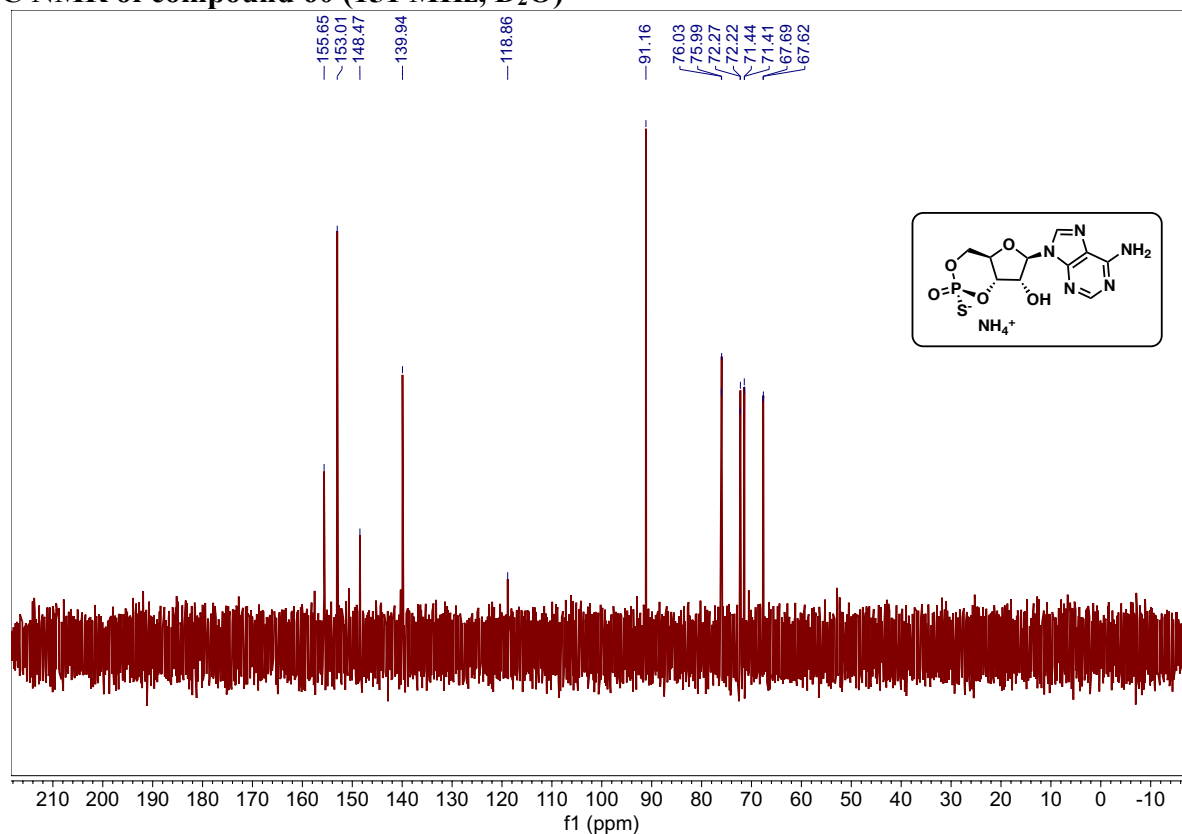
^{19}F NMR of compound 59 (565 MHz, D_2O)



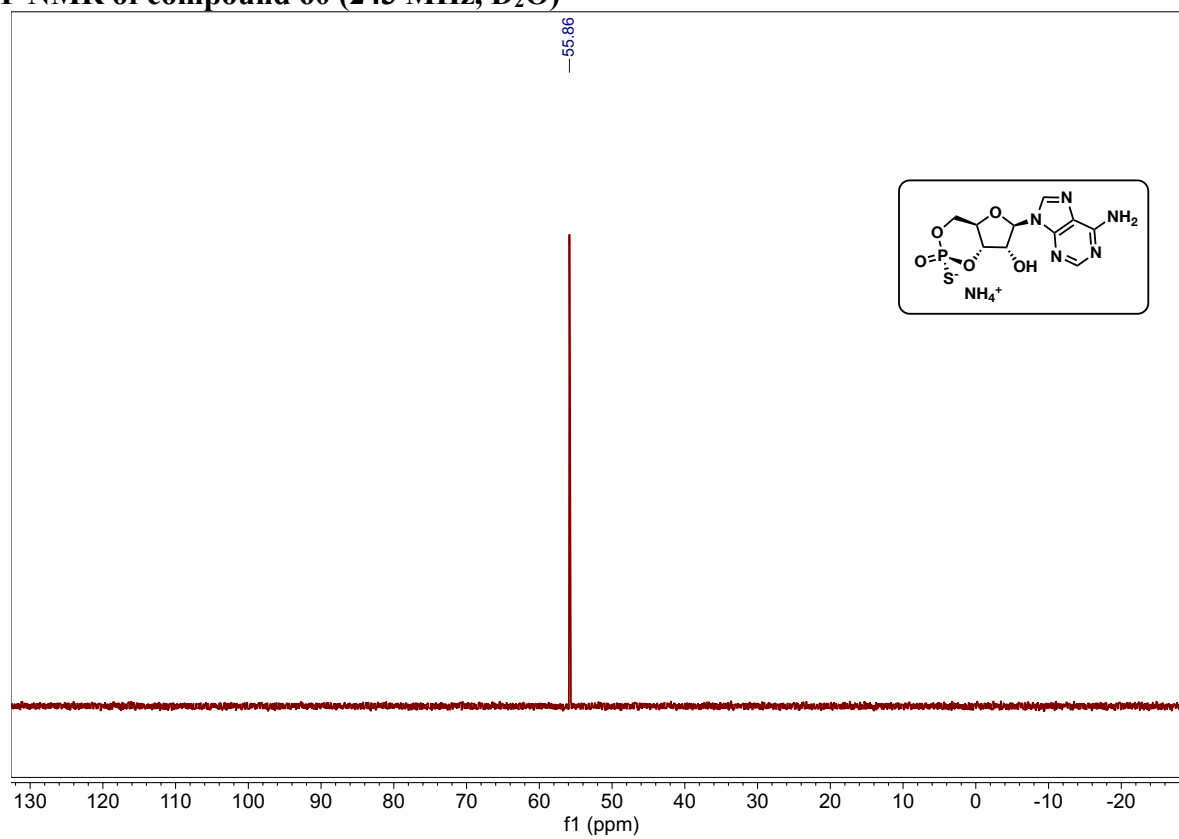
¹H NMR of compound 60 (600 MHz, D₂O)



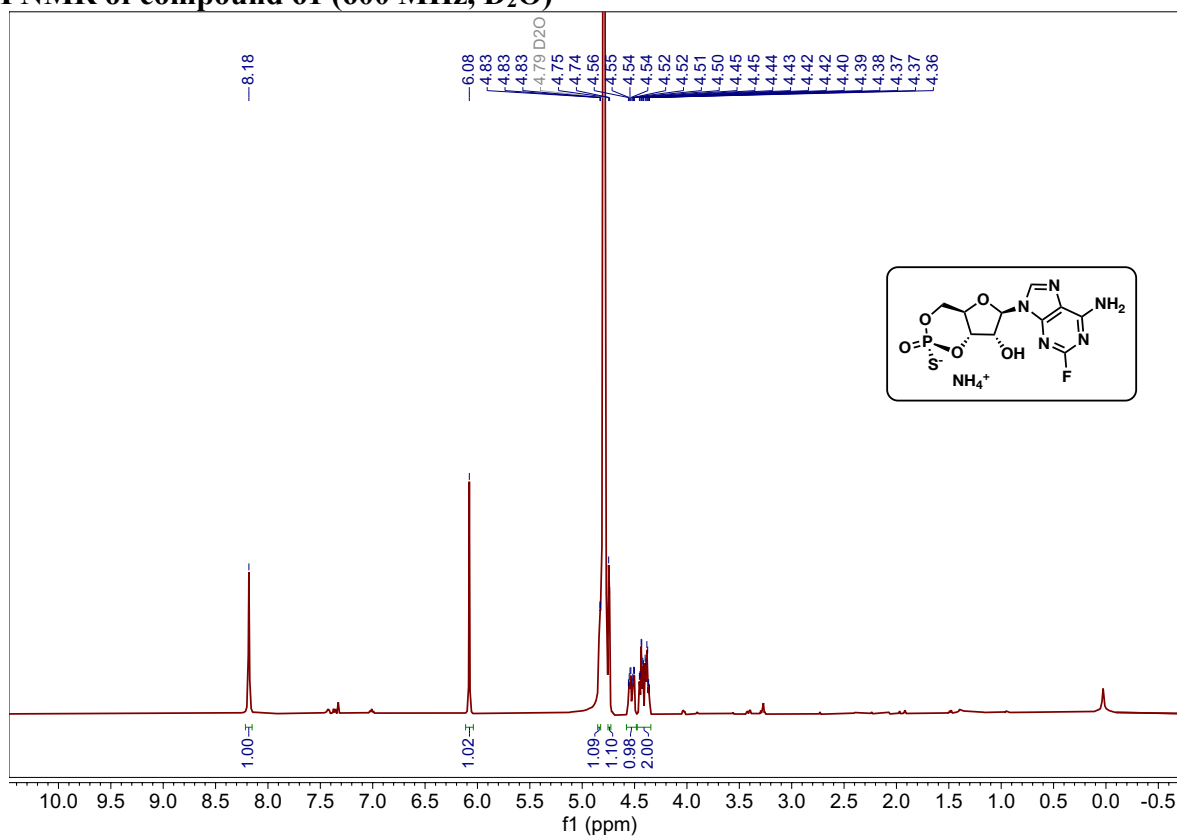
¹³C NMR of compound 60 (151 MHz, D₂O)



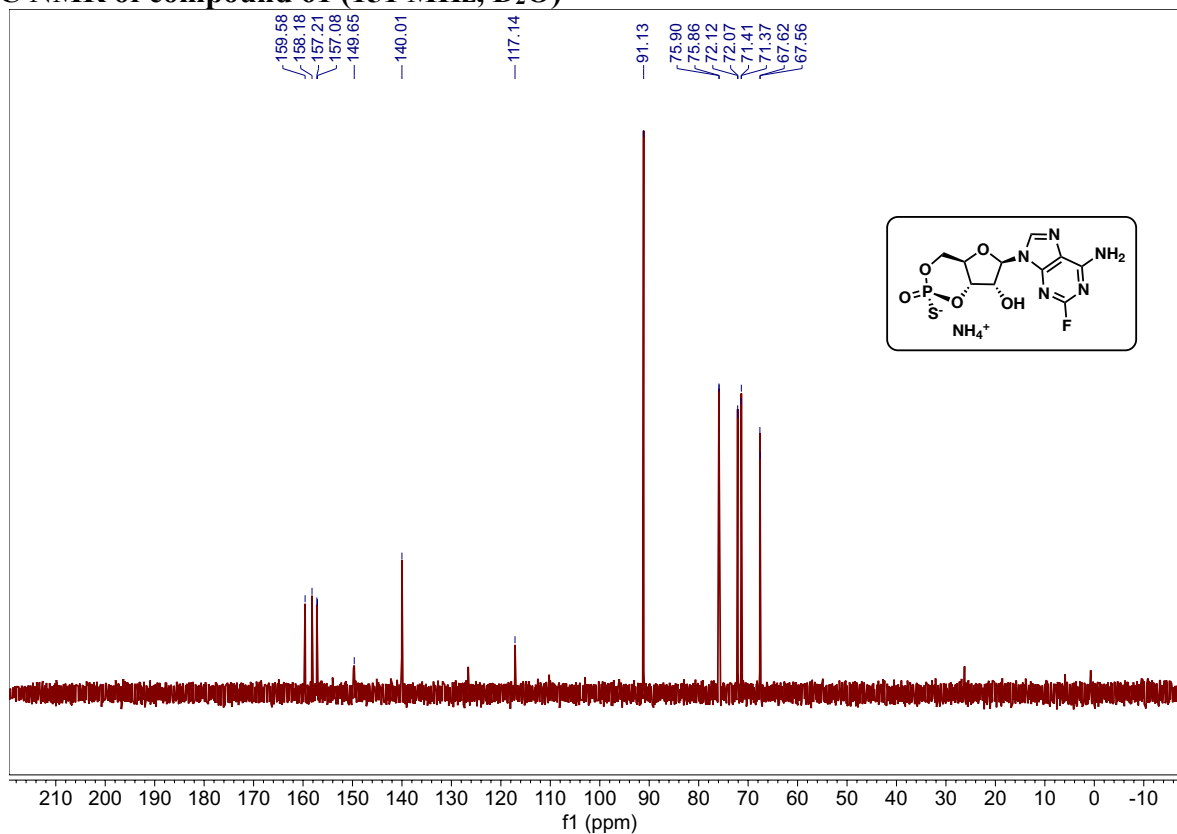
^{31}P NMR of compound 60 (243 MHz, D_2O)



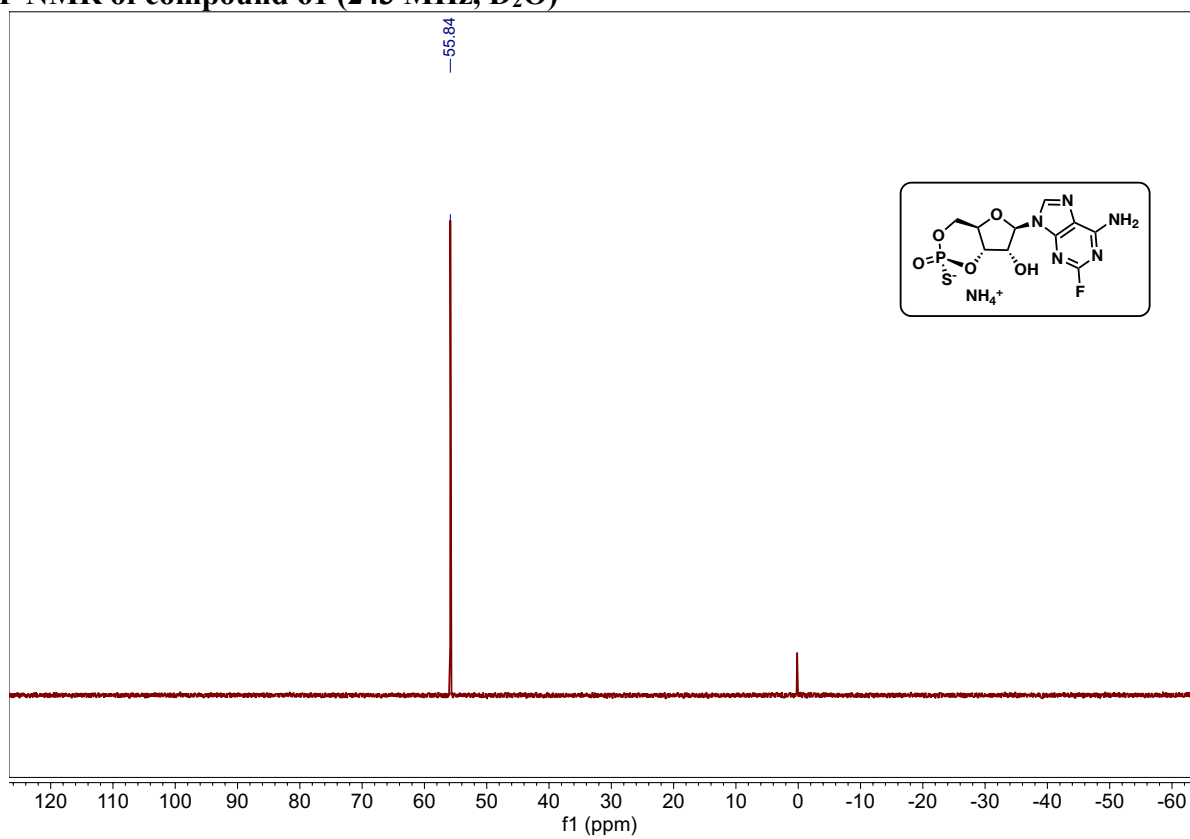
¹H NMR of compound 61 (600 MHz, D₂O)



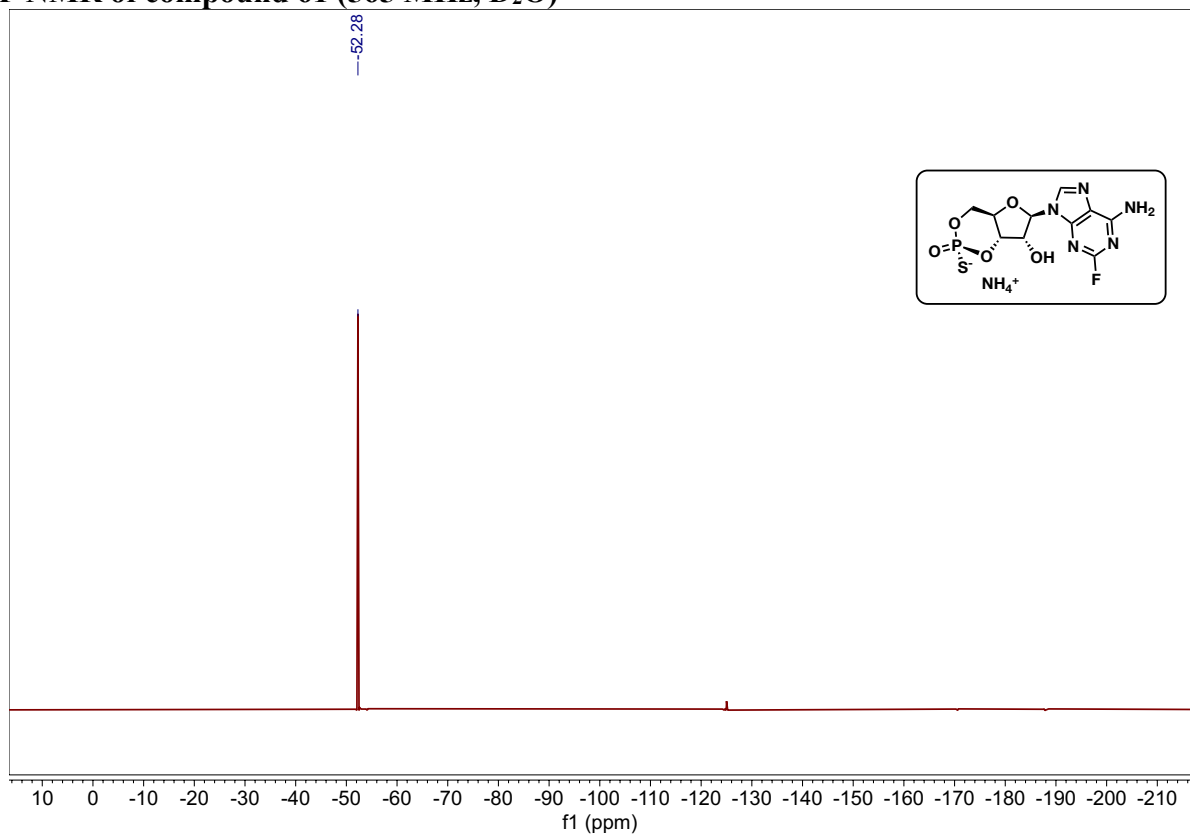
¹³C NMR of compound 61 (151 MHz, D₂O)



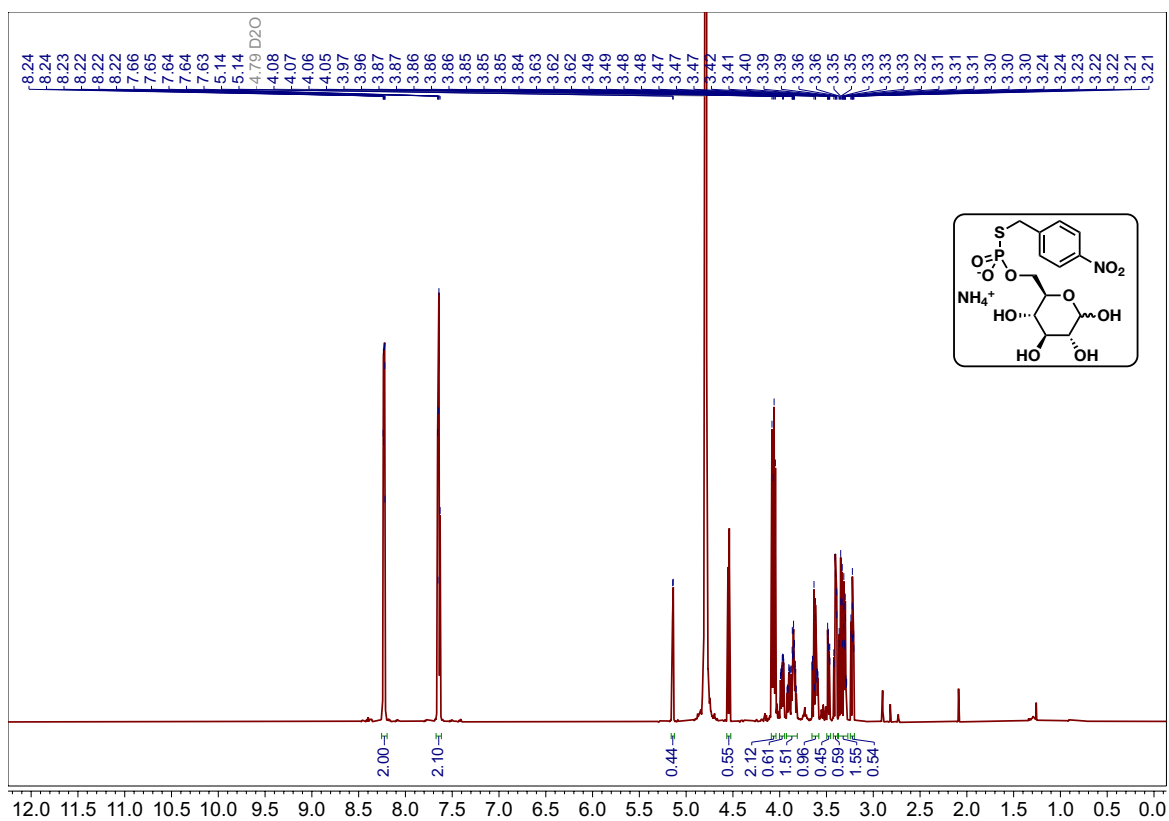
^{31}P NMR of compound 61 (243 MHz, D_2O)



