Supporting Information

Next-Generation Reduction Sensitive Lipid Conjugates of Tenofovir: Antiviral Activity and Mechanism of Release

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Biological Assays

Anti-HIV Assay

All HIV assays were performed by ImQuest BioSciences (Frederick, MD). All compounds were solubilized at 40 mM in DMSO and stored at -20ºC. Test materials were evaluated up to 100 μM, and five serial logarithmic dilutions. AZT and 3TC were obtained from the NIH AIDS Research and Reference Reagent Program and used as controls in the anti-HIV and anti-HBV assay, respectively. Fresh human PBMCs were obtained from a commercial source and determined to be HIV and HBV negative. The leukophoresed blood cells were washed repeatedly with PBS, then diluted 1:1 with Dulbecco's phosphate buffered saline (PBS) and layered over 15 ml of Ficoll-Hypaque density gradient in a 50 ml conical centrifuge tube. The tubes were centrifuged for 30 min at 600 g. Banded PBMCs were gently aspirated from the resulting interface and washed three times with PBS. After the final wash, cell number was determined by Trypan Blue dye exclusion and cells re-suspended at 1×10^6cells/mL in RPMI 1640 with 15% Fetal Bovine Serum (FBS), 2 mmol/L L-glutamine, 2 μg/mL PHA-P, 100 U/ml penicillin and 100 μg/mL streptomycin and allowed to incubate for 48-72 hr at 37℃. After incubation, PBMCs were centrifuged and re-suspended in tissue culture medium (RPMI 1640 with 15% Fetal Bovine Serum (FBS), 2 mmol/L L-glutamine, 2 μg/mL PHA-P, 100 U/ml penicillin and 100 μg/mL streptomycin, 3.6 ng/mL recombinant human IL-2). The cultures were maintained until use by half-volume culture changes with fresh IL-2 containing tissue culture medium every 3 days. Assays were initiated with PBMCs at 72 hr post PHA-P stimulation. Immediately prior to use, target cells were re-suspended in fresh tissue culture medium at 1×10^6 cells/ml and plated in the interior wells of a 96-well round bottom microliter plate at 50 μL/well. Then, 100 μL of 2X concentrations of compound-containing medium was transferred to the 96-well plate containing the cells in 50 μL of the medium. Following addition of test compound to the wells, 50 μL of a predetermined dilution of HIV virus (prepared at 4x of final desired in-well concentration) was added, and mixed well. For infection, 50-150 $TCID_{50}$ of each virus was added per well (final MOI approximately 0.002). PBMCs were exposed in triplicate to virus and cultured in the presence or absence of the test compound at varying concentrations as described for the 96-well microliter plates. After 7 days, HIV-1 replication was quantified in the tissue culture supernatant by measurement of reverse transcriptase activity. Wells with cells and virus only served as virus controls. Separate plates were identically prepared without virus for cytoxicity studies. Reverse transcriptase activity was measured in cell-free supernatants using a standard radioactive incorporation polymerization assay.

Anti-HBV Assay

One hundred microliters (100 μL) of wells of a 96-well flat-bottom plate at a density of $1x10^4$ cells per well were incubated at 37ºC in 5% CO_2 for 24 hours. Following incubation, six ten-fold serial dilutions of test compound prepared in RPMI1640 medium with 10% fetal bovine serum were added to individual wells of the plate in triplicate. Six wells in the plate received medium alone as a virus control only. The plate was incubated for 6 days at 37ºC at 5% CO_2. The culture medium was changed on day 3 with medium containing the indicated concentration of each compound. One hundred microliters (100 μL) of supernatant was collected from each well for analysis of viral DNA by qPCR and cytotoxicity was evaluated by XTT staining of the cell culture monolayer on the sixth day (see below). Ten microliters (10 μL) of cell culture supernatant collected on the sixth

day was diluted in qPCR dilution buffer (40 μg/ml sheared salmon sperm DNA) and boiled for 15 minutes. Quantitative real time PCR was performed in 386 well plates using an Applied Biosystems 7900HT Sequence Detection System and the supporting SDS 2.4 software. Five microliters (5 μL) of boiled DNA for each sample and serial 10-fold dilutions of a quantitative DNA standard were subjected to real time Q-PCR using Platinum Quantitative PCR SuperMix-UDG (Invitrogen) and specific DNA oligonucleotide primers (IDT, Coralville, ID) HBV-AD38-qF1 (5'-CCG TCT GTG CCT TCT CAT CTG-3'), HBV-AD38-qR1 (5'-AGT CCA AGA GTY CTC TTA TRY AAG ACC TT-3') and HBV-AD38-qP1 (5'-FAM-CCG TGT GCA /ZEN/CTT GCG TTC ACC TCT GC-3'BHQ1) at a final concentration of 0.2 μM for each primer in a total reaction volume of 15 μl. The final HBV DNA copy number in each sample was interpolated from the standard curve by the SDS2.4 software and the data were analyzed by Excel.

Cytotoxicity Studies

Cytotoxicity was evaluated by staining uninfected cells with tetrazolium dye XXT with spectrophotometric readings at 450/650 nm with a Molecular Devices Vmax plate reader. These experiments were run in triplicate for each compound tested.