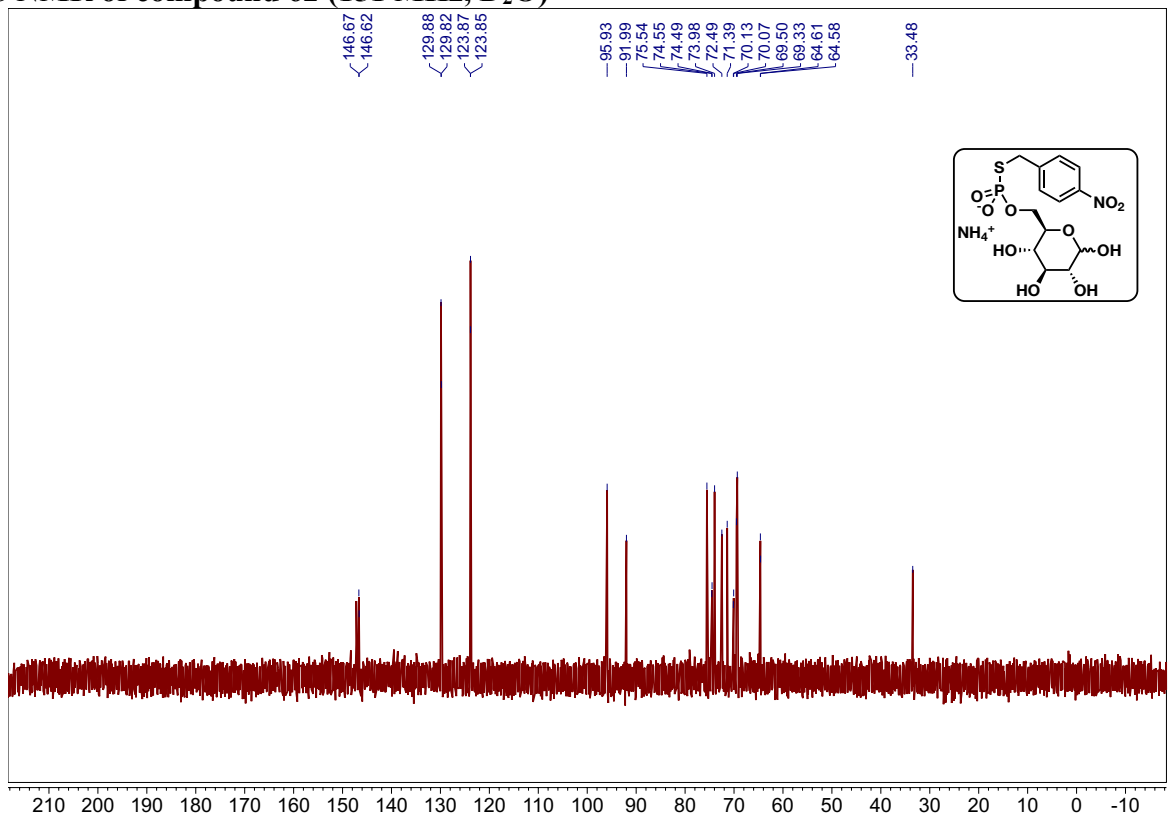
^{19}F NMR of compound 61 (565 MHz, D_2O)



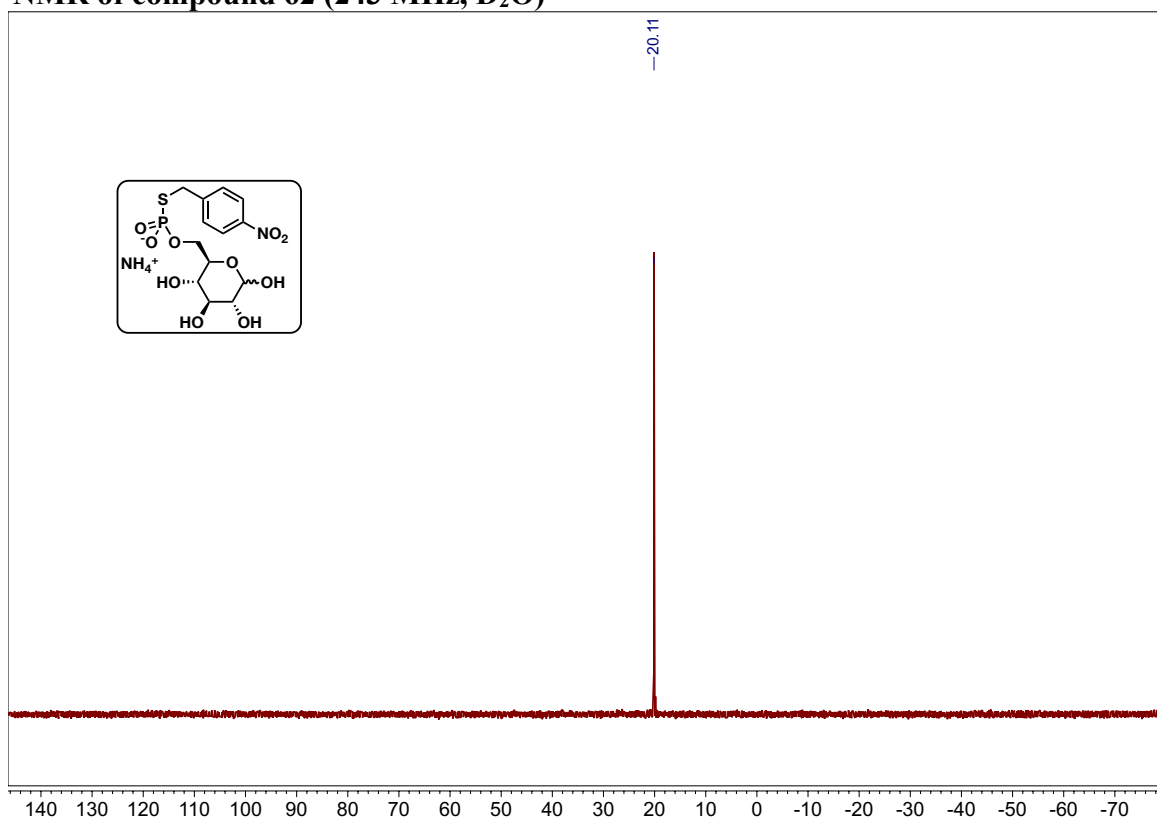
^1H NMR of compound 62 (600 MHz, D_2O)



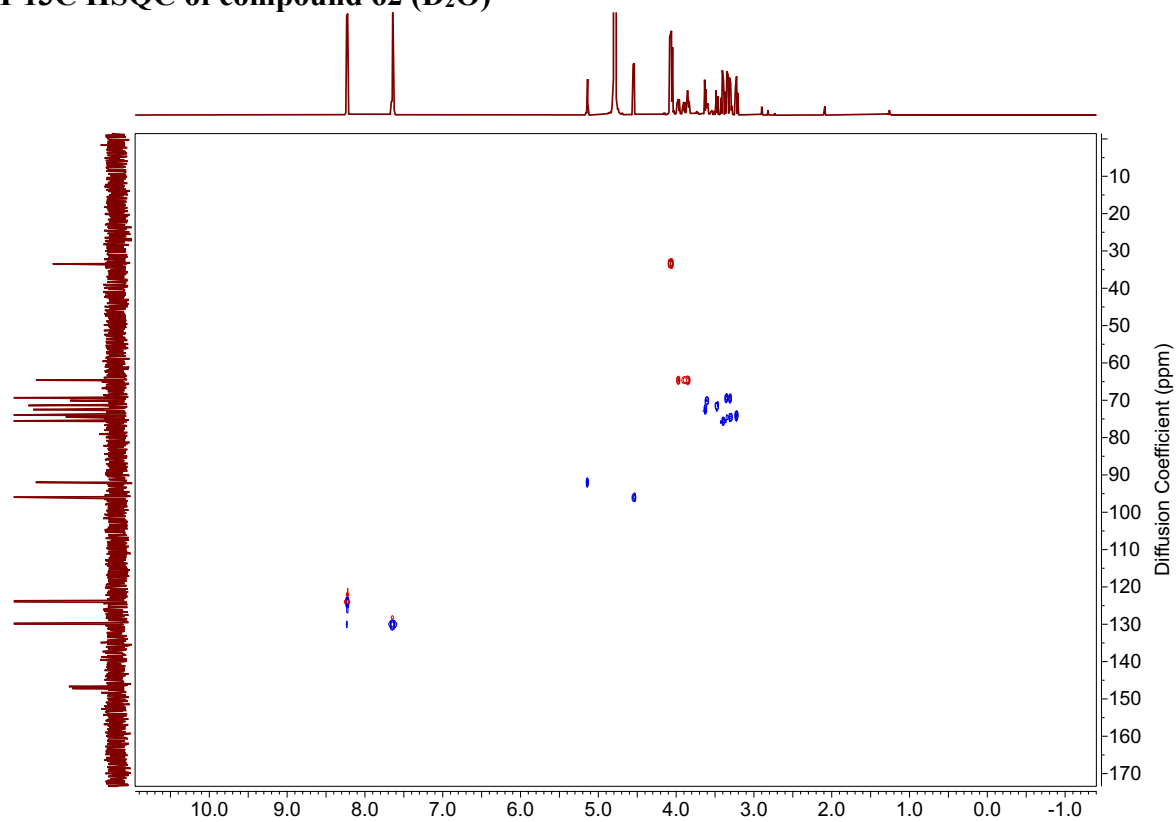
^{13}C NMR of compound 62 (151 MHz, D_2O)



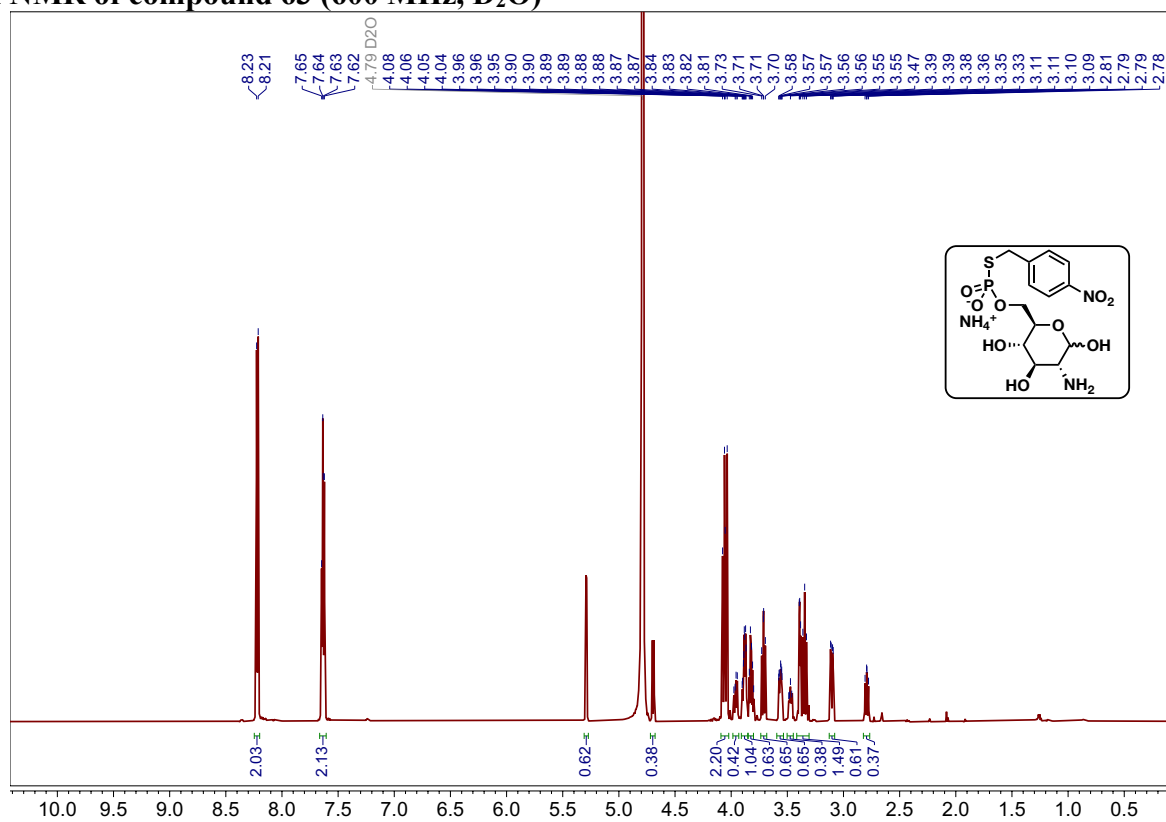
^{31}P NMR of compound 62 (243 MHz, D_2O)



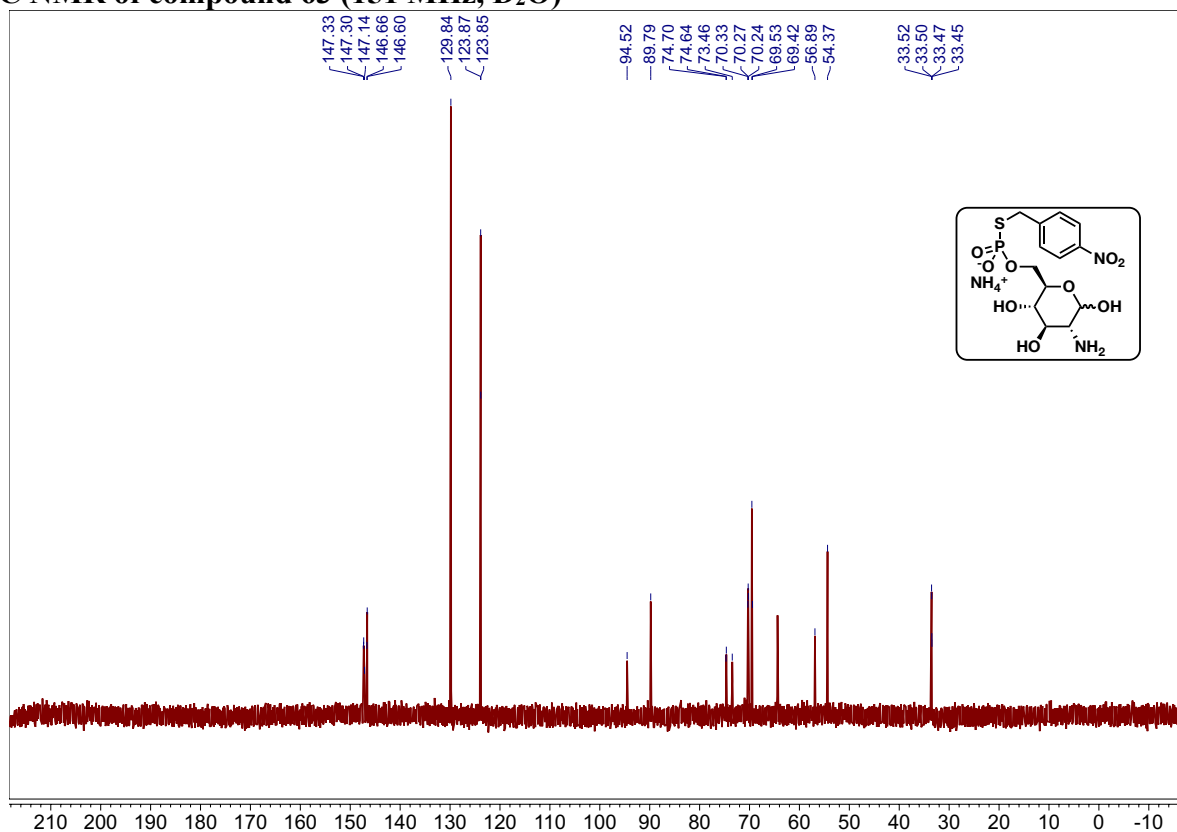
^1H - ^{13}C HSQC of compound 62 (D_2O)



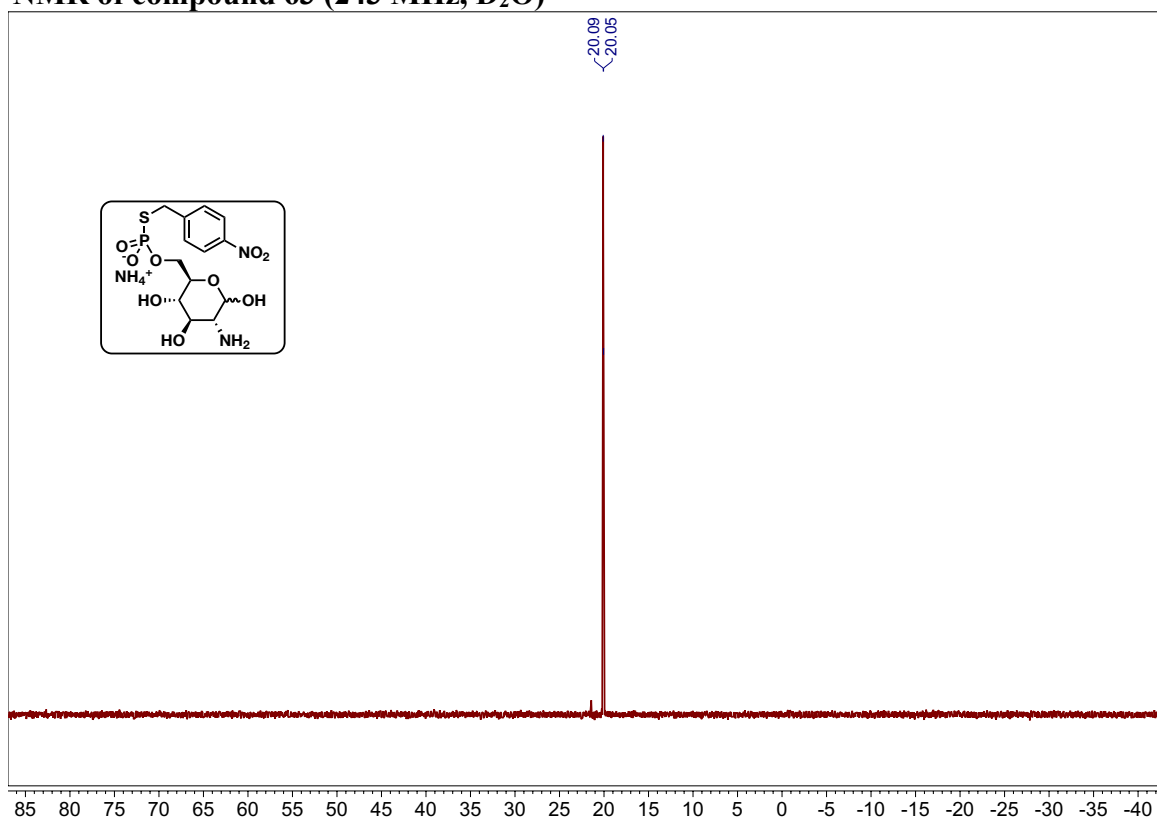
¹H NMR of compound 63 (600 MHz, D₂O)



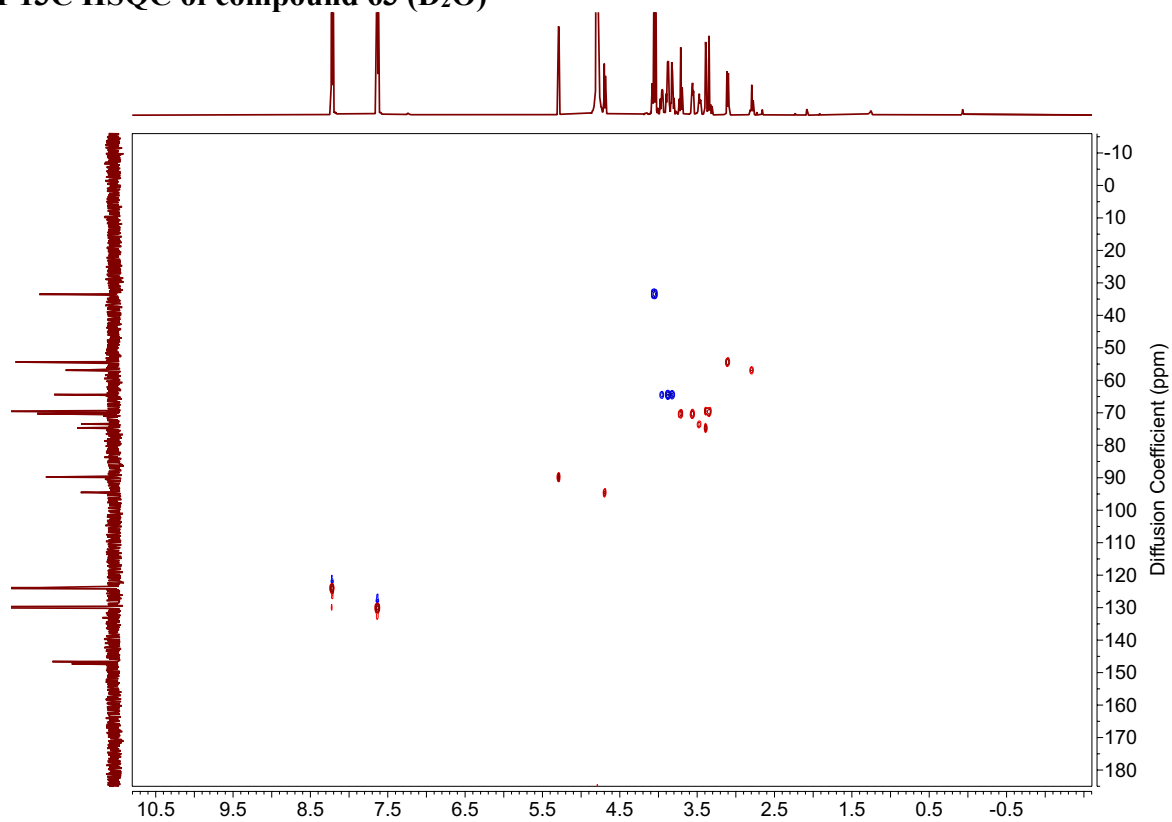
¹³C NMR of compound 63 (151 MHz, D₂O)



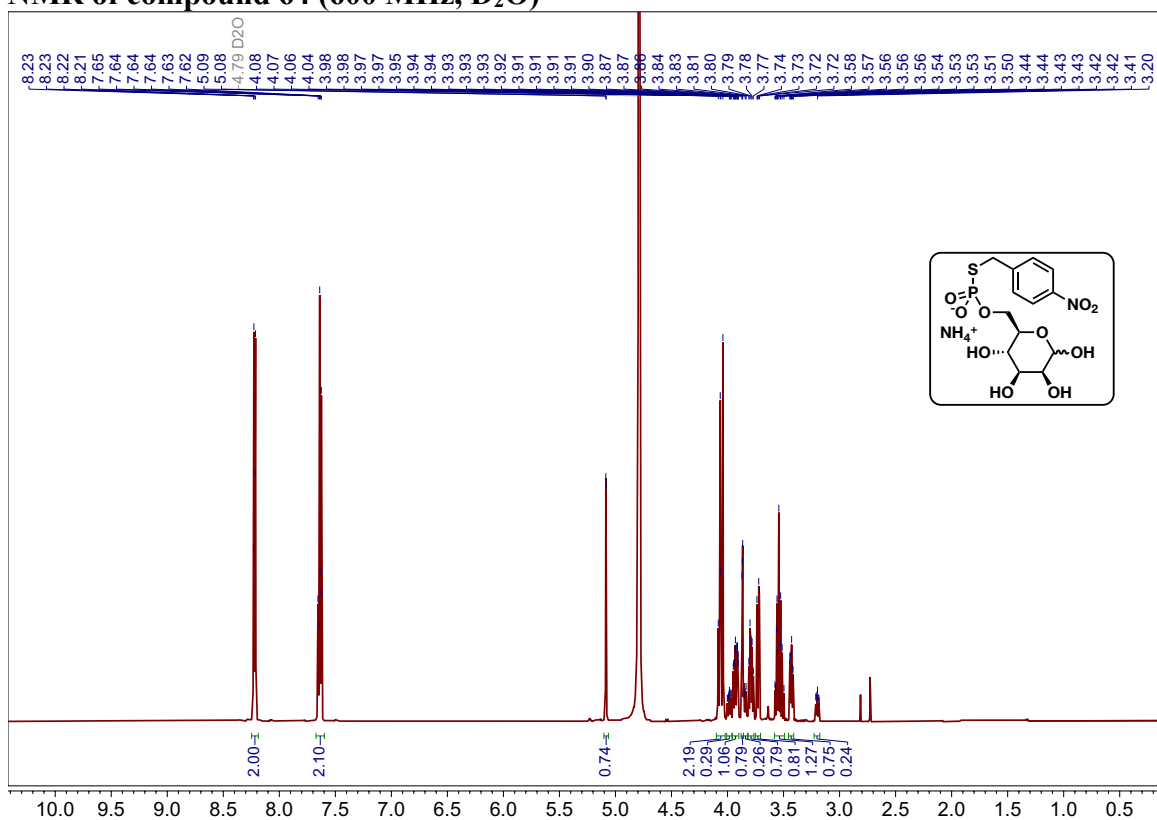
^{31}P NMR of compound 63 (243 MHz, D_2O)



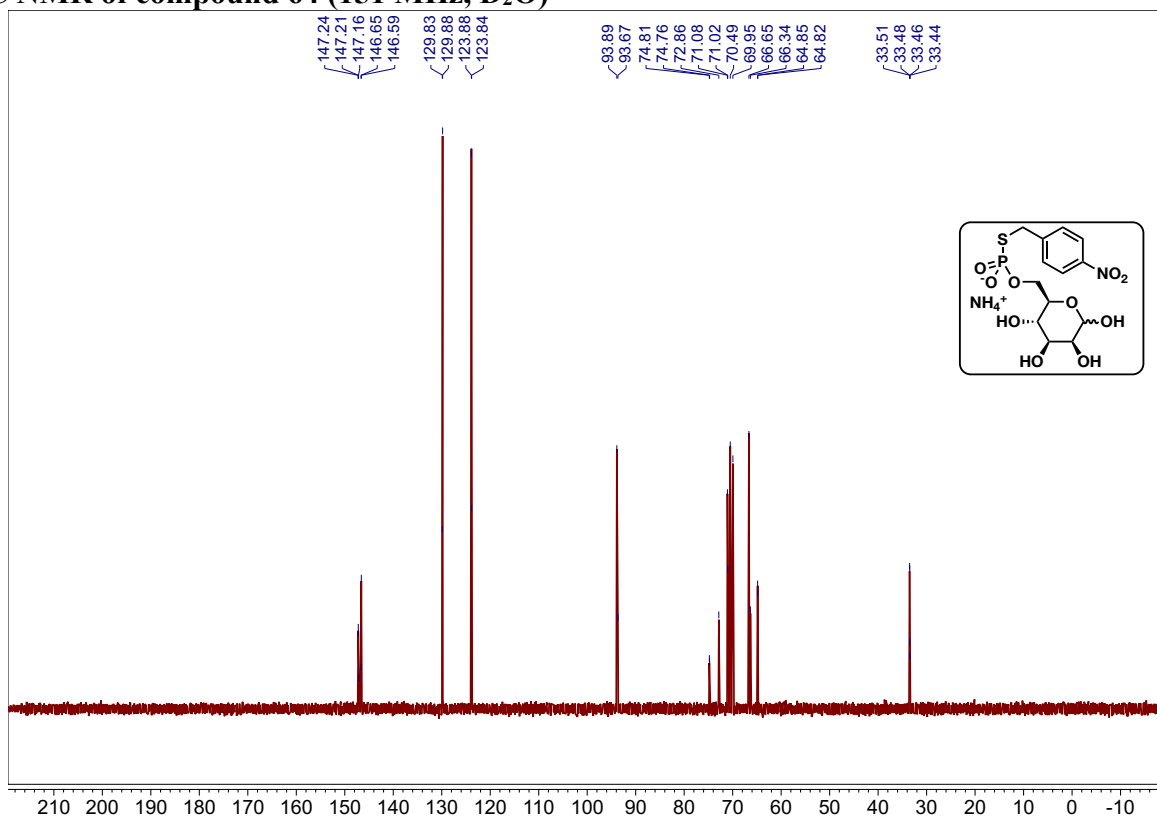
^1H - ^{13}C HSQC of compound 63 (D_2O)



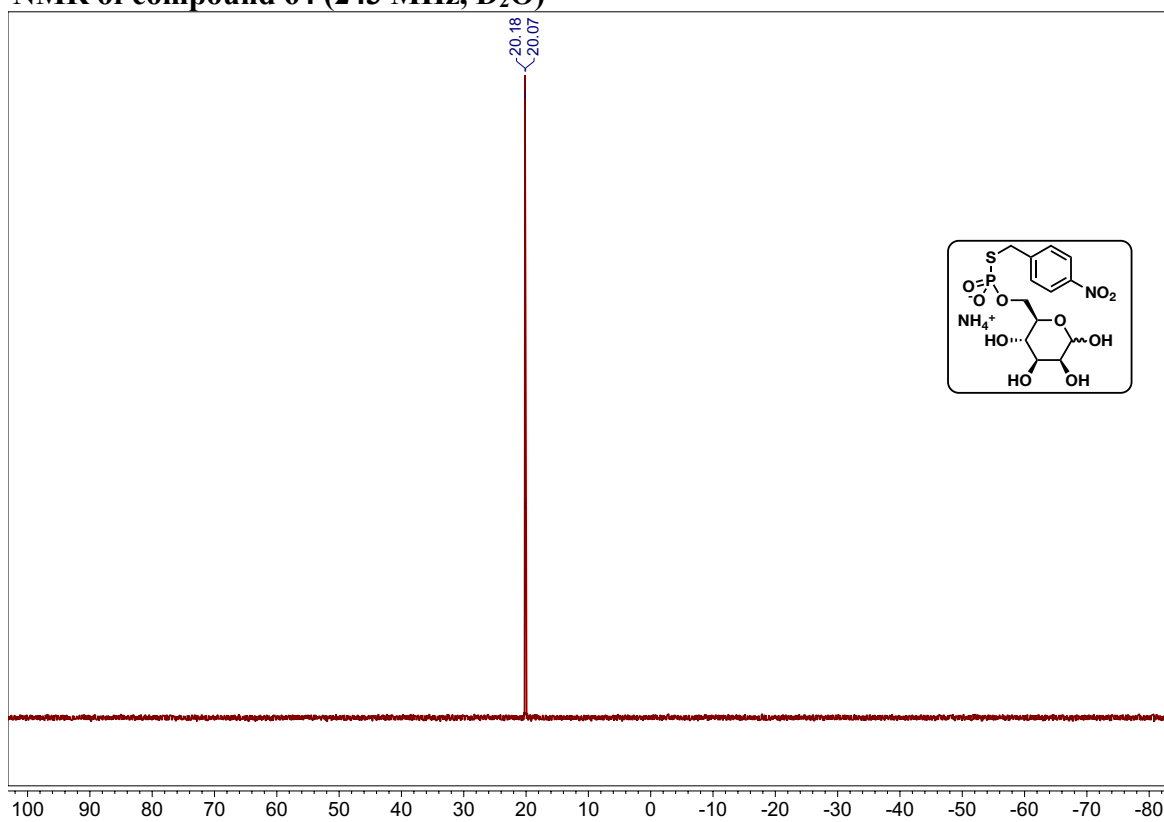
¹H NMR of compound 64 (600 MHz, D₂O)



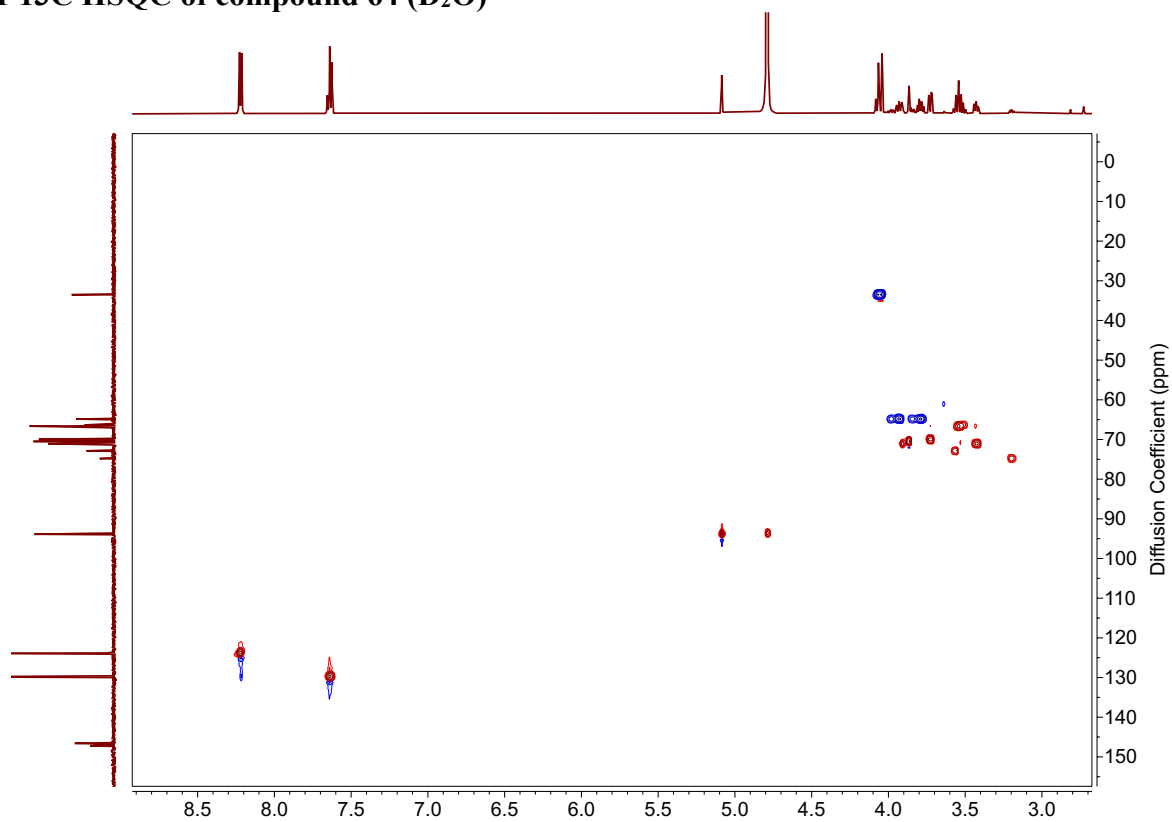
¹³C NMR of compound 64 (151 MHz, D₂O)



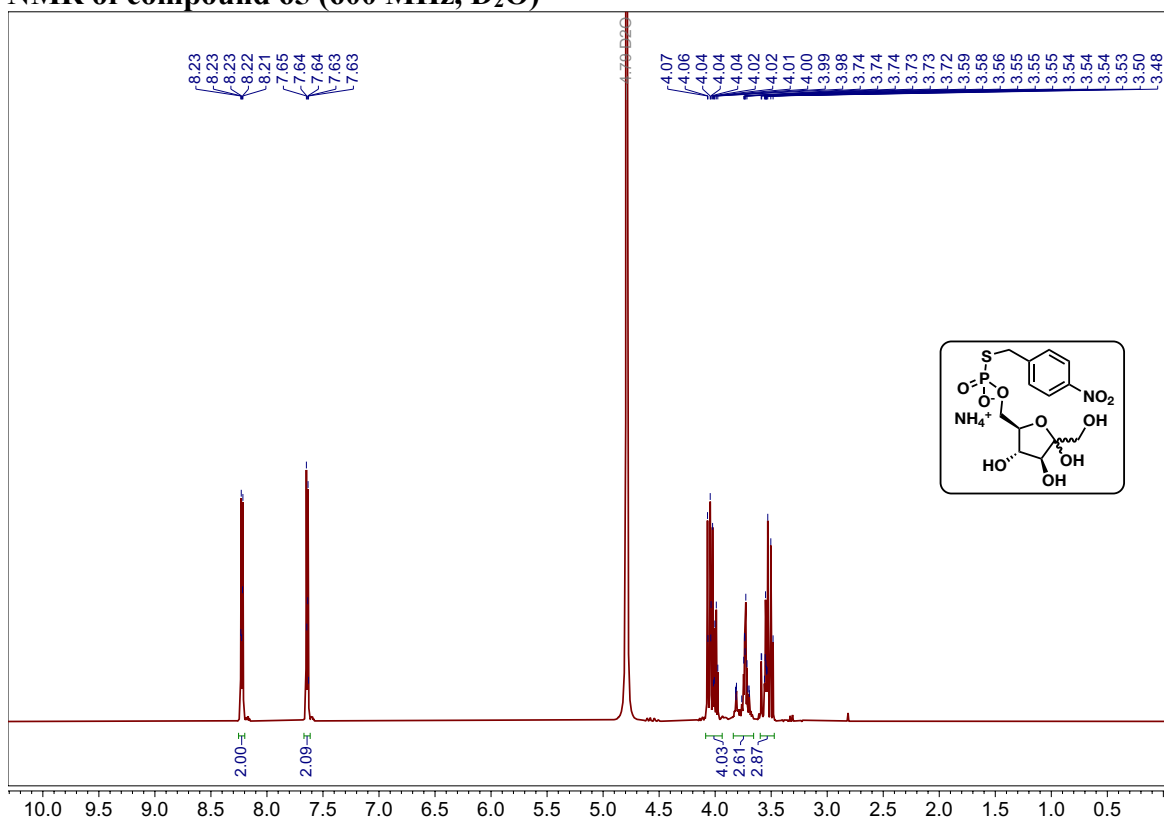
^{31}P NMR of compound 64 (243 MHz, D_2O)



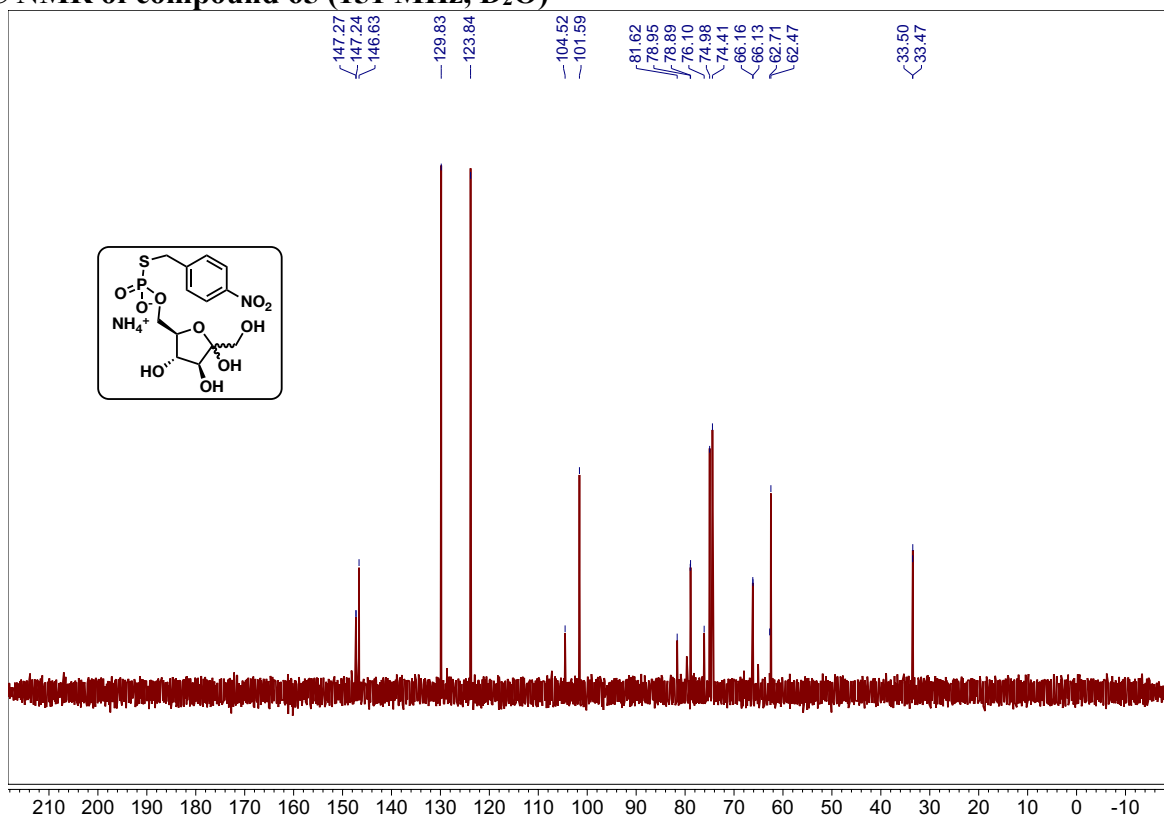
^1H - ^{13}C HSQC of compound 64 (D_2O)



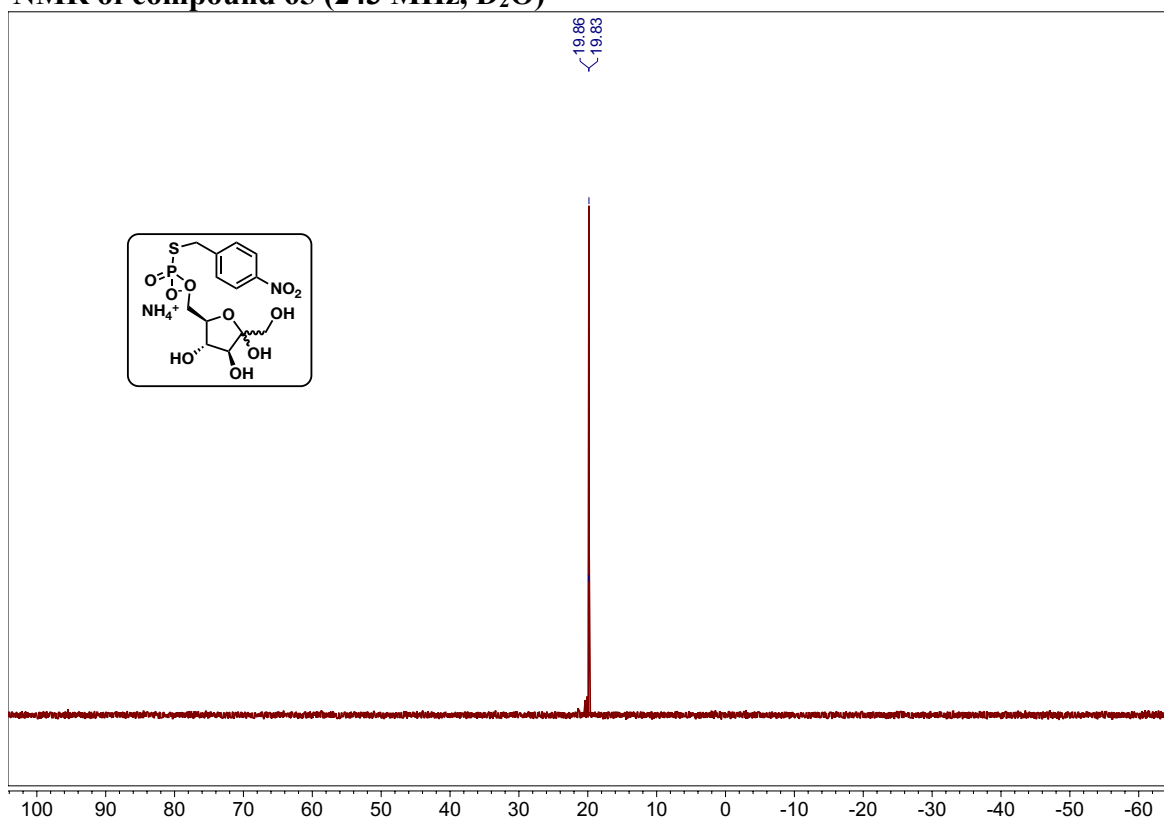
¹H NMR of compound 65 (600 MHz, D₂O)