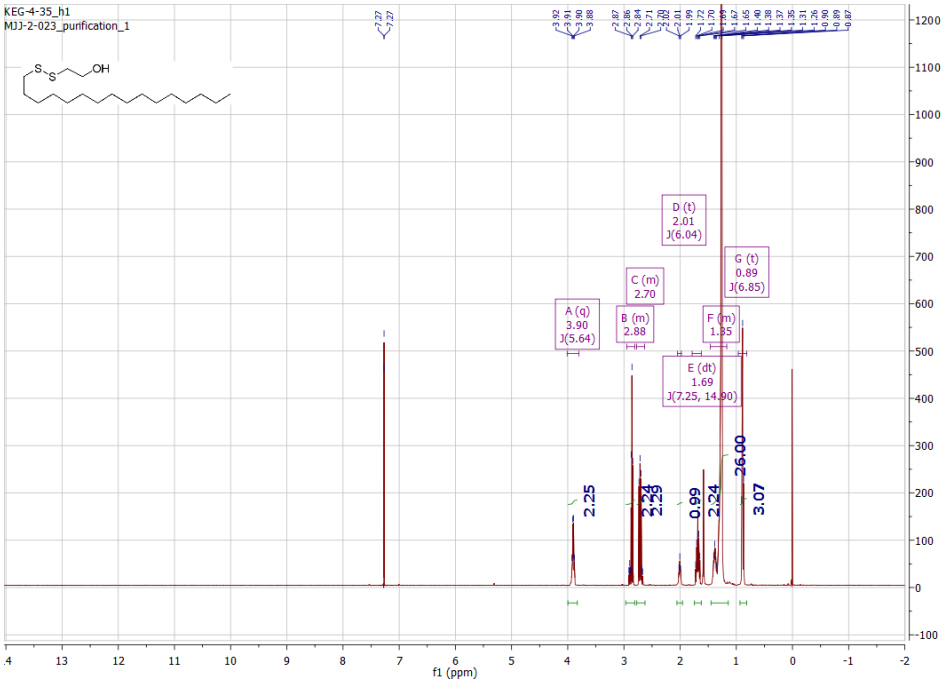
Kinetic Studies

All buffer solutions (pH = 2, 7.4, 9.19, 10.16) were freshly prepared and the pH of each solution was measured with an Accumet Basic AB15 Plus pH meter at 23 ℃. Quantitative LC-MS (QLC-MS) was performed with an Agilent Technologies 6100 quadrupole instrument equipped with UV detection at 254 and 210 nm and a Varian C8 analytical column using a H_2O/MeOH gradient (35-75% MeOH) over three minutes with intermittent washing (35% MeOH isocratic, 1 min) between runs. For analysis, a four dram vial equipped with a magnetic stir bar was charged with the desired buffer (3.0 ml) and incubated in a water bath at 37.4 $\pm$0.2 ℃ prior to the introduction of analyte (compound **6** or **7**). Dithiothreotol (DTT) was also added so that the concentration of DTT was ~0.1 M. After stirring for 10 minutes, 60 μL of a stock solution of **6** or **7** (dissolved in water and MeOH) was added to the buffer solution. Aliquots of this solution (25 μL) were removed at time points t = 0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h. Shorter time points were required for some analyses. Note that the samples were not removed from the water bath during aliquot acquisition. The aliquots were diluted with an aqueous solution containing H_2O/MeOH/HCOOH (75:25:0.1, respectively) to a fixed volume of 1025 μL and analyzed by LC-MS. Integration of the 254 nm absorption signal was the preferred method of quantitation. Linear and exponential regressions were performed with IgorPro v.6.36 and Microsoft Excel, where convenient.

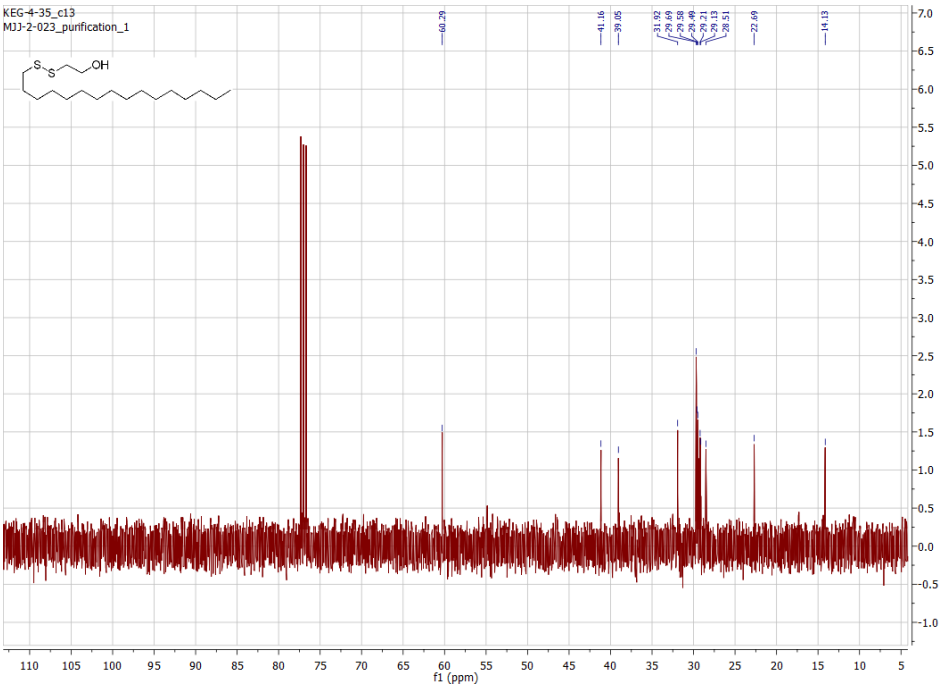
Associated Spectra

2-(Hexadecyldisulfanyl)ethanol (1a)

^{1}H NMR (400 MHz in $CDCl_3$)

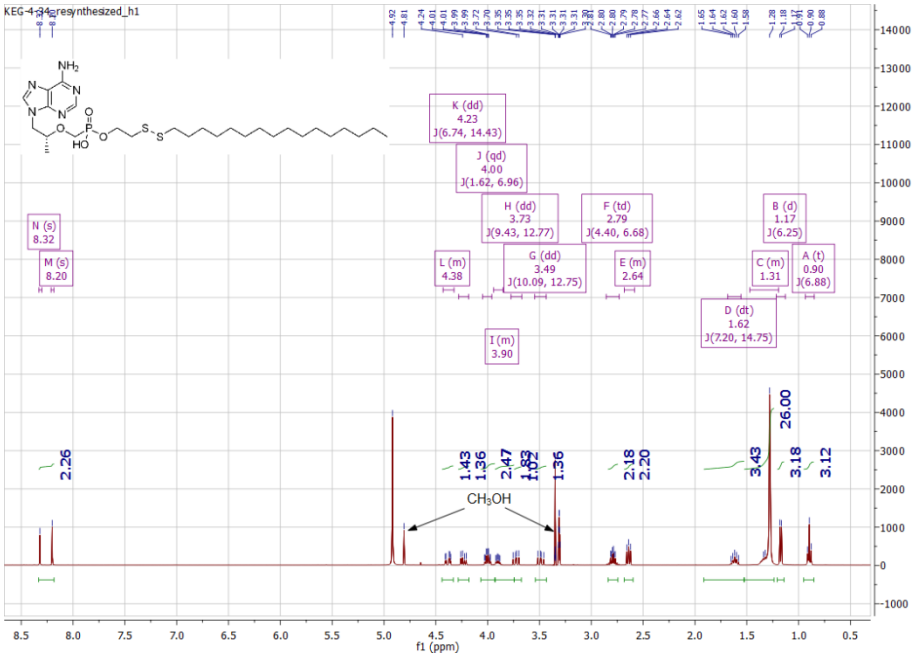


^{13}C NMR (101 MHz in $CDCl_3$)

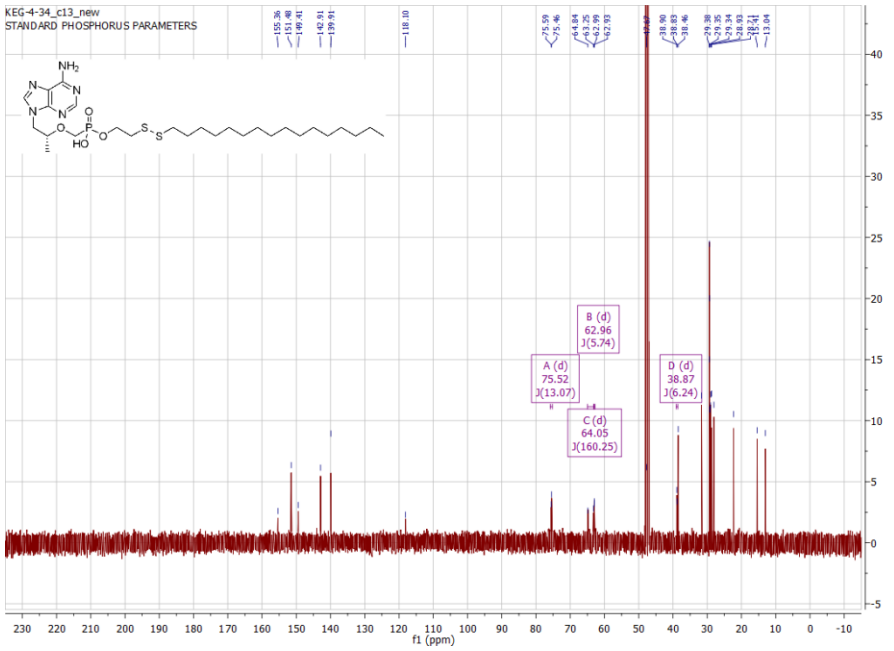


2-(Hexadecyldisulfanyl)ethyl hydrogen ((((*R*)-1-(6-amino-9H-purin-9-yl)propan-2-yl)oxy)methyl)phosphonate (1b)