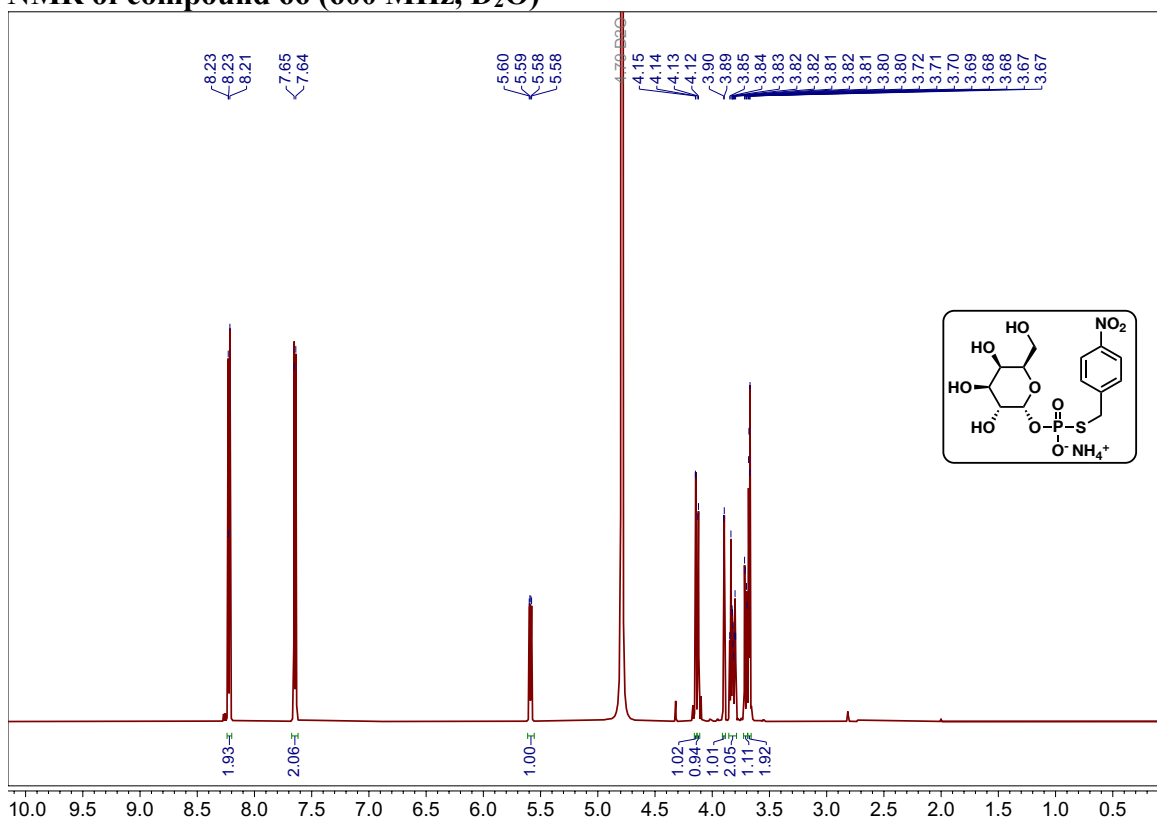
¹³C NMR of compound 65 (151 MHz, D₂O)



^{31}P NMR of compound 65 (243 MHz, D_2O)



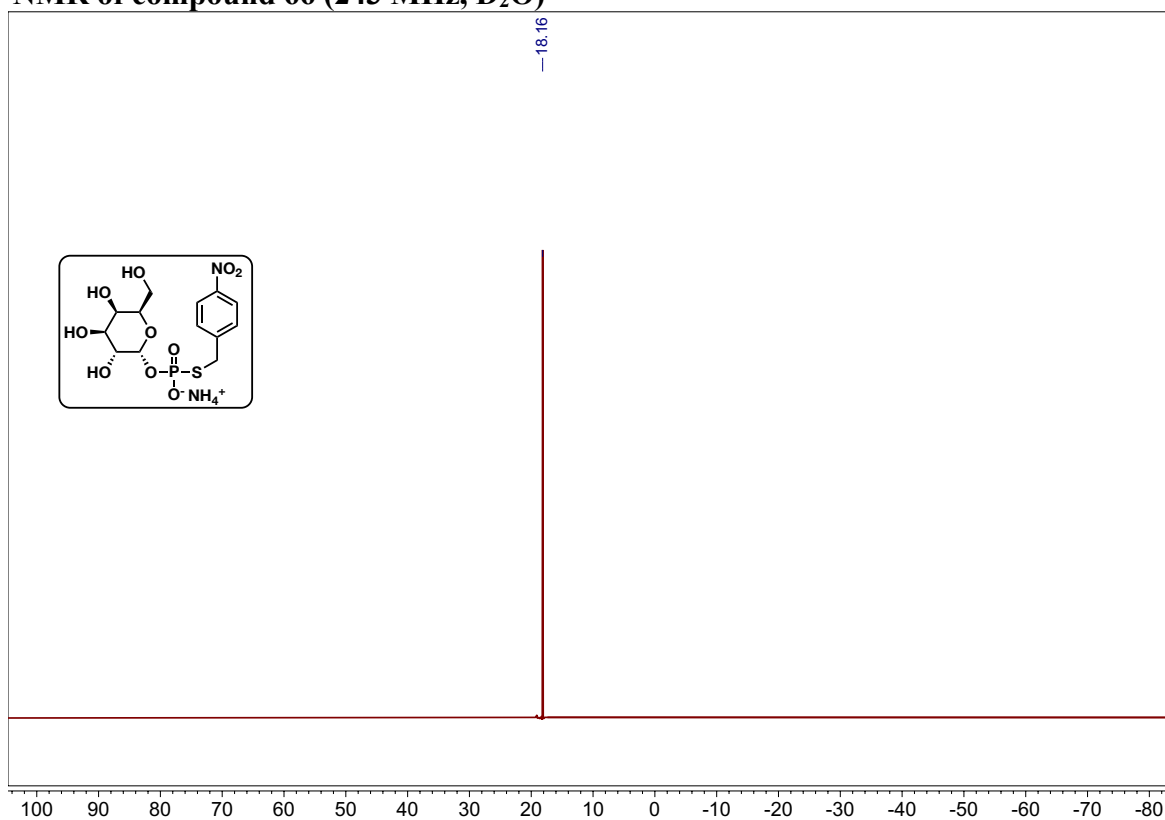
^1H NMR of compound 66 (600 MHz, D_2O)



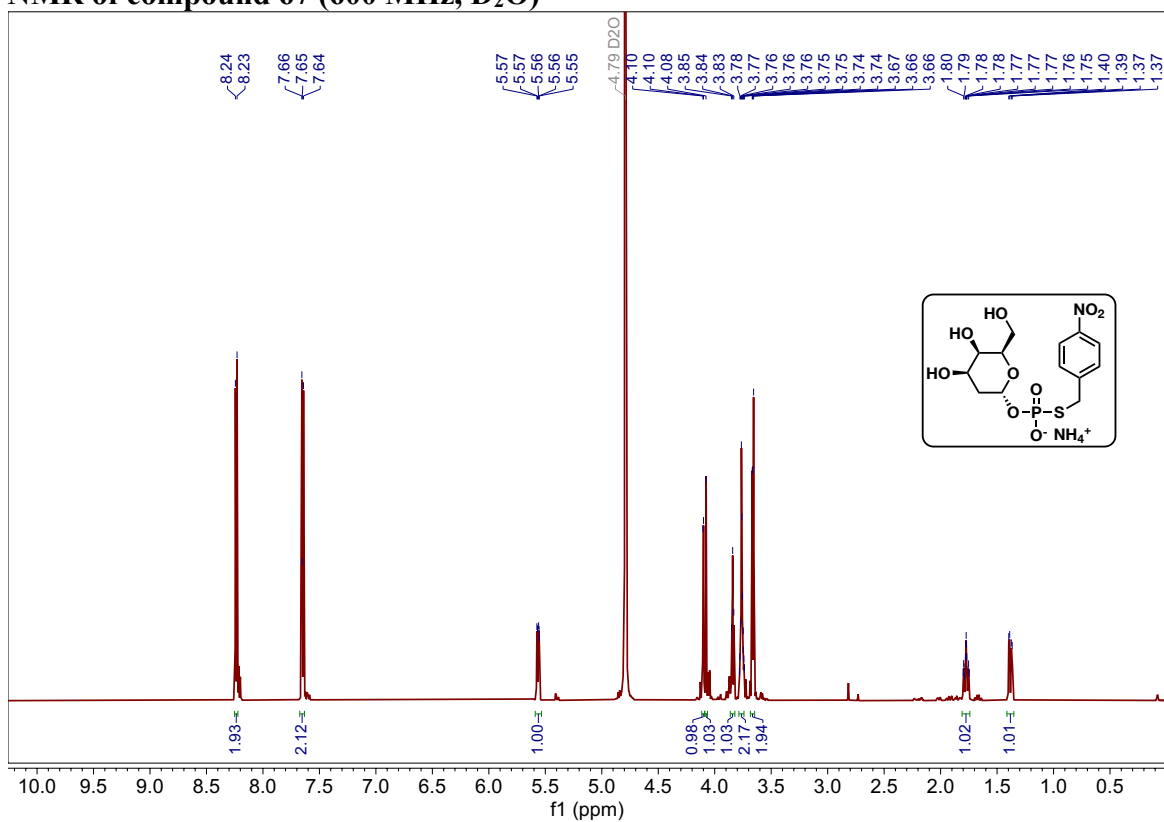
^{13}C NMR of compound 66 (151 MHz, D_2O)



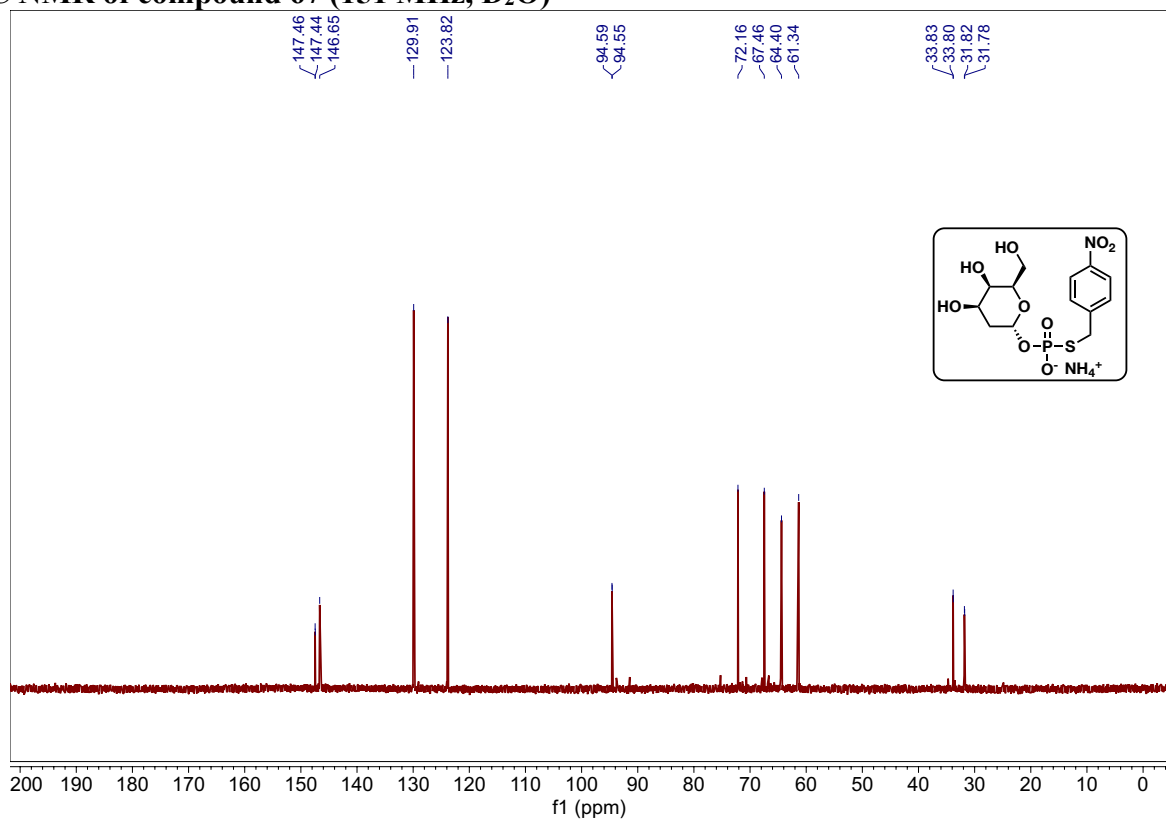
^{31}P NMR of compound 66 (243 MHz, D_2O)



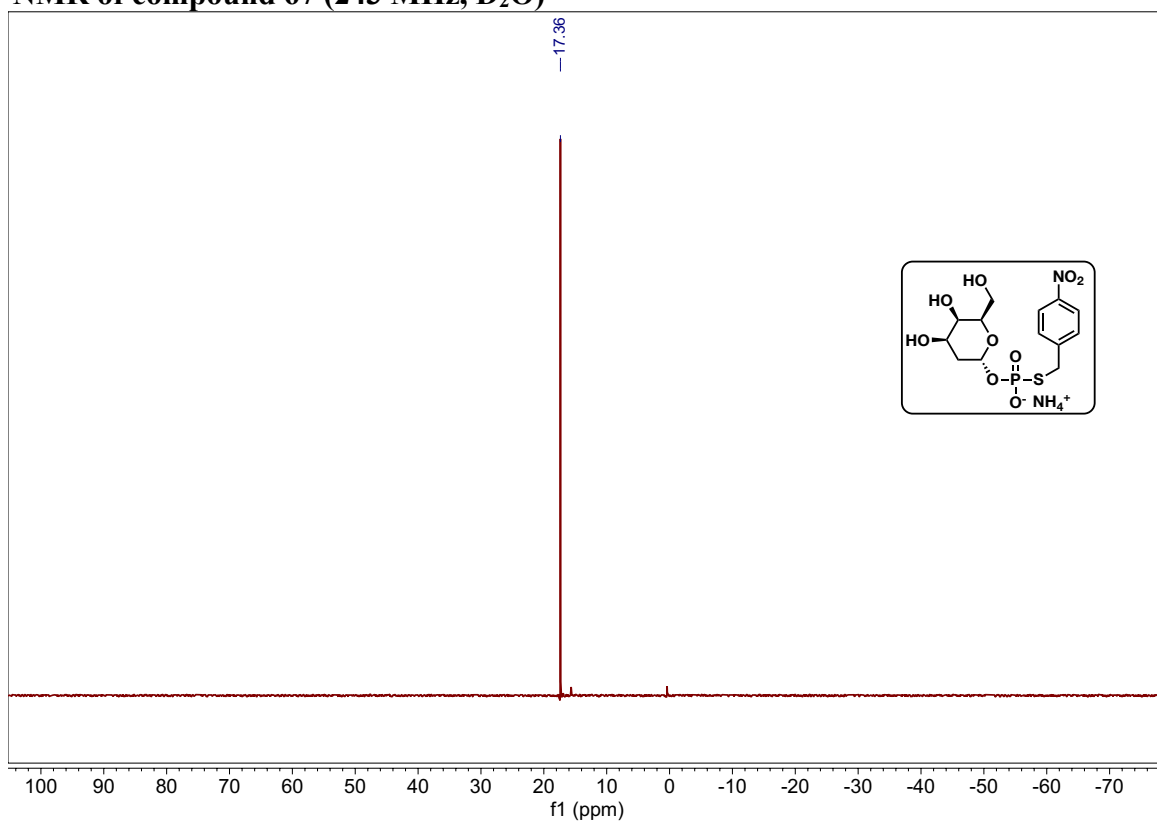
¹H NMR of compound 67 (600 MHz, D₂O)



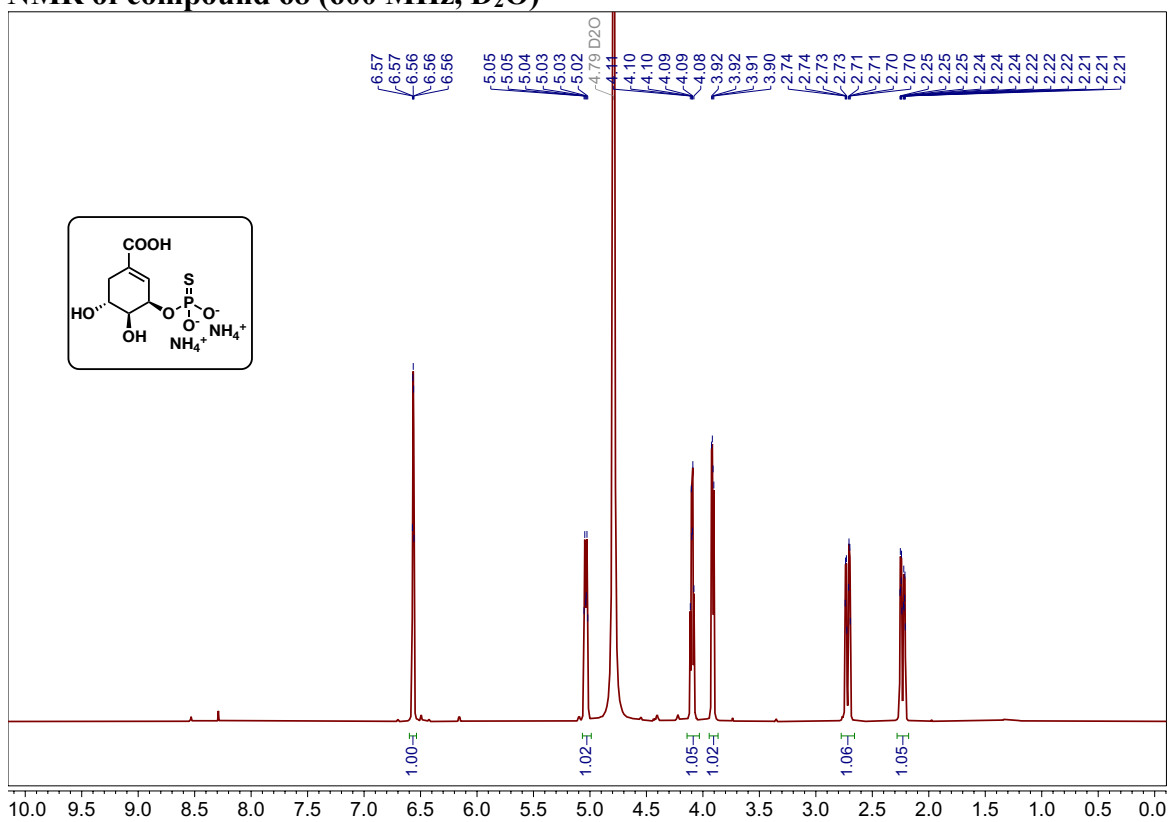
¹³C NMR of compound 67 (151 MHz, D₂O)



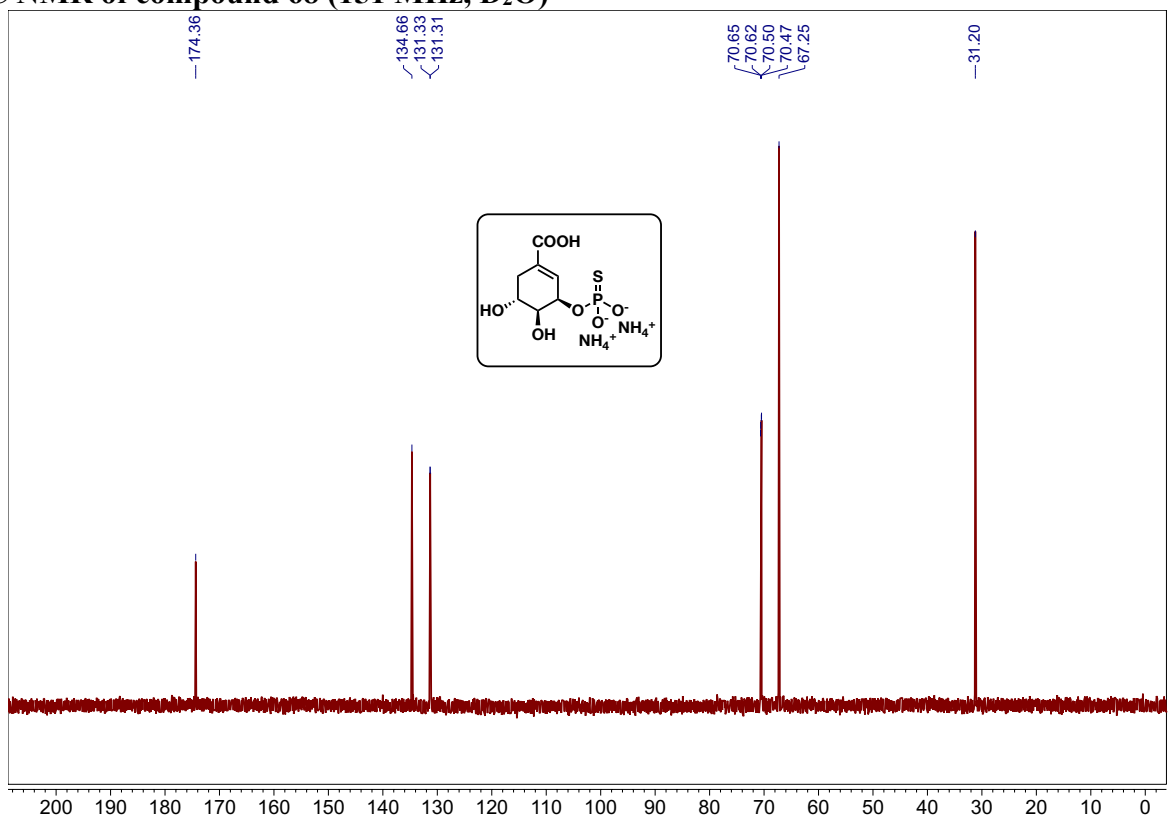
^{31}P NMR of compound 67 (243 MHz, D_2O)