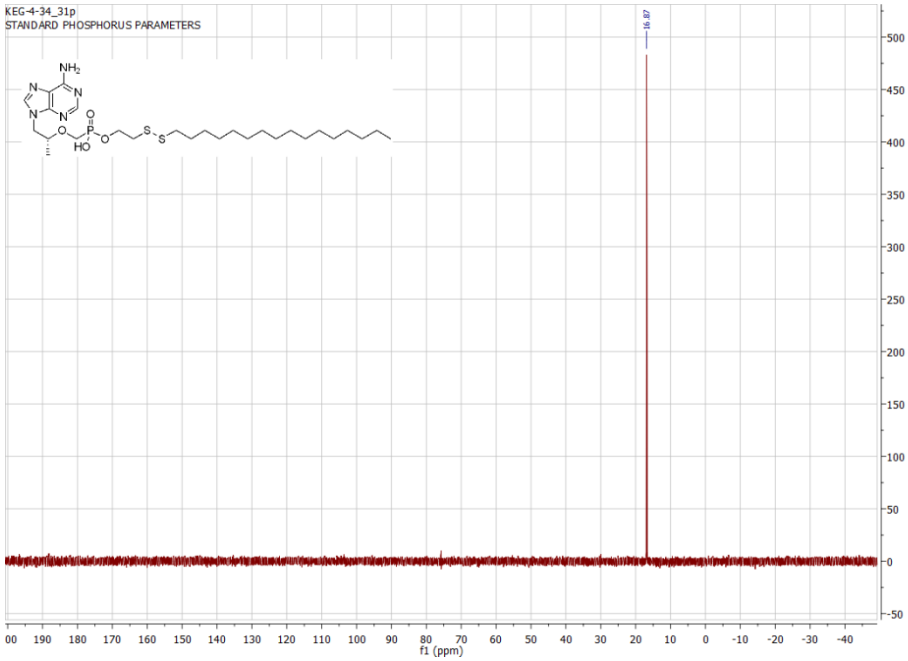
^{1}H NMR (400 MHz, CD_3OD)



^{13}C NMR (101 MHz, CD_3OD)



^{31}P NMR (162 MHz, CD_3OD)



4-(Hexadecyldisulfanyl)butan-1-ol (2a)

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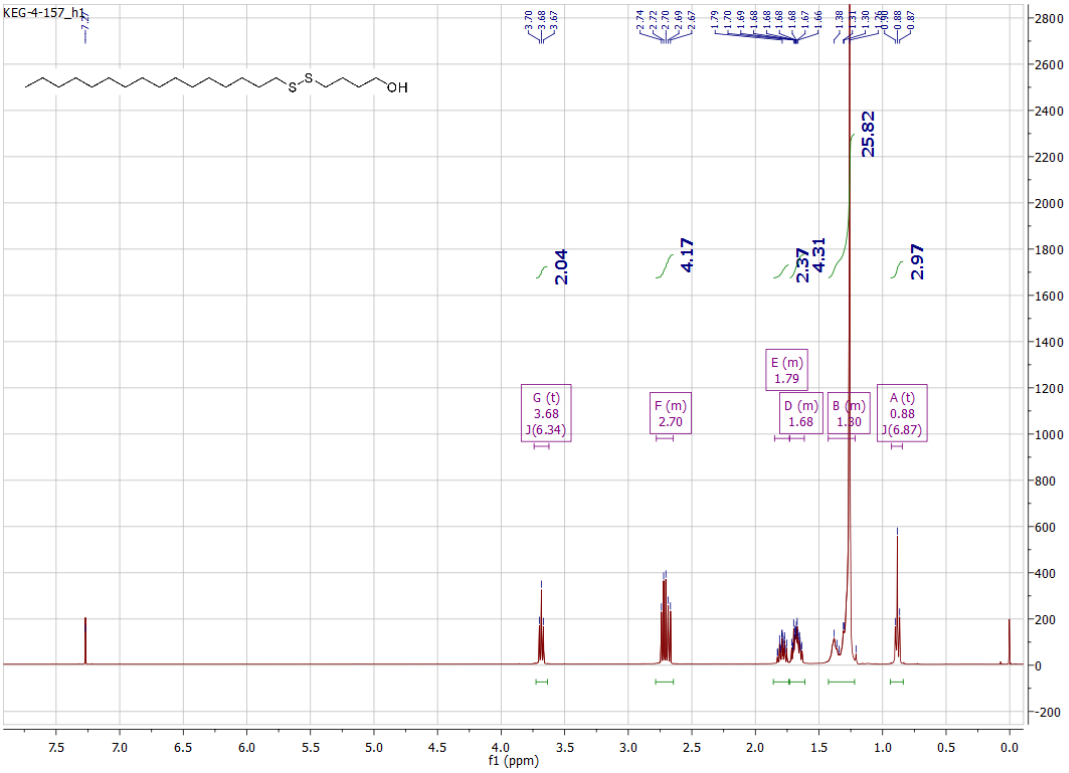
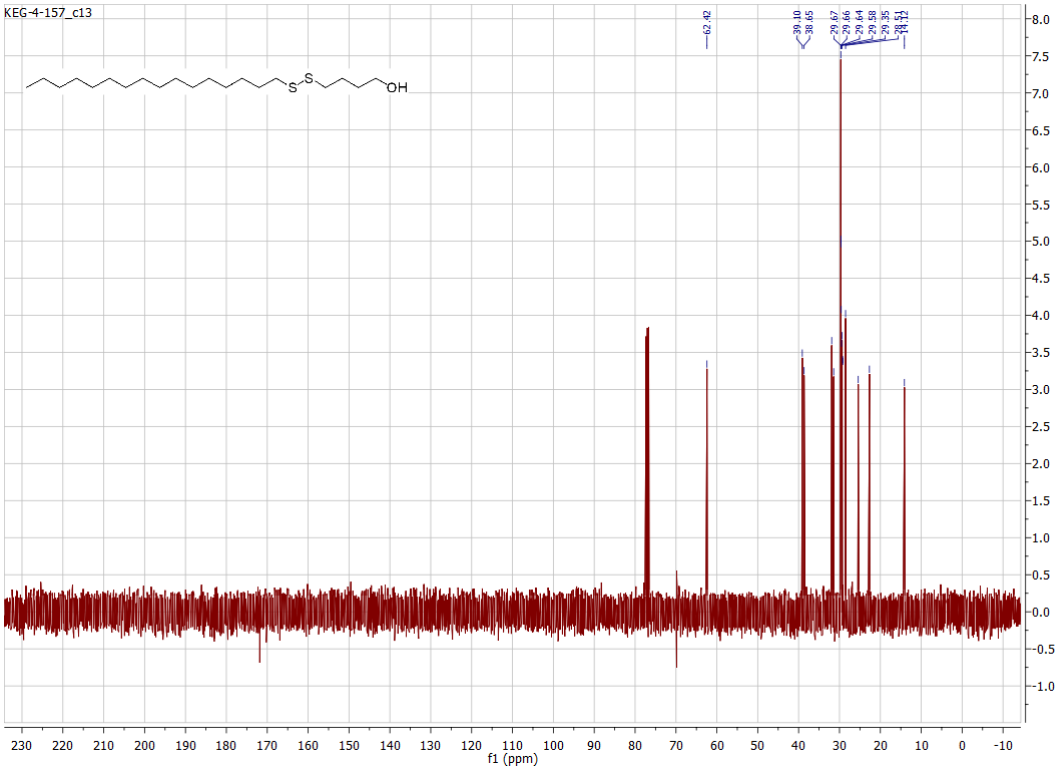
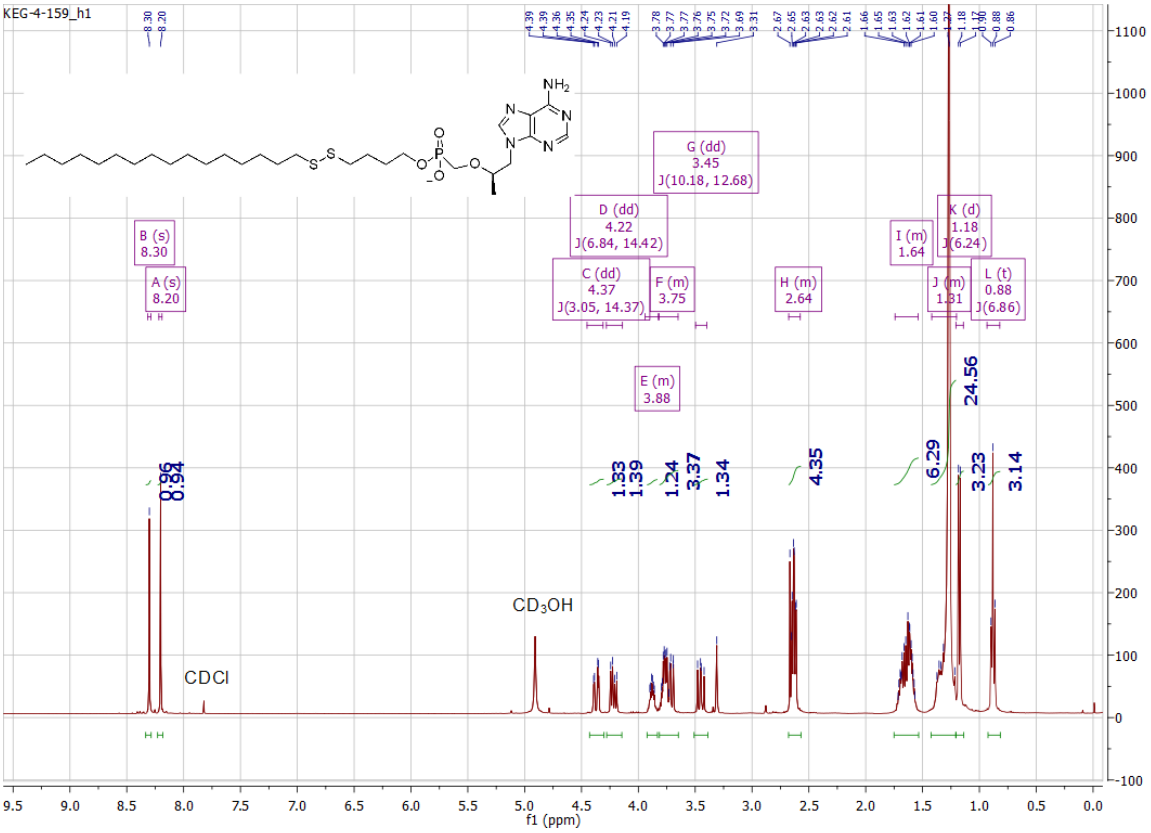


^{13}C NMR (400 MHz, $CDCl_3$)

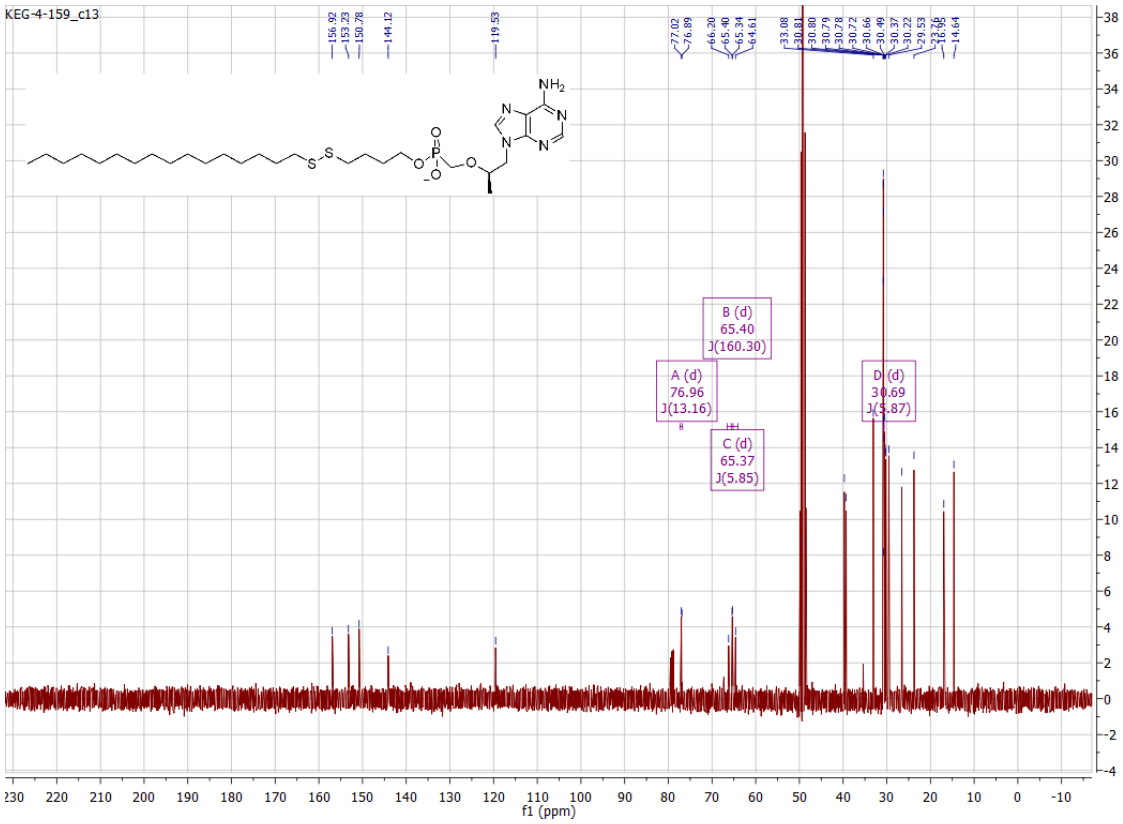


(*R*)-4-(Hexadecyldisulfanyl)butyl (((1-(6-amino-9H-purin-9-yl)propan-2-yl)oxy)methyl)phosphonate (2b)