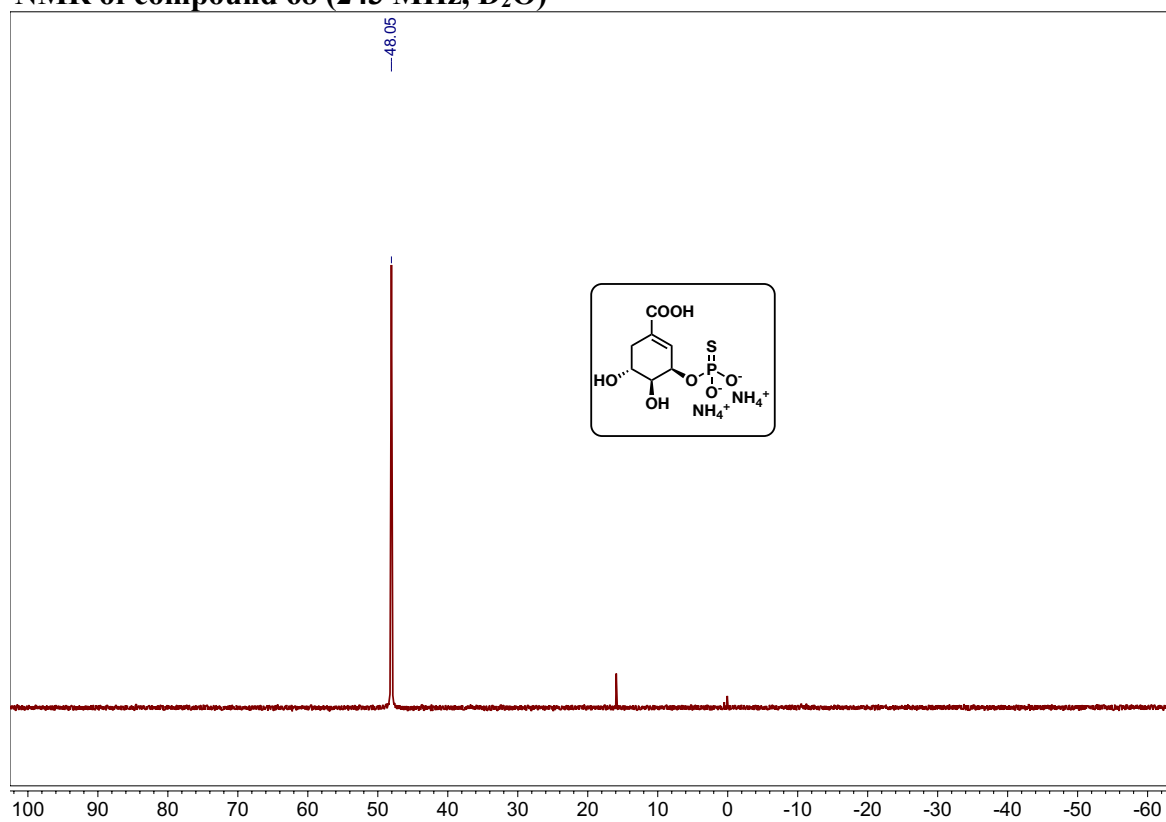
^1H NMR of compound 68 (600 MHz, D_2O)



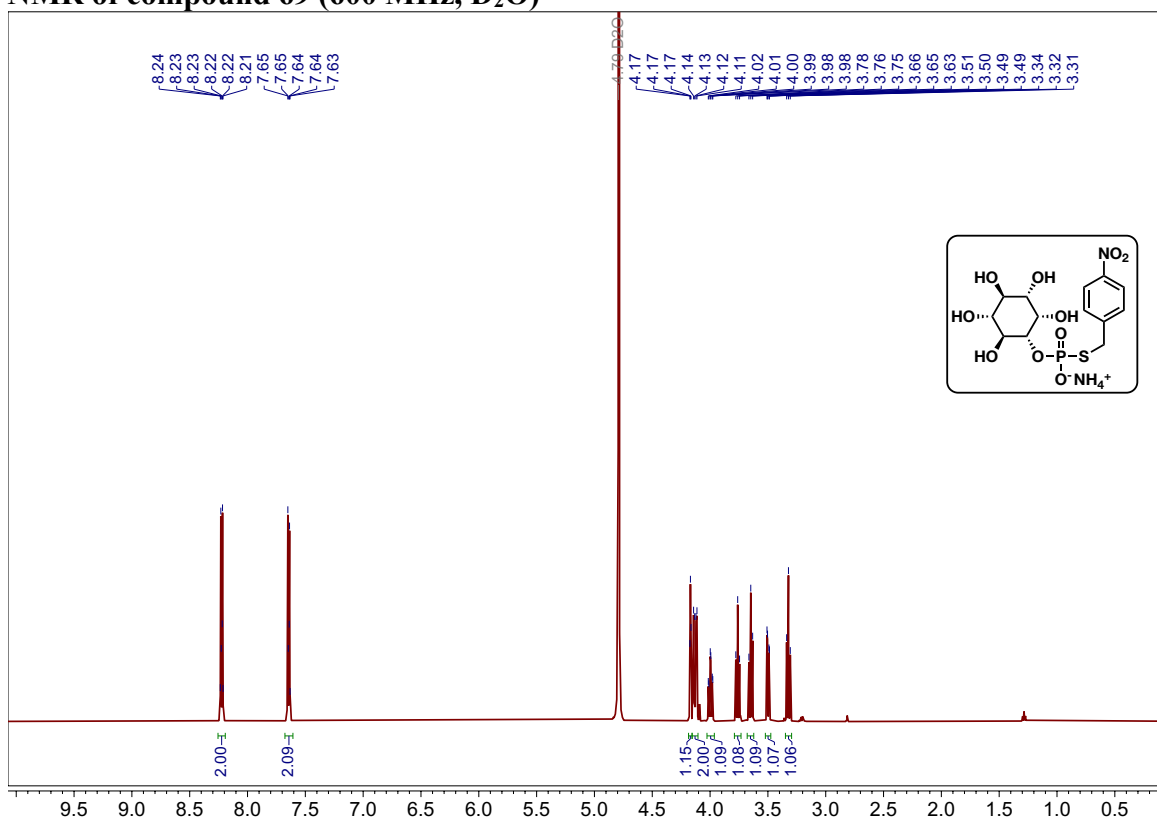
^{13}C NMR of compound 68 (151 MHz, D_2O)



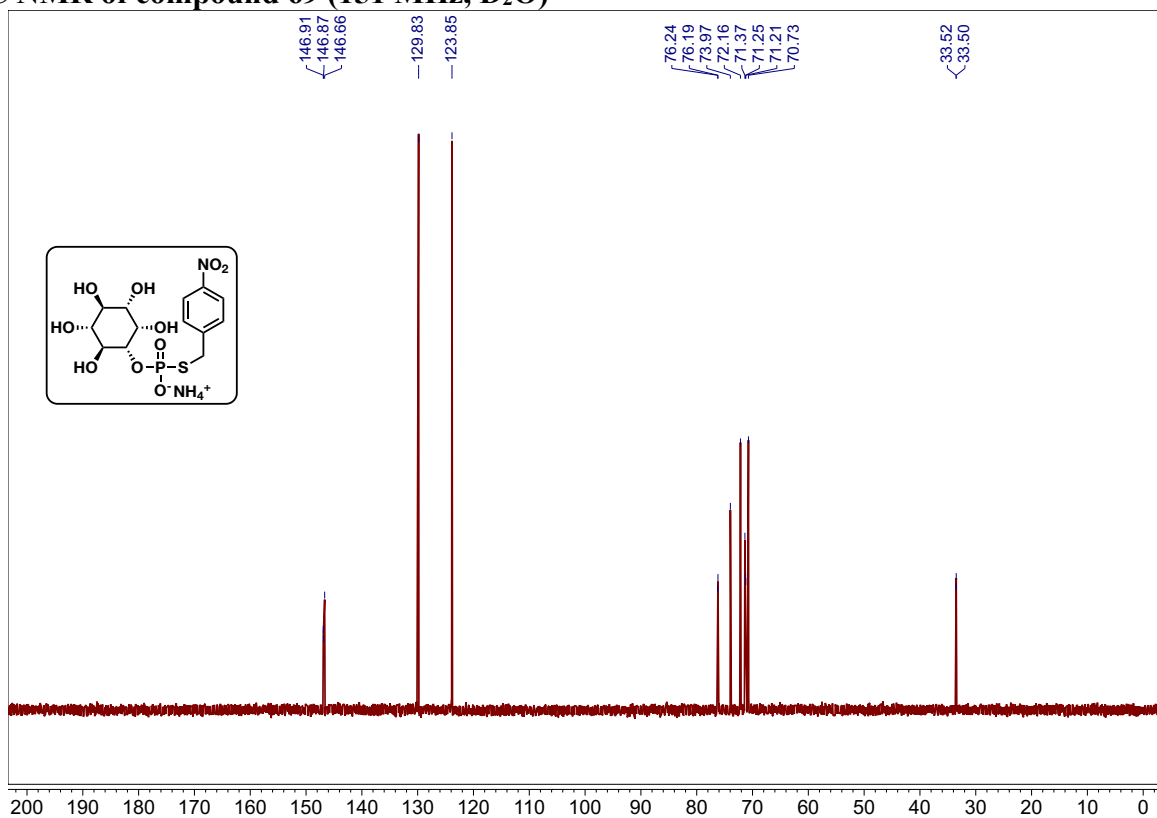
^{31}P NMR of compound 68 (243 MHz, D_2O)



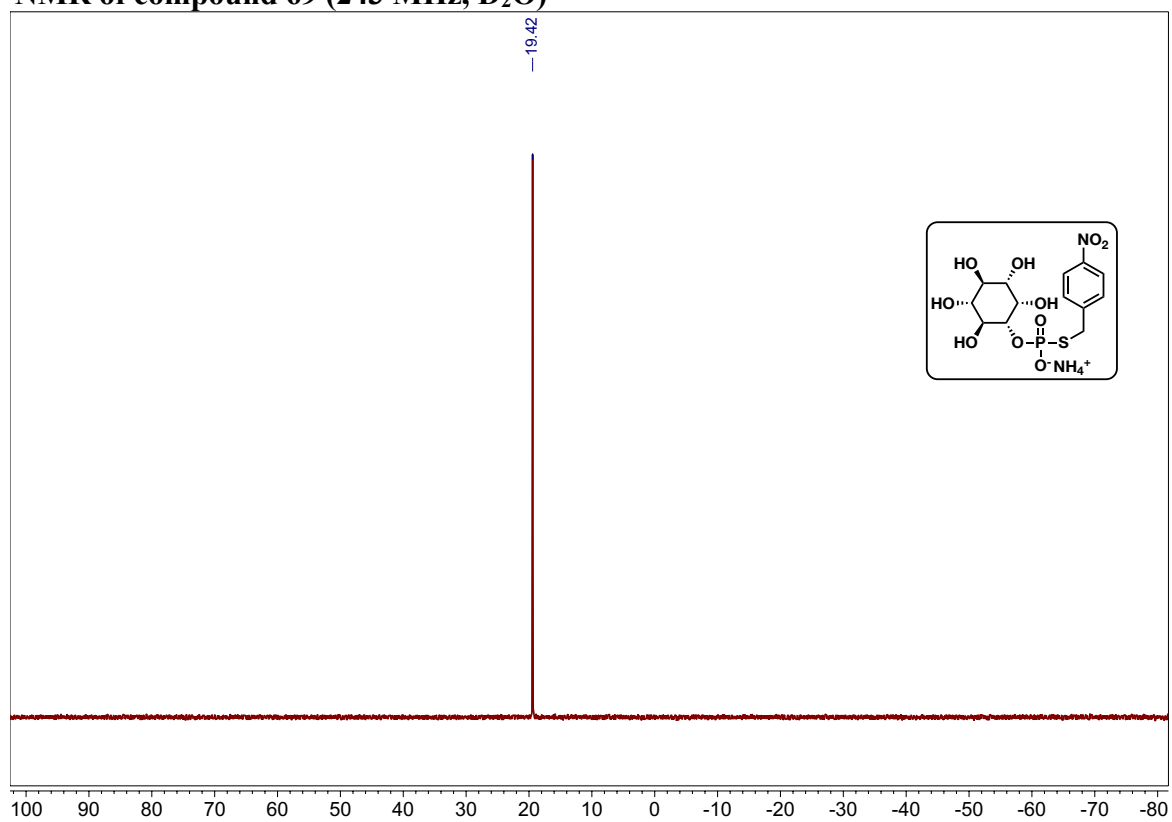
^1H NMR of compound 69 (600 MHz, D_2O)



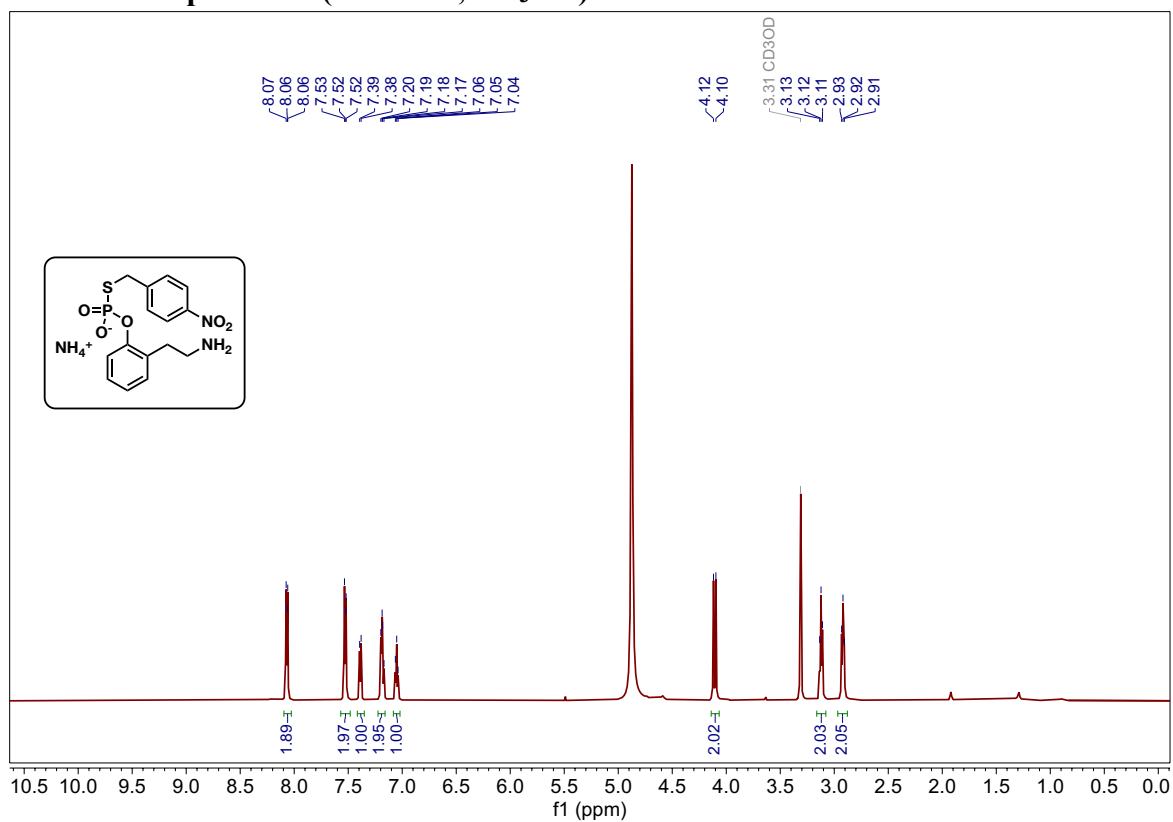
^{13}C NMR of compound 69 (151 MHz, D_2O)



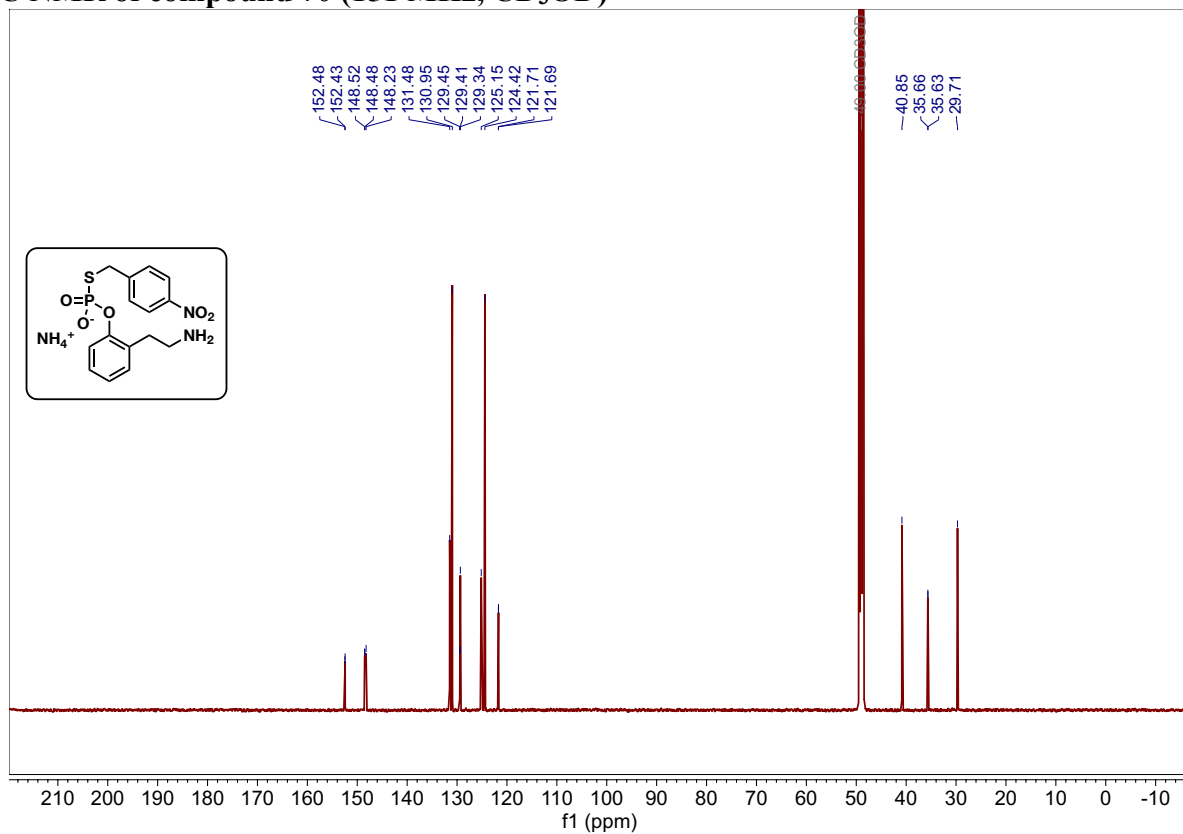
^{31}P NMR of compound 69 (243 MHz, D_2O)



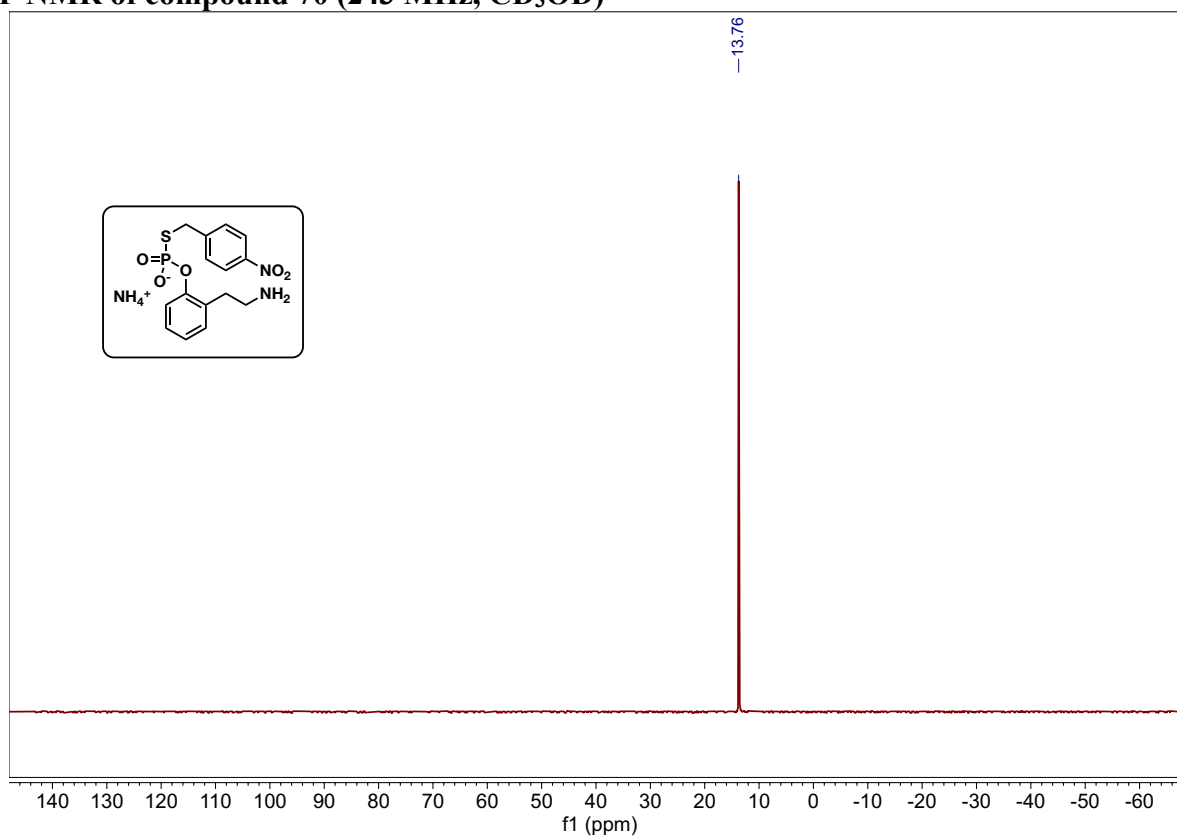
¹H NMR of compound 70 (600 MHz, CD₃OD)