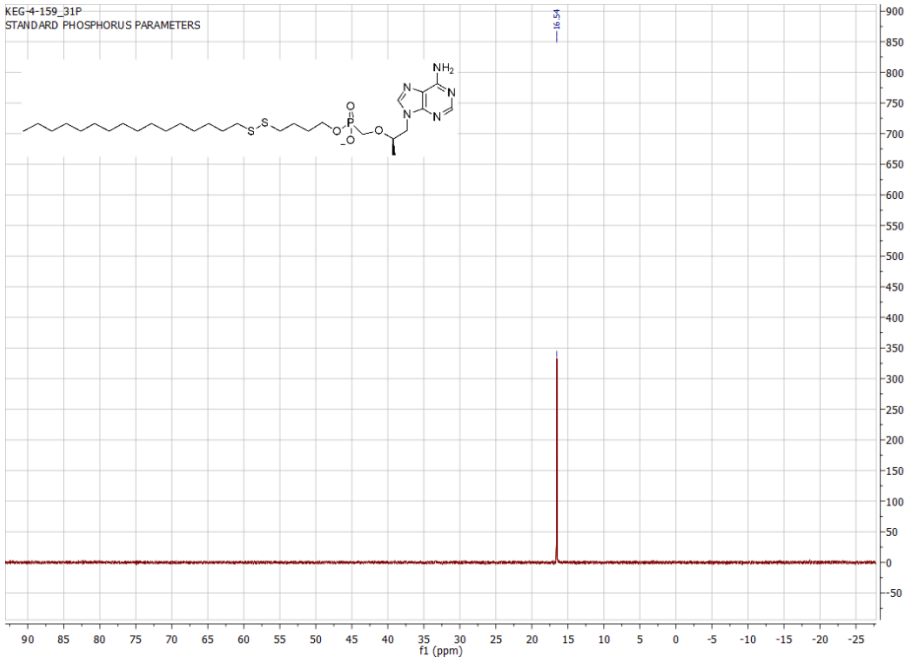
^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$)



^{13}C NMR (400 MHz, $CDCl_3$/CD_3OD)

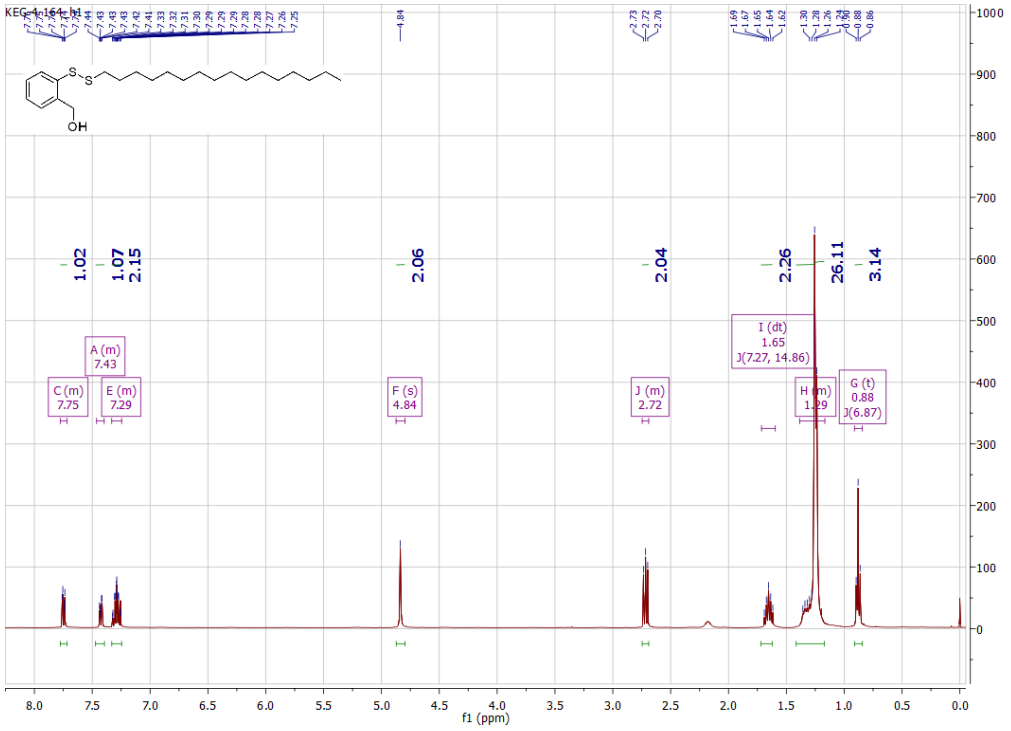


^{31}P NMR (400 MHz, $CDCl_3$/CD_3OD)

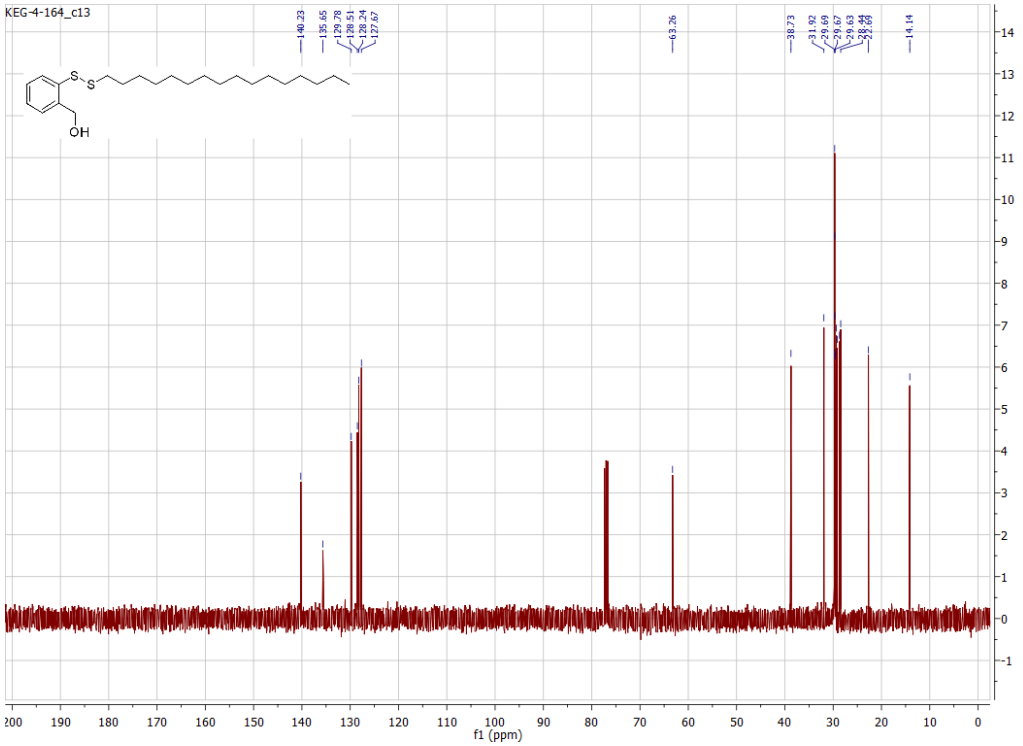


(2-(Hexadecyldisulfanyl)phenyl)methanol (3a)

^{1}H NMR (400 MHz in $CDCl_3$)

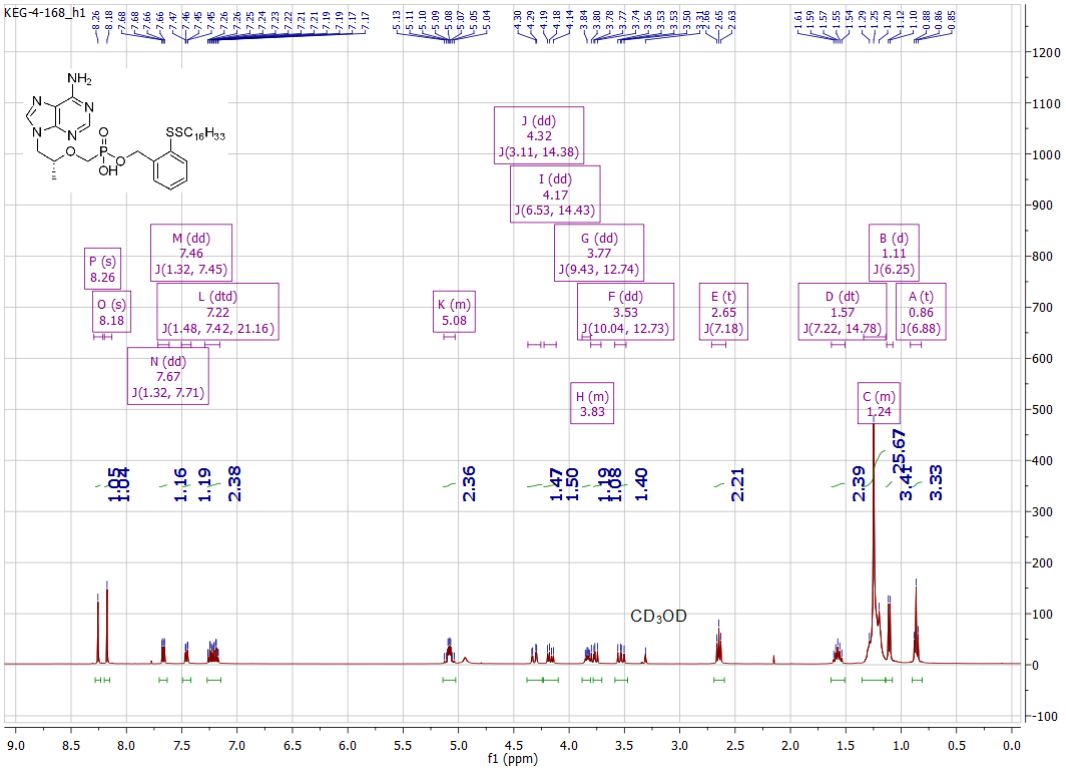


^{13}C NMR (400 MHz in $CDCl_3$)

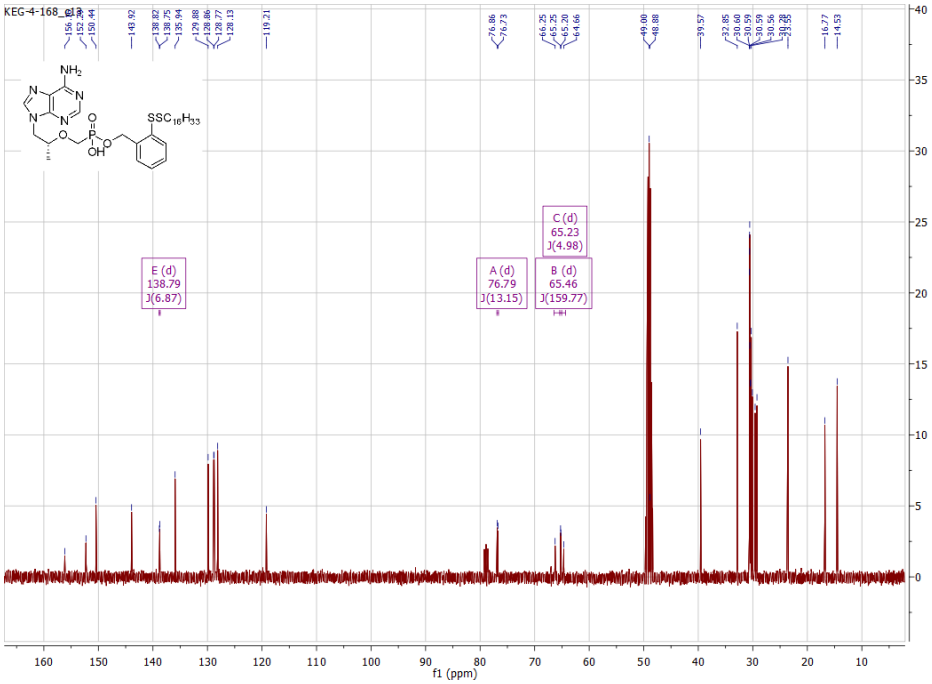


(*R*)-2-(Hexadecyldisulfanyl)benzyl (((1-(6-amino-9H-purin-9-yl)propan-2-yl)oxy)methyl)phosphonate (3b)

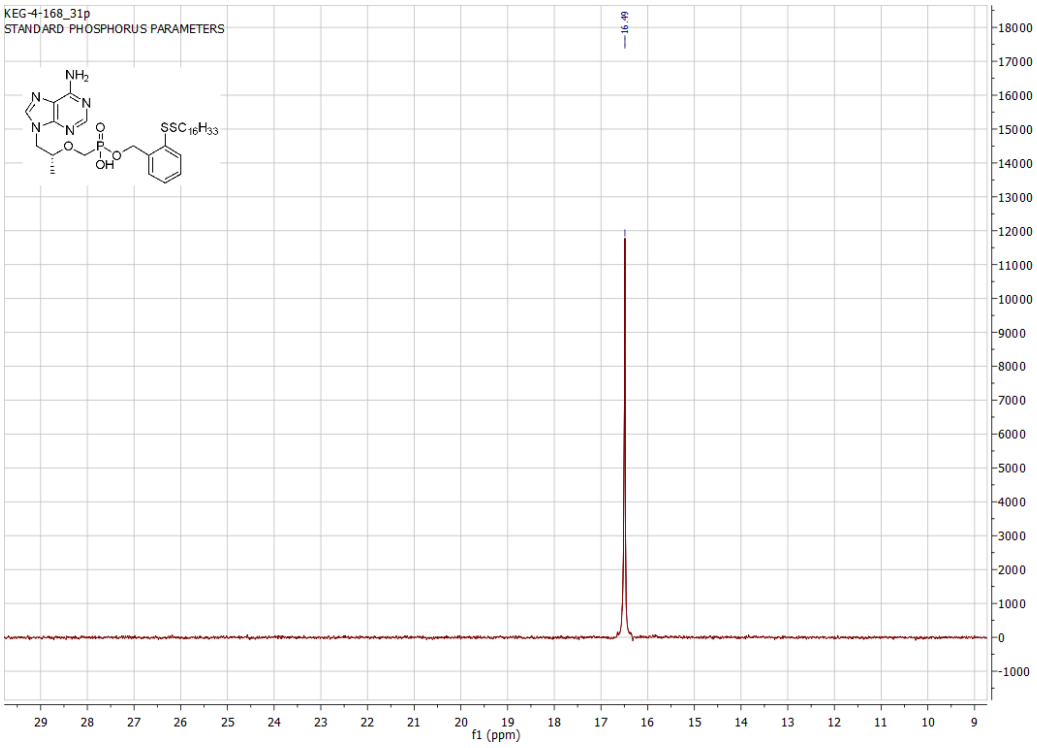
^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD)



^{13}C NMR (400 MHz, $CDCl_3$/CD_3OD)