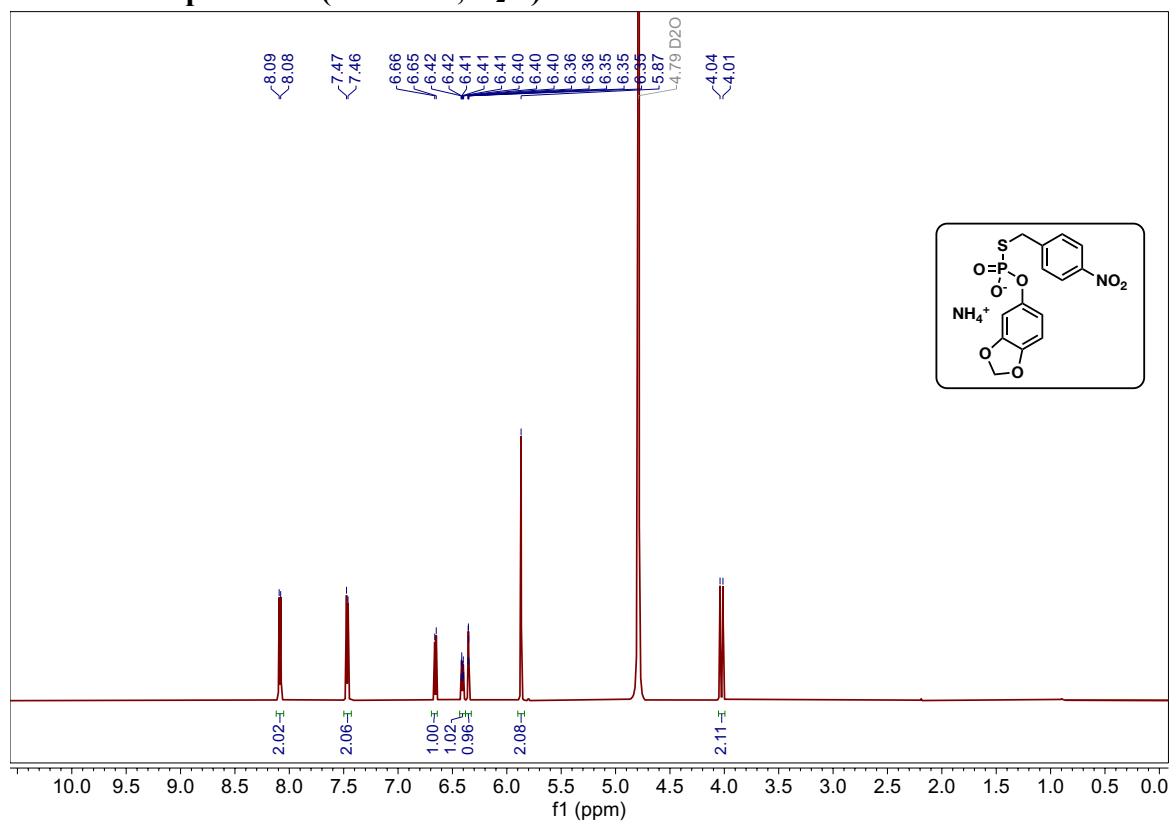
¹³C NMR of compound 70 (151 MHz, CD₃OD)



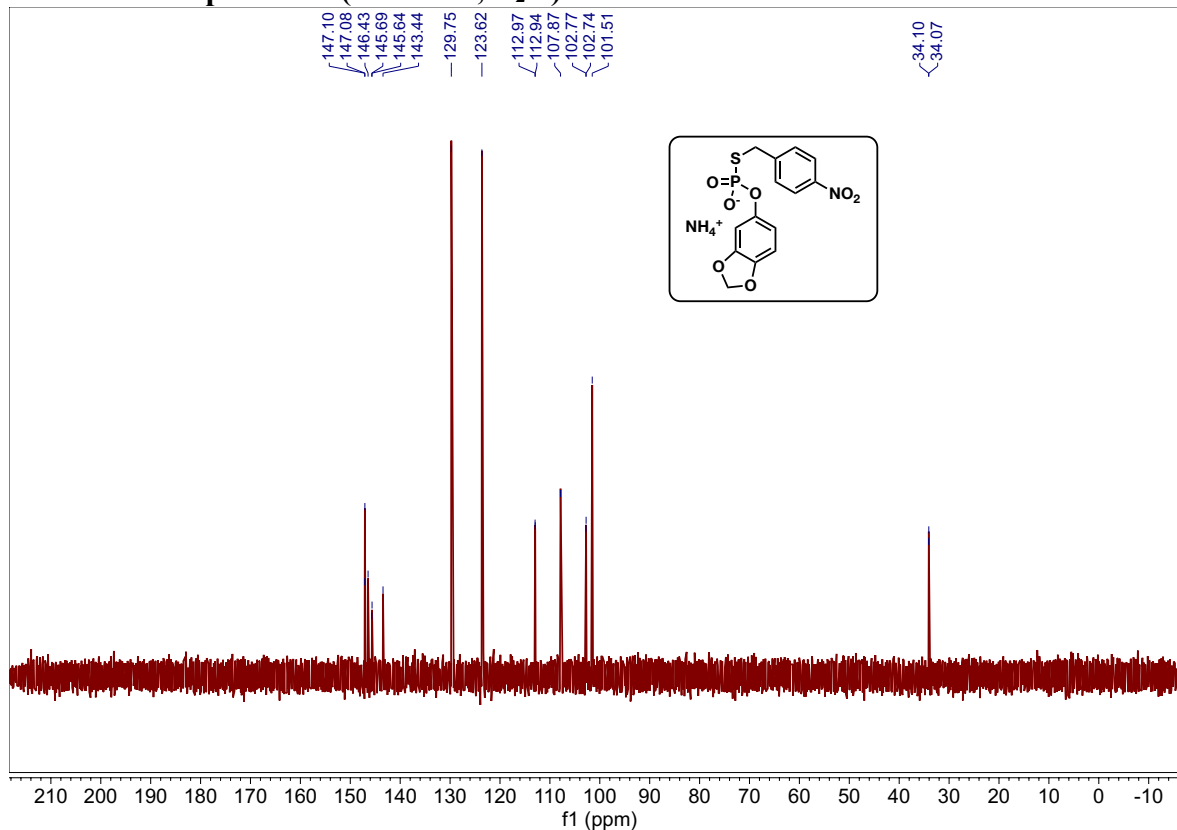
³¹P NMR of compound 70 (243 MHz, CD₃OD)



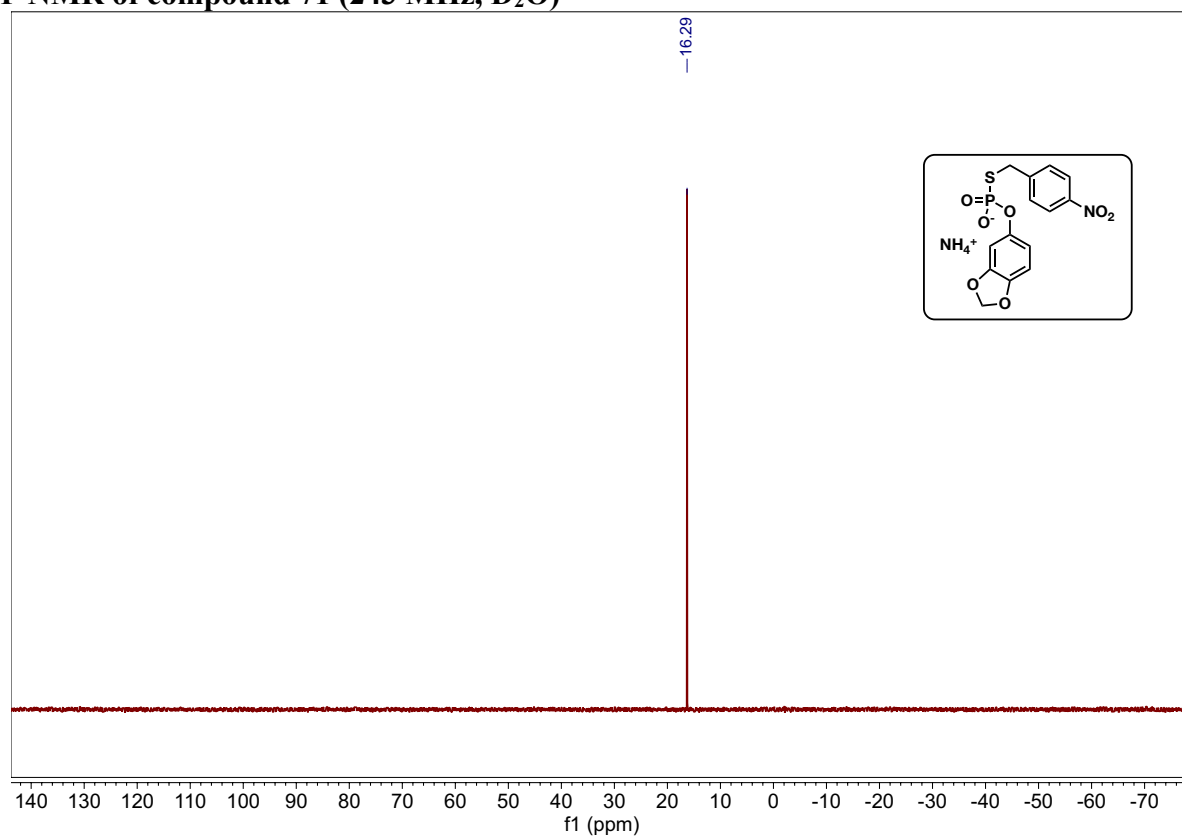
¹H NMR of compound 71 (600 MHz, D₂O)



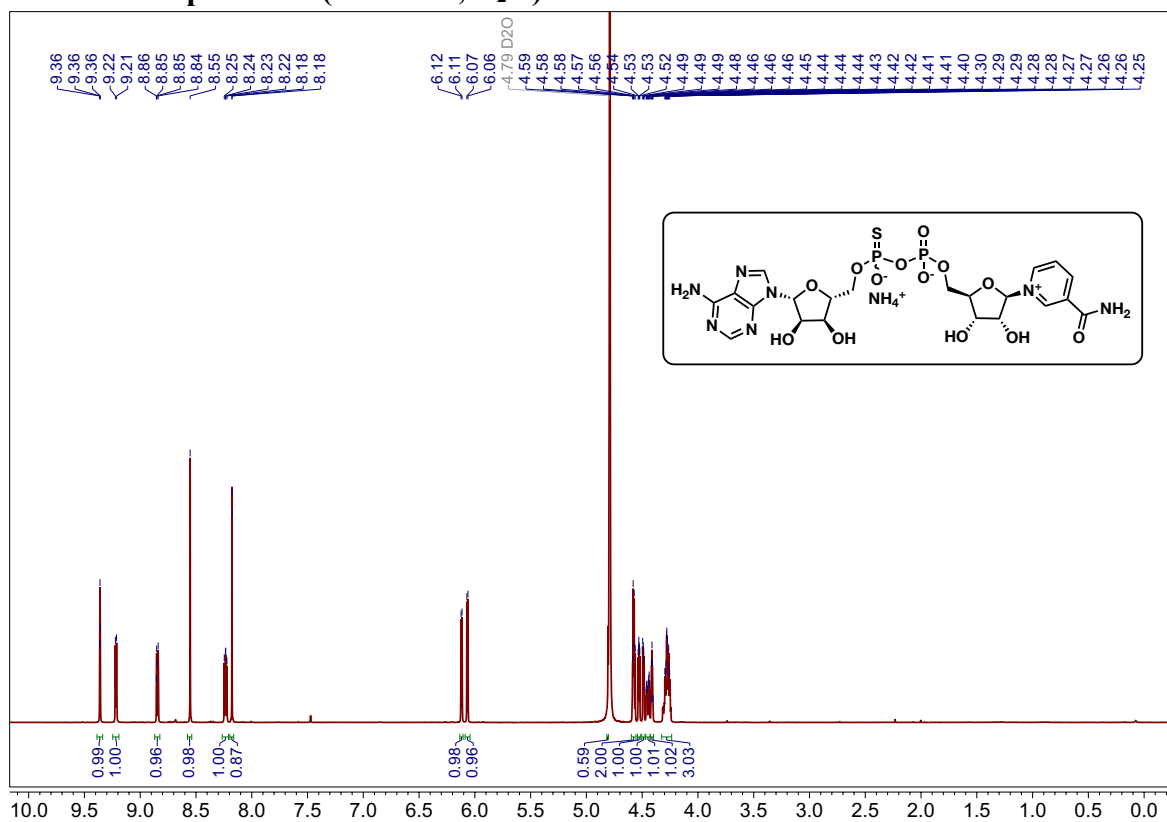
¹³C NMR of compound 71 (151 MHz, D₂O)



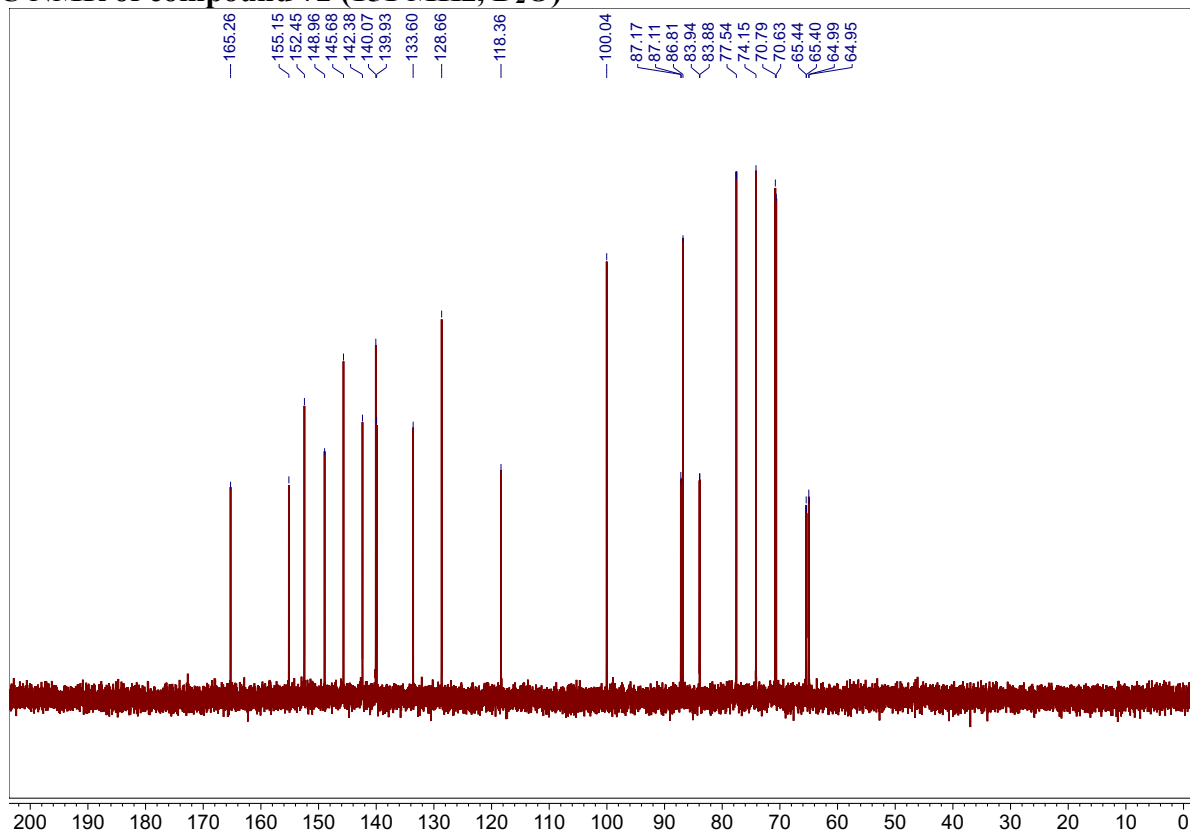
^{31}P NMR of compound 71 (243 MHz, D_2O)



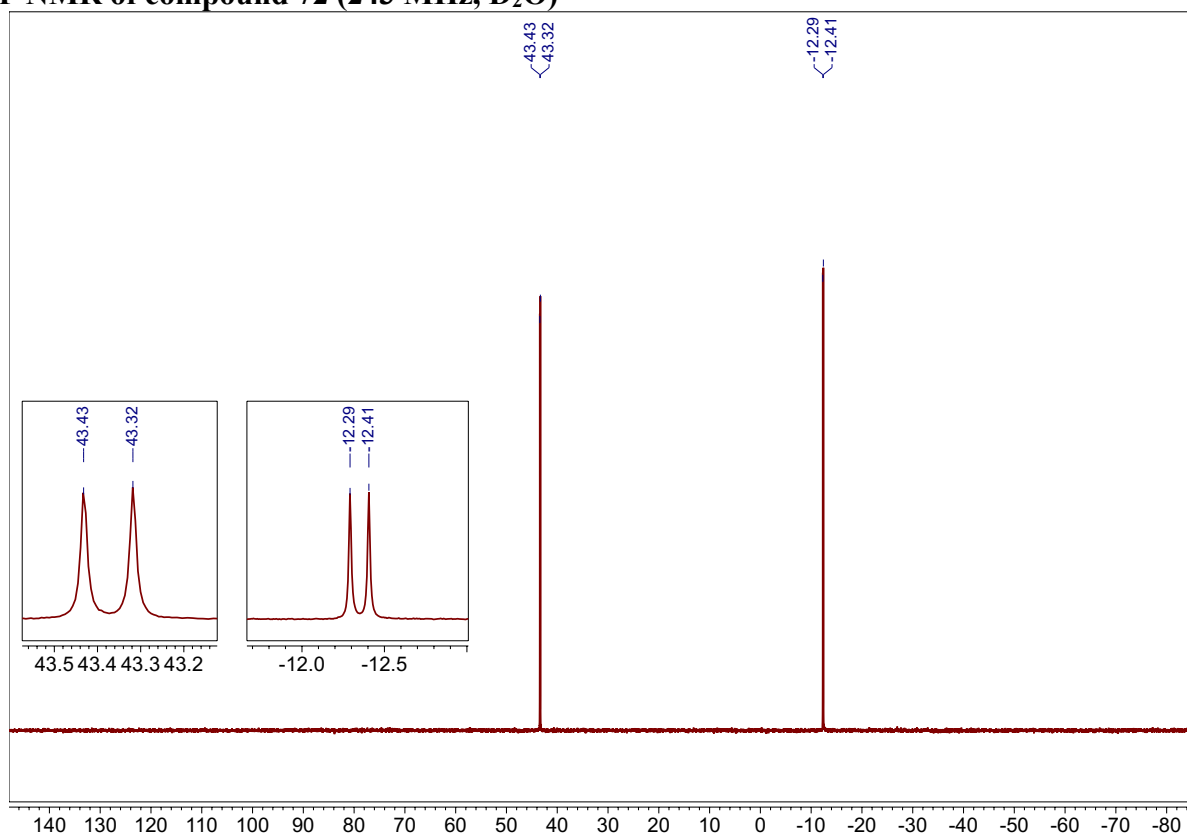
¹H NMR of compound 72 (600 MHz, D₂O)



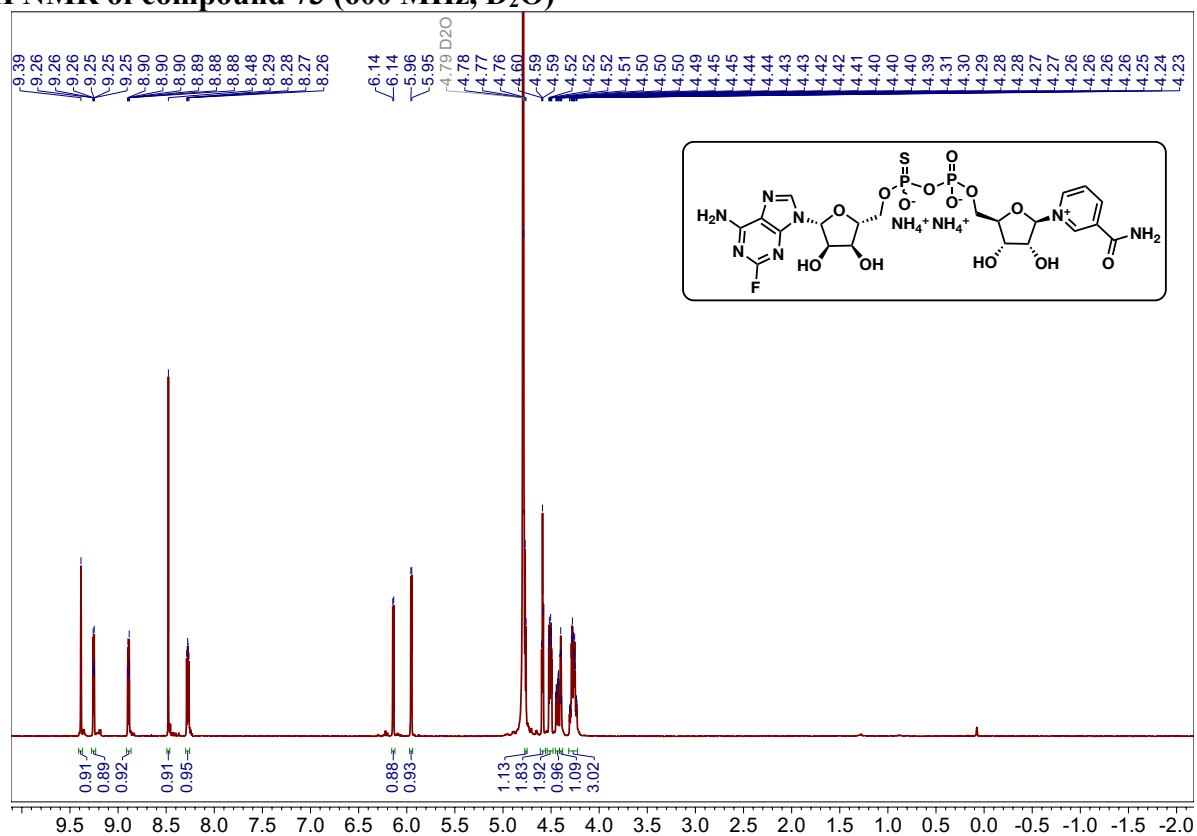
¹³C NMR of compound 72 (151 MHz, D₂O)