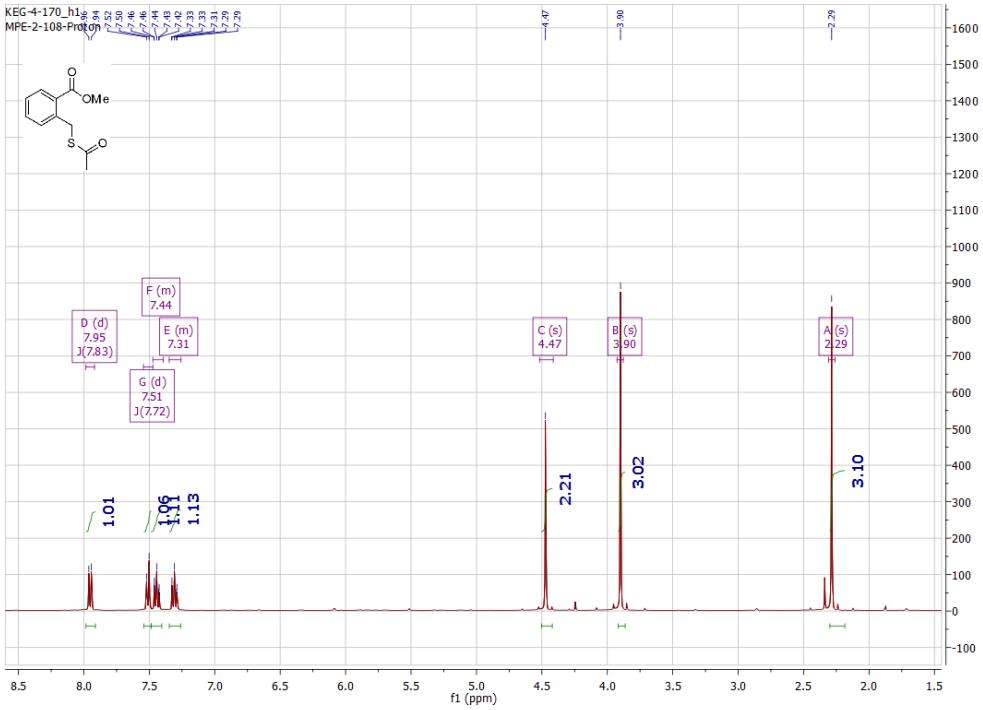


^{31}P NMR (400 MHz, $CDCl_3$/CD_3OD)

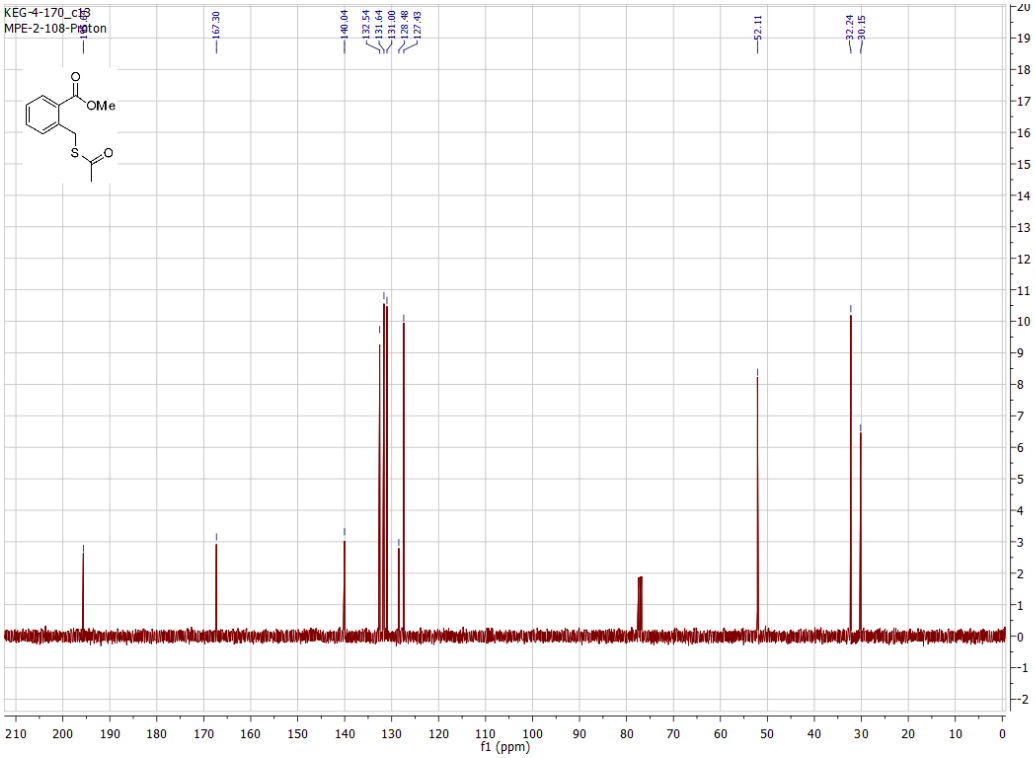


Methyl 2-((acetylthio)methyl)benzoate

^{1}H NMR (400 MHz, $CDCl_3$)

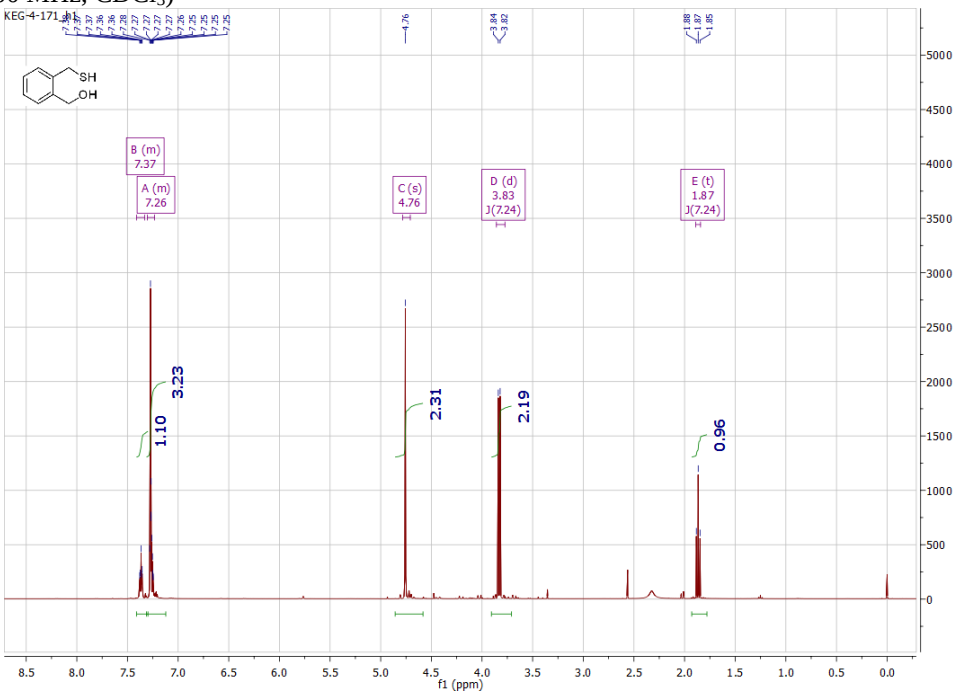


^{13}C NMR (400 MHz, $CDCl_3$)