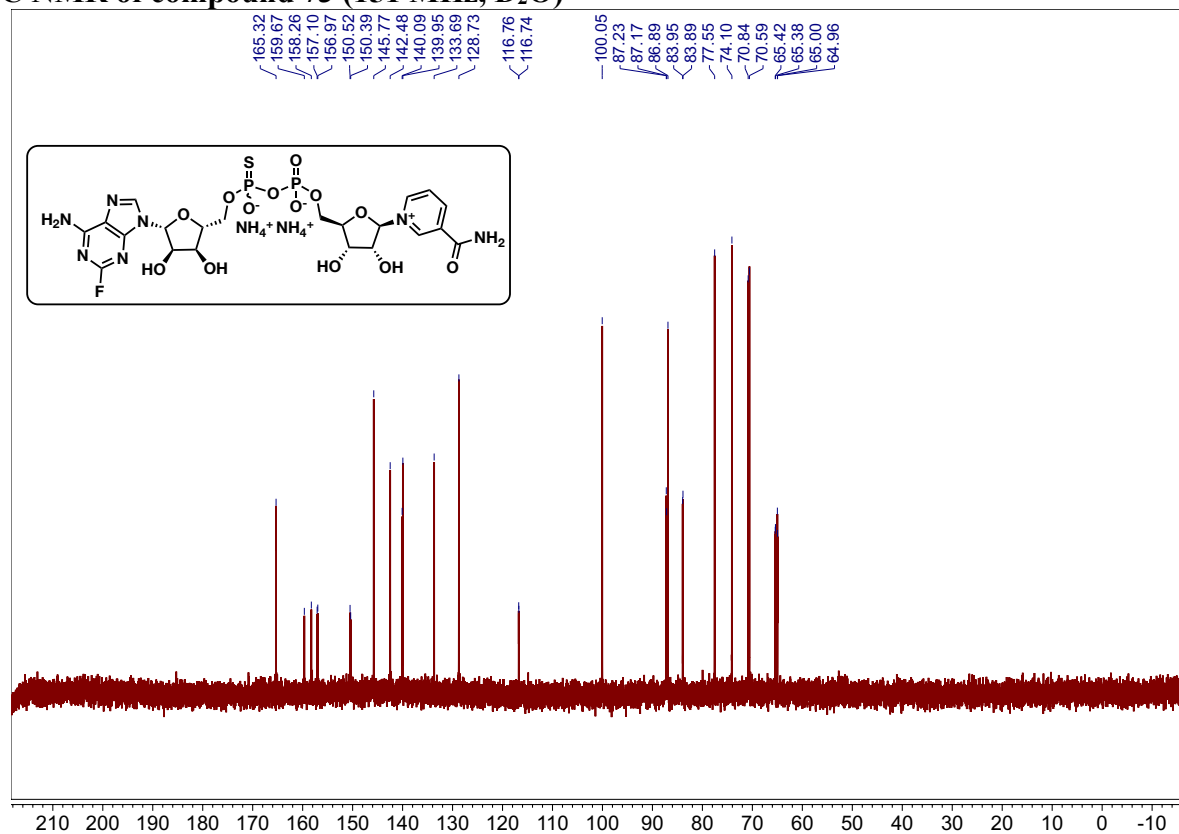
^{31}P NMR of compound 72 (243 MHz, D_2O)



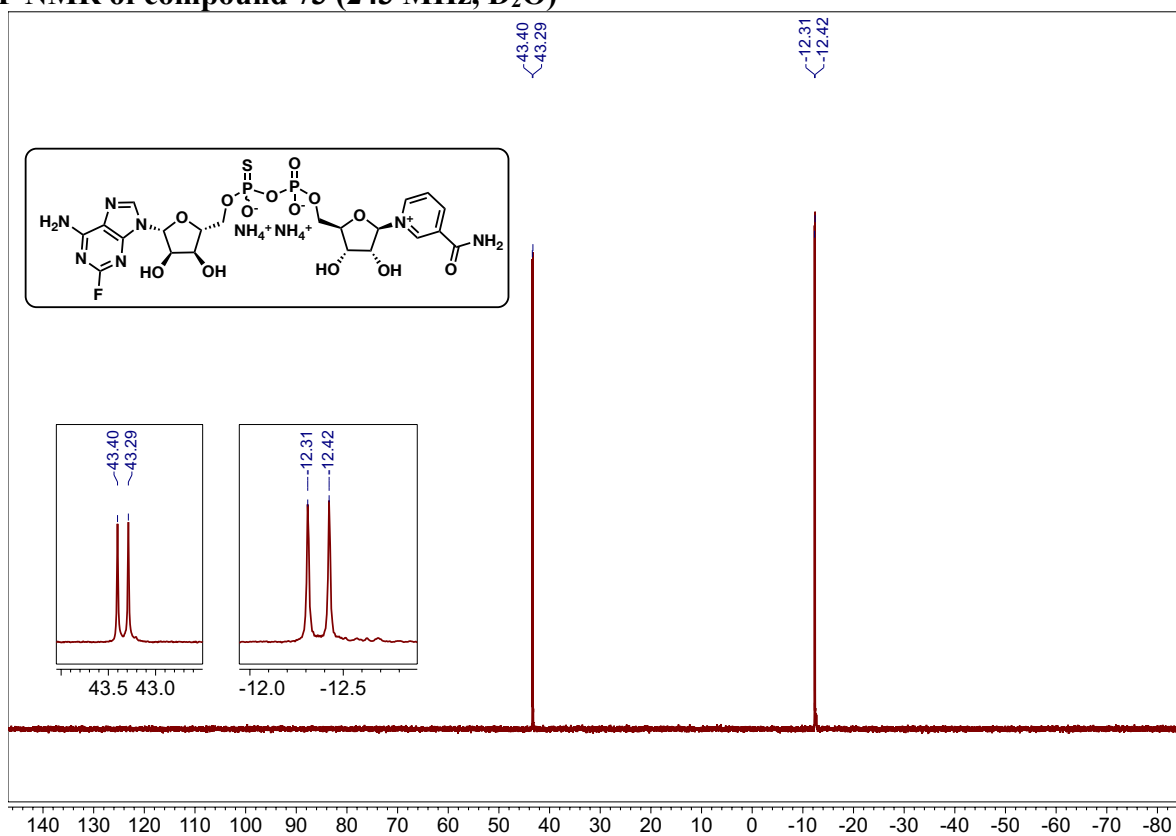
¹H NMR of compound 73 (600 MHz, D₂O)



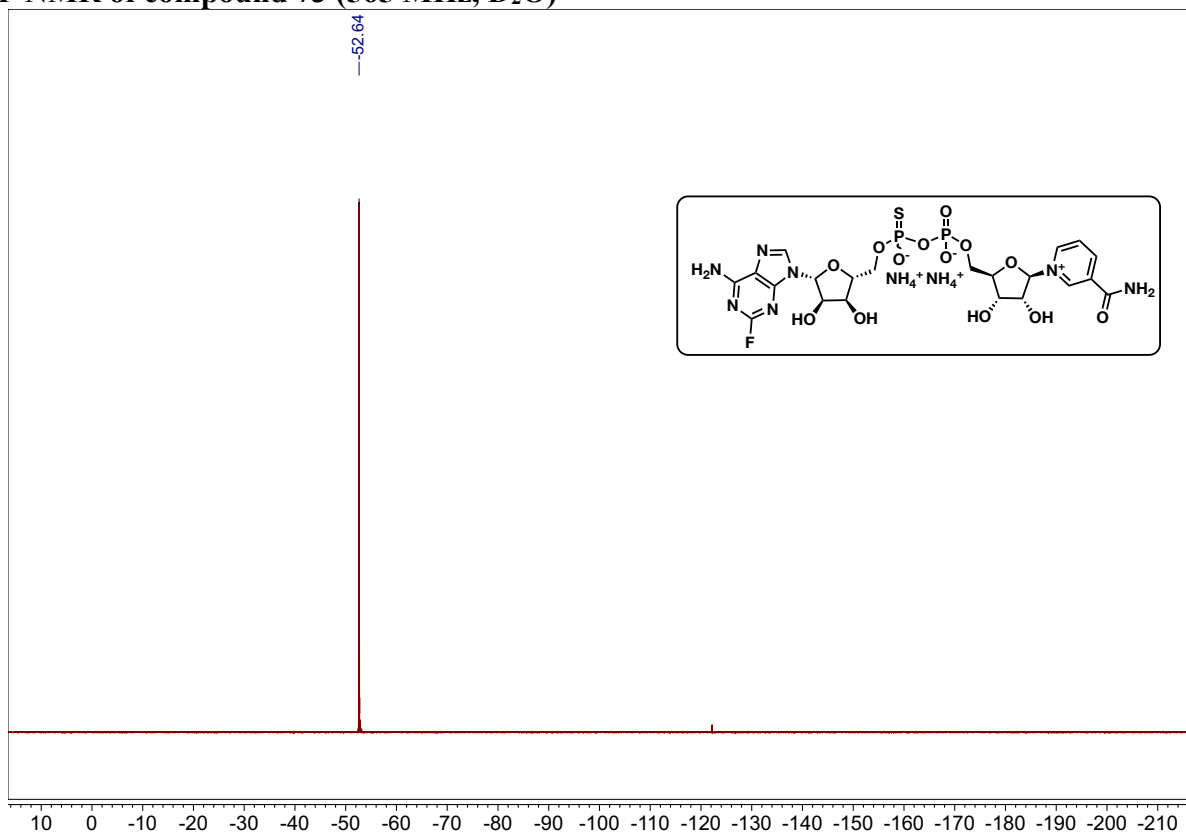
¹³C NMR of compound 73 (151 MHz, D₂O)



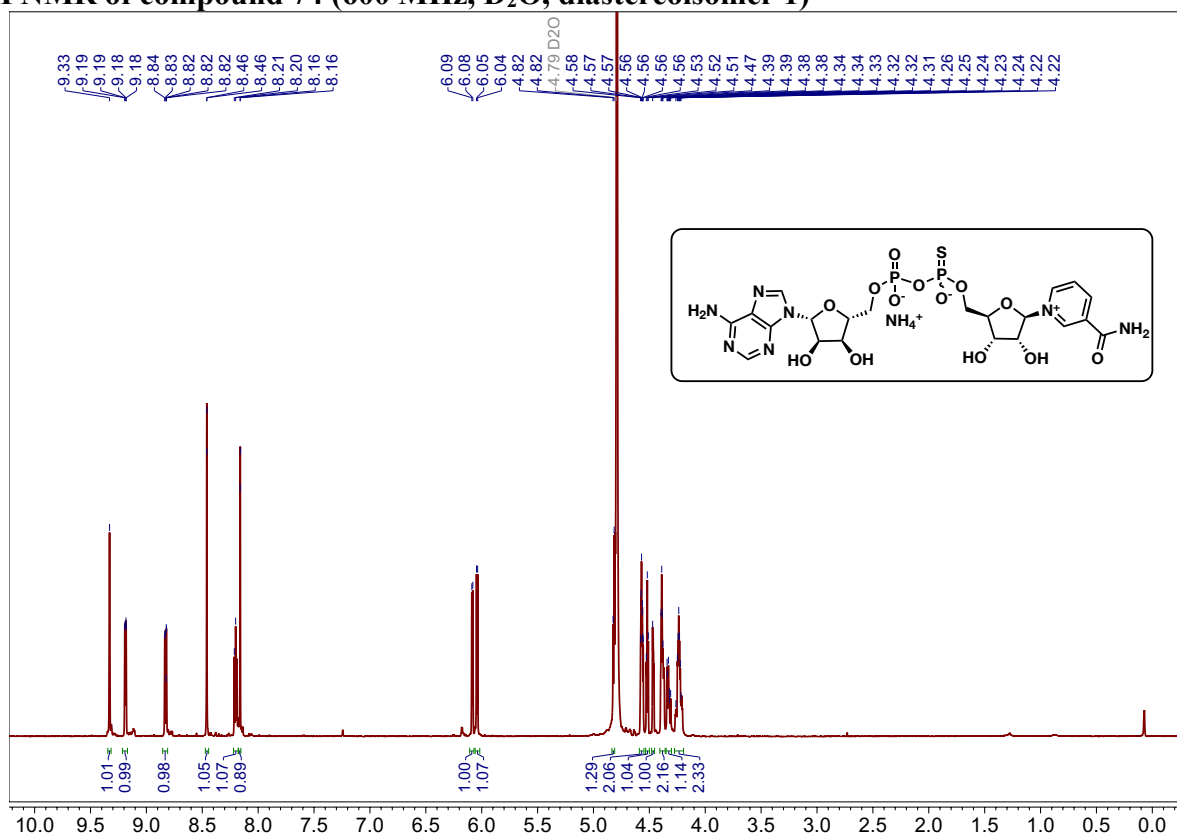
^{31}P NMR of compound 73 (243 MHz, D_2O)



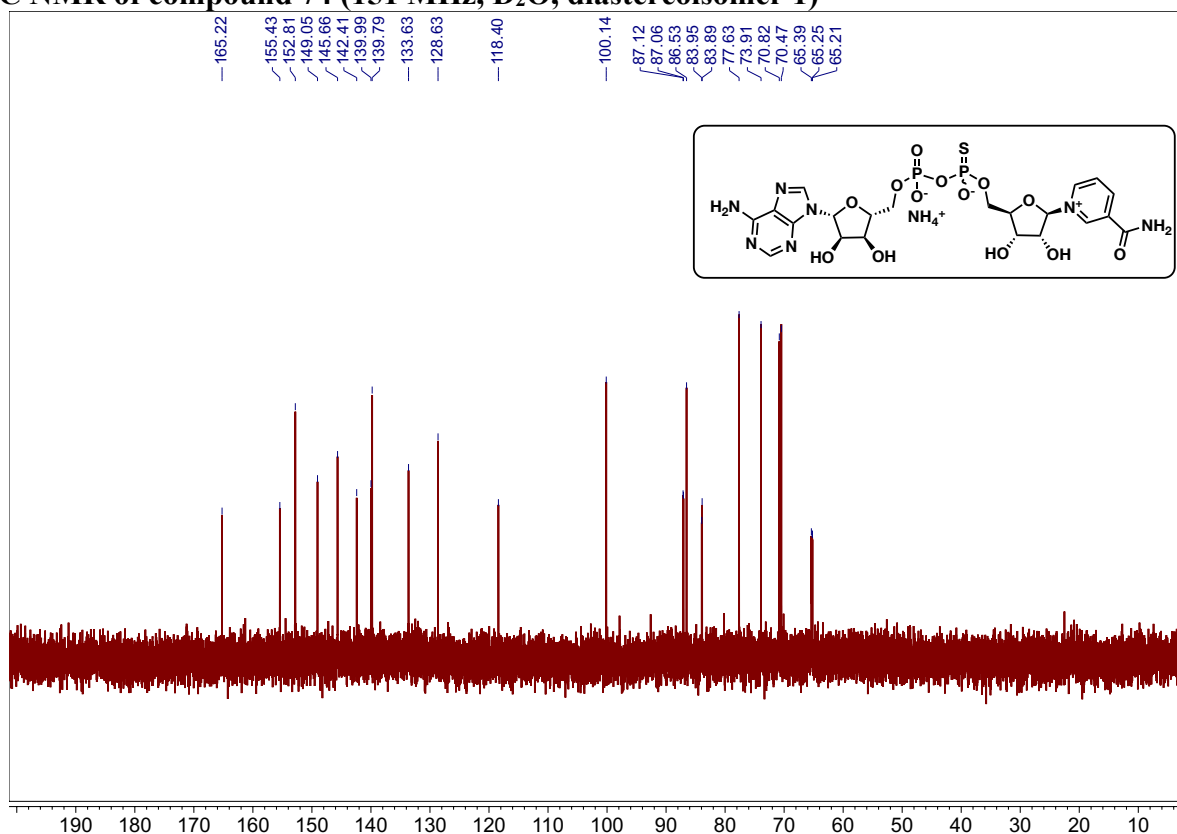
^{19}F NMR of compound 73 (565 MHz, D_2O)



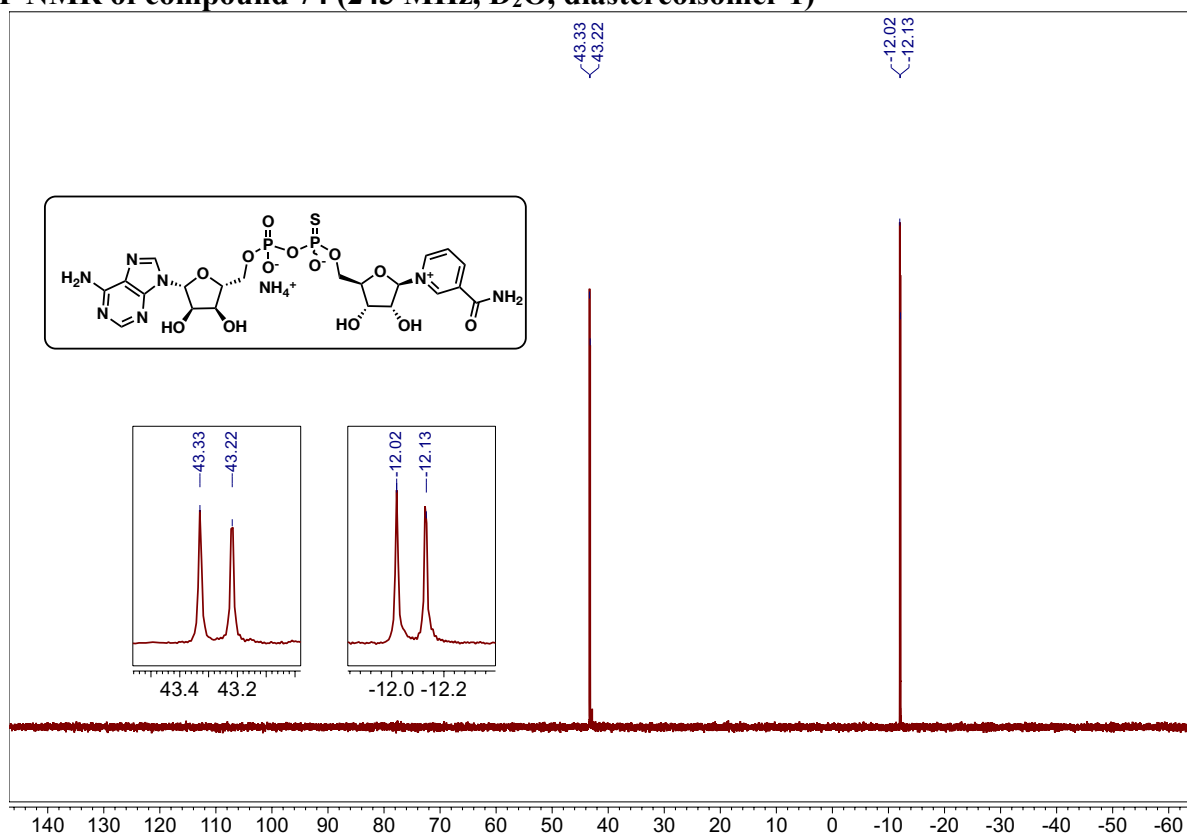
^1H NMR of compound 74 (600 MHz, D_2O , diastereoisomer 1)



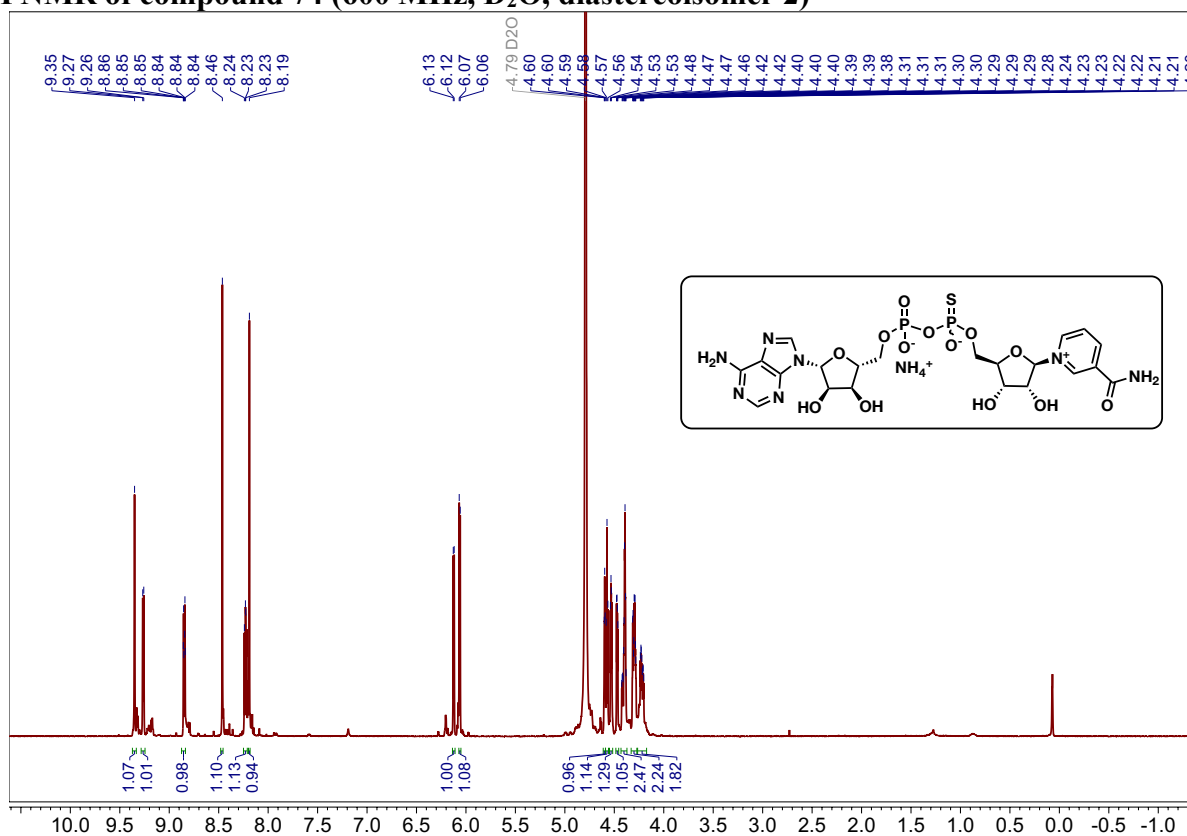
^{13}C NMR of compound 74 (151 MHz, D_2O , diastereoisomer 1)



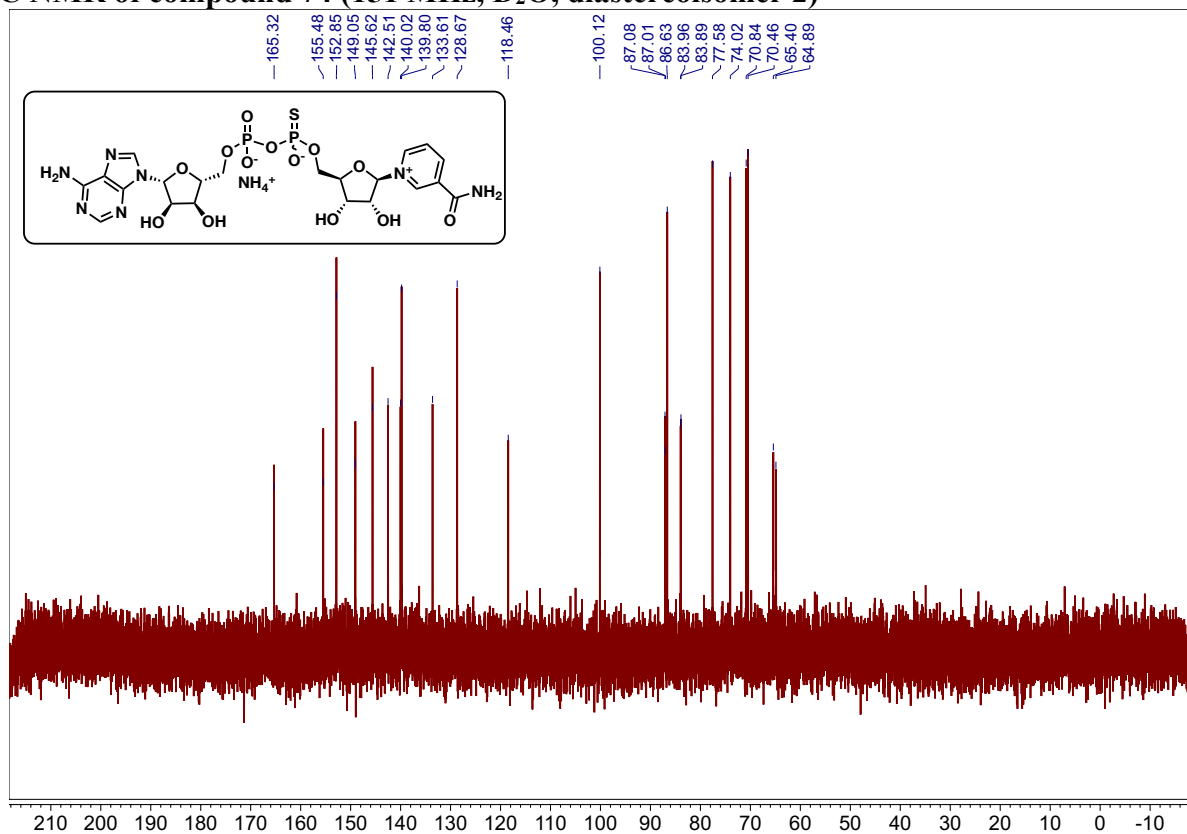
^{31}P NMR of compound 74 (243 MHz, D_2O , diastereoisomer 1)



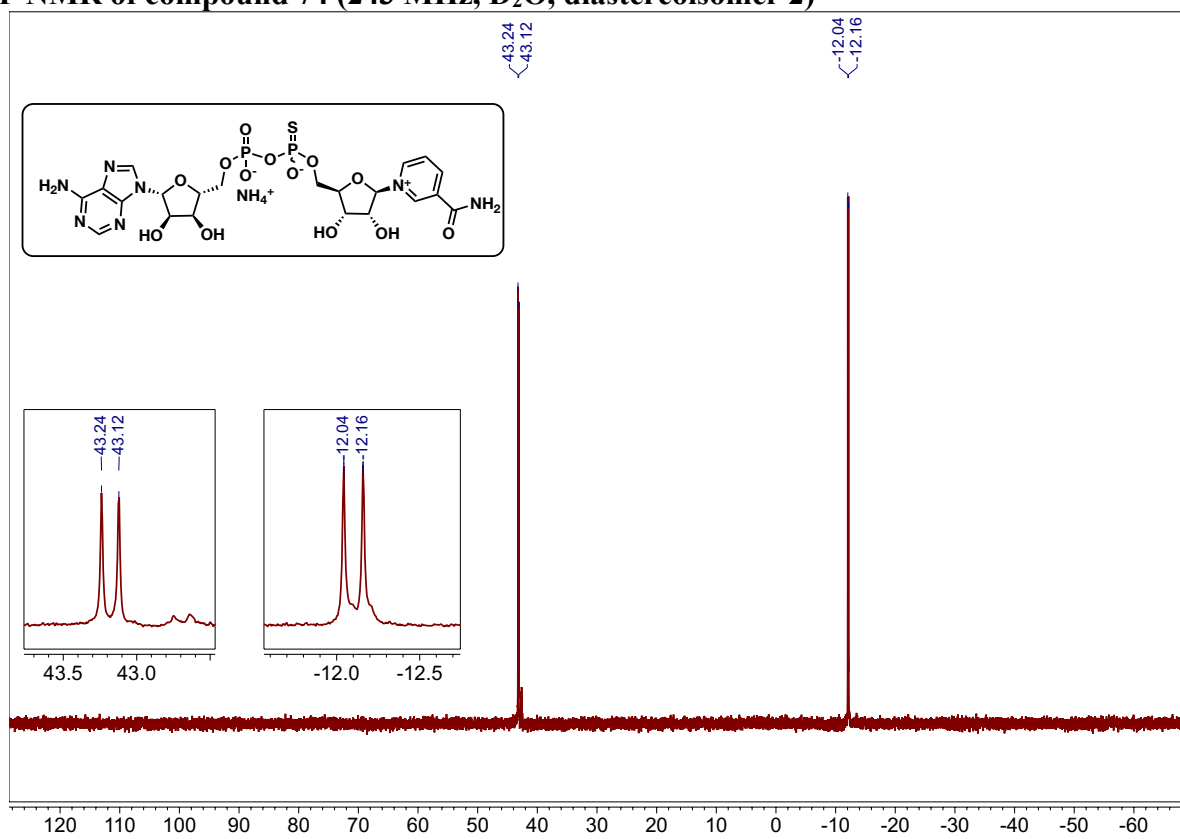
^1H NMR of compound 74 (600 MHz, D_2O , diastereoisomer 2)



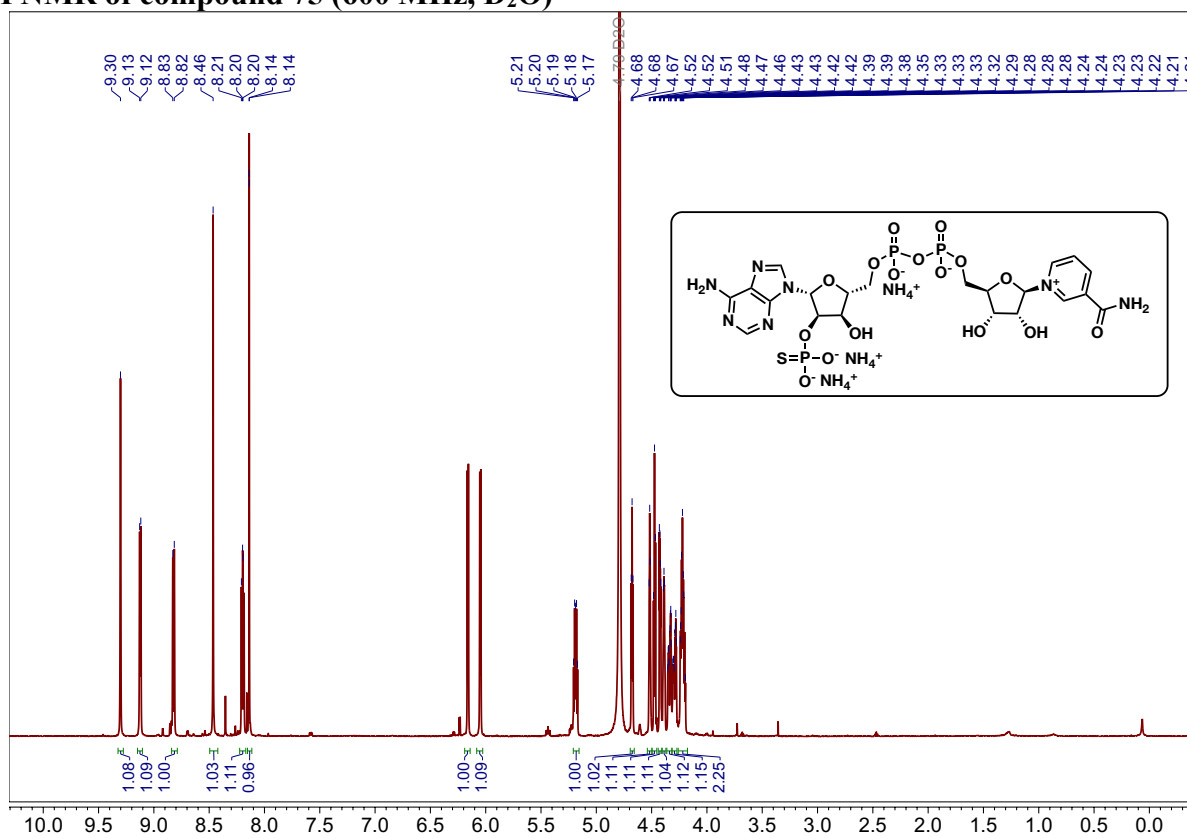
^{13}C NMR of compound 74 (151 MHz, D_2O , diastereoisomer 2)



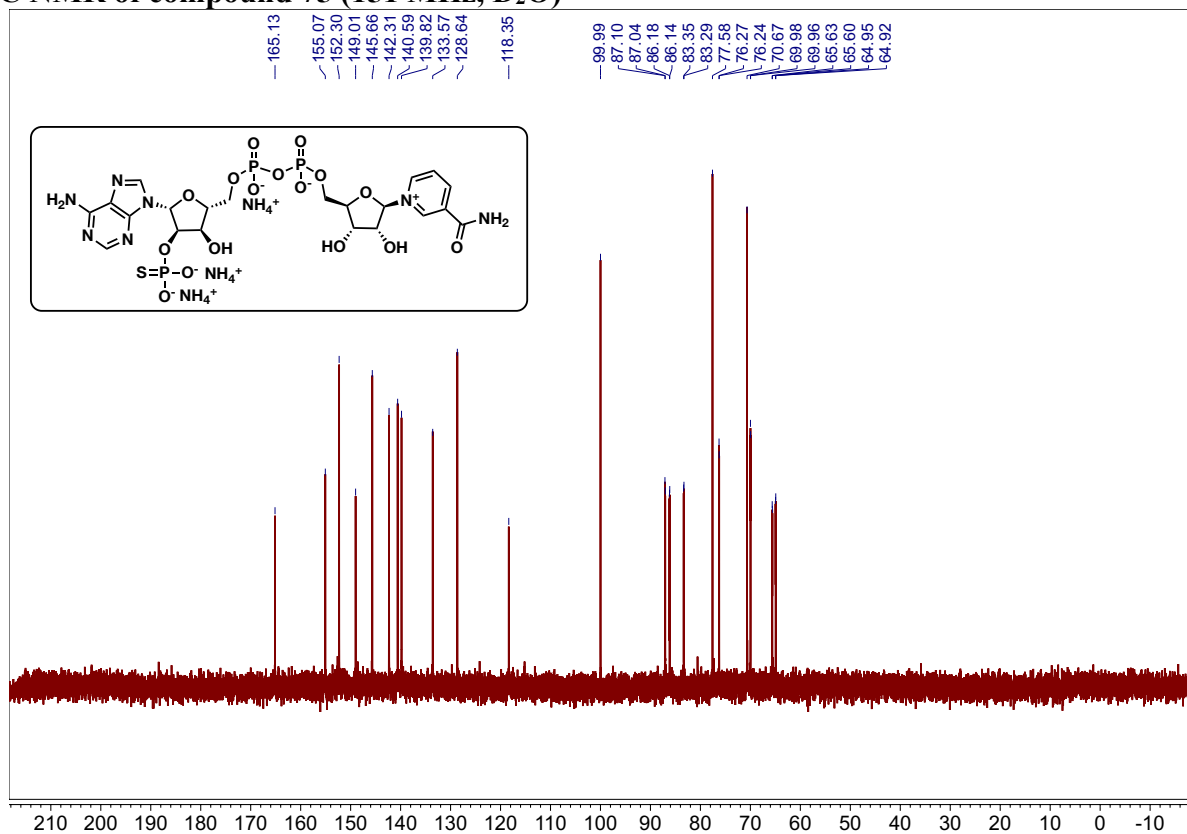
^{31}P NMR of compound 74 (243 MHz, D_2O , diastereoisomer 2)



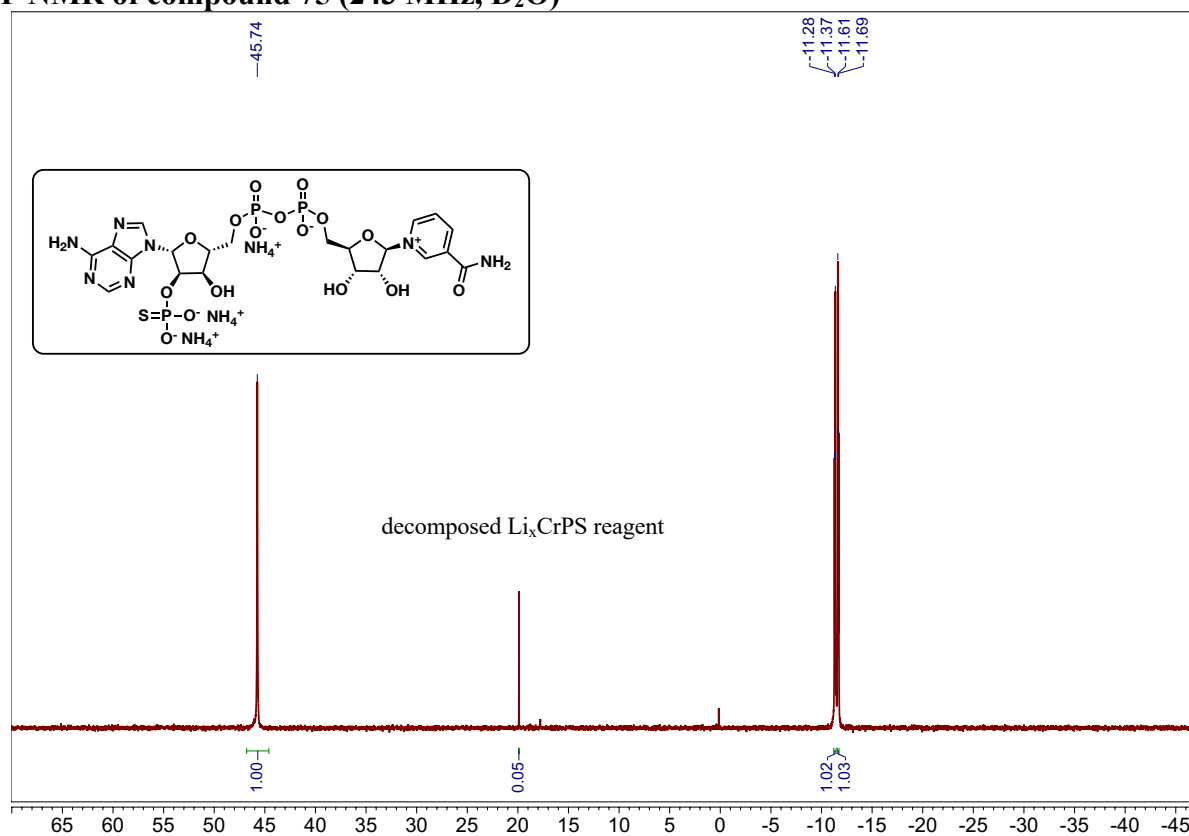
¹H NMR of compound 75 (600 MHz, D₂O)



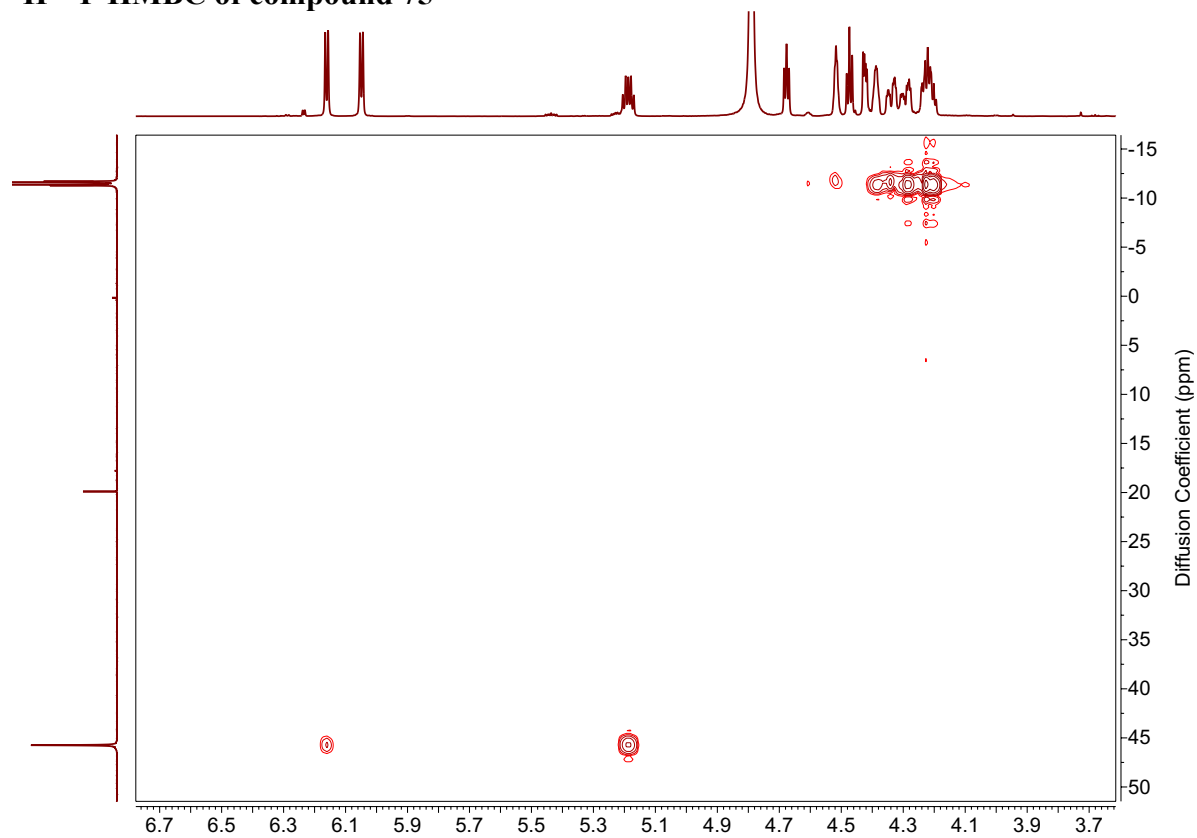
¹³C NMR of compound 75 (151 MHz, D₂O)



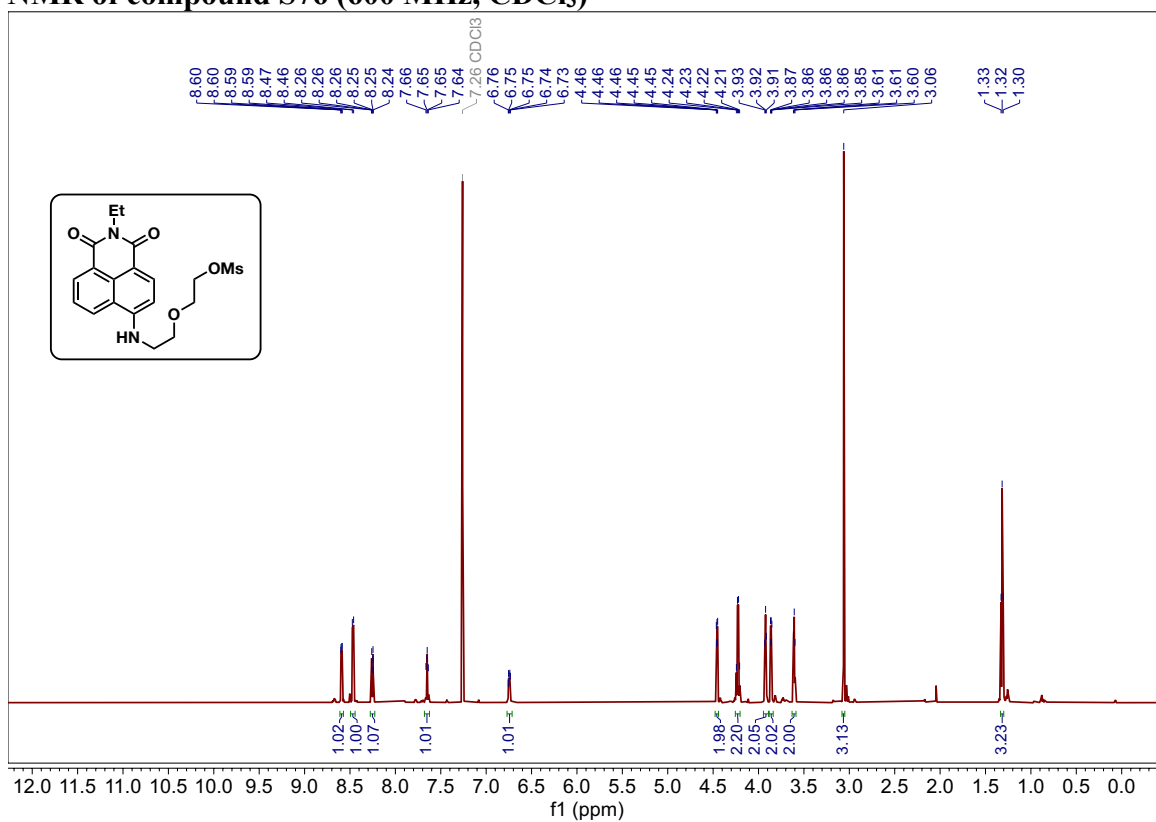
^{31}P NMR of compound 75 (243 MHz, D_2O)



^1H - ^{31}P HMBC of compound 75



¹H NMR of compound S76 (600 MHz, CDCl₃)



¹³C NMR of compound S76 (151 MHz, CDCl₃)