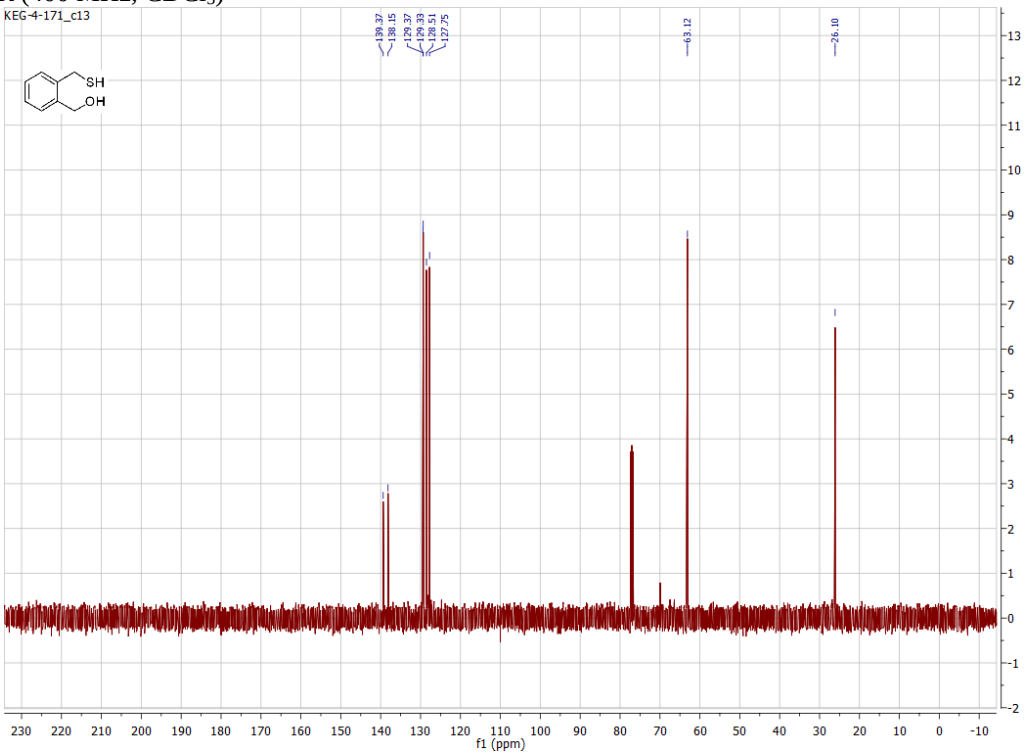


(2-(Mercaptomethyl)phenyl)methanol

^{1}H NMR (400 MHz, $CDCl_3$)

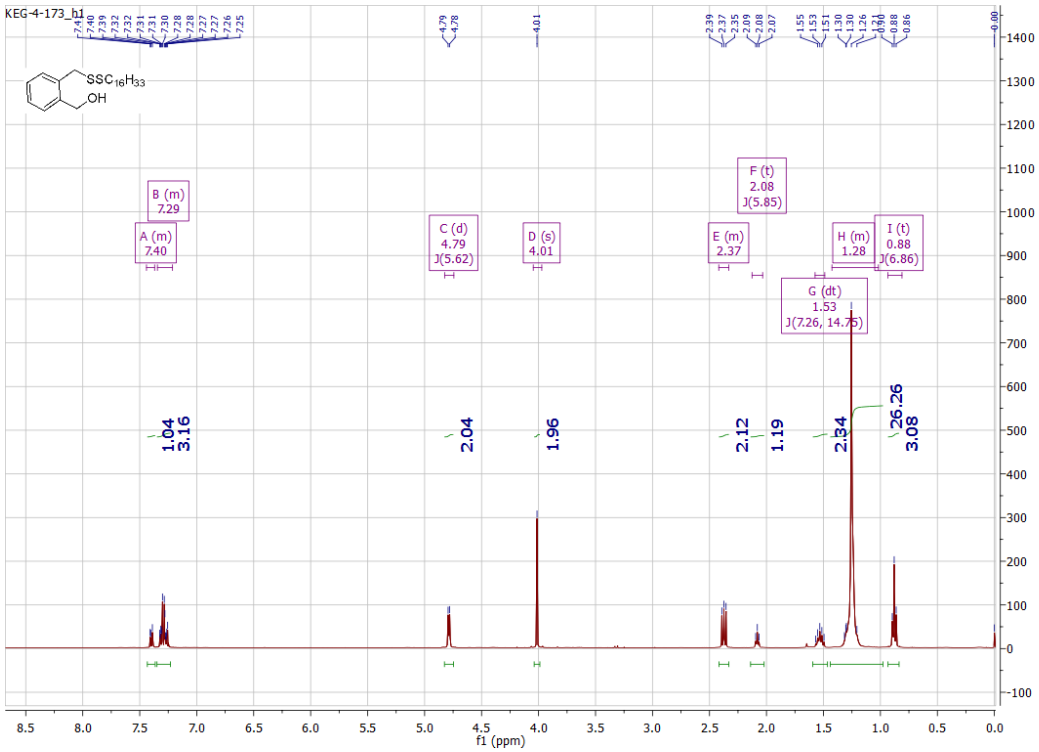


^{13}C NMR (400 MHz, $CDCl_3$)

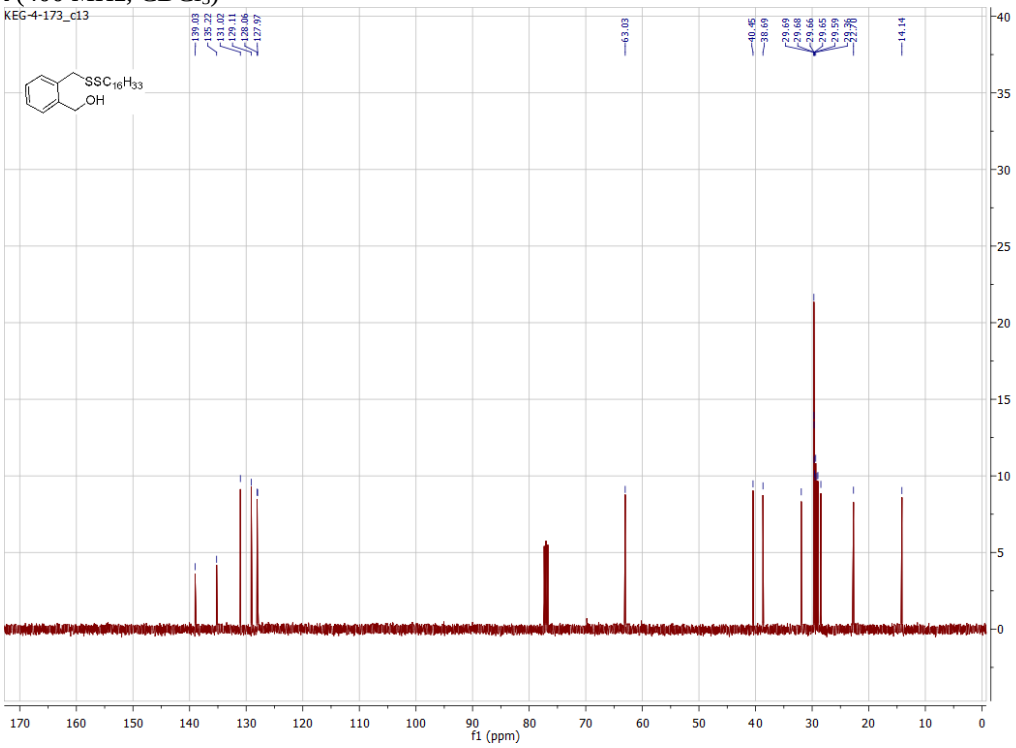


(2-((Hexadecyldisulfanyl)methyl)phenyl)methanol (4a)

^{1}H NMR (400 MHz, $CDCl_3$)

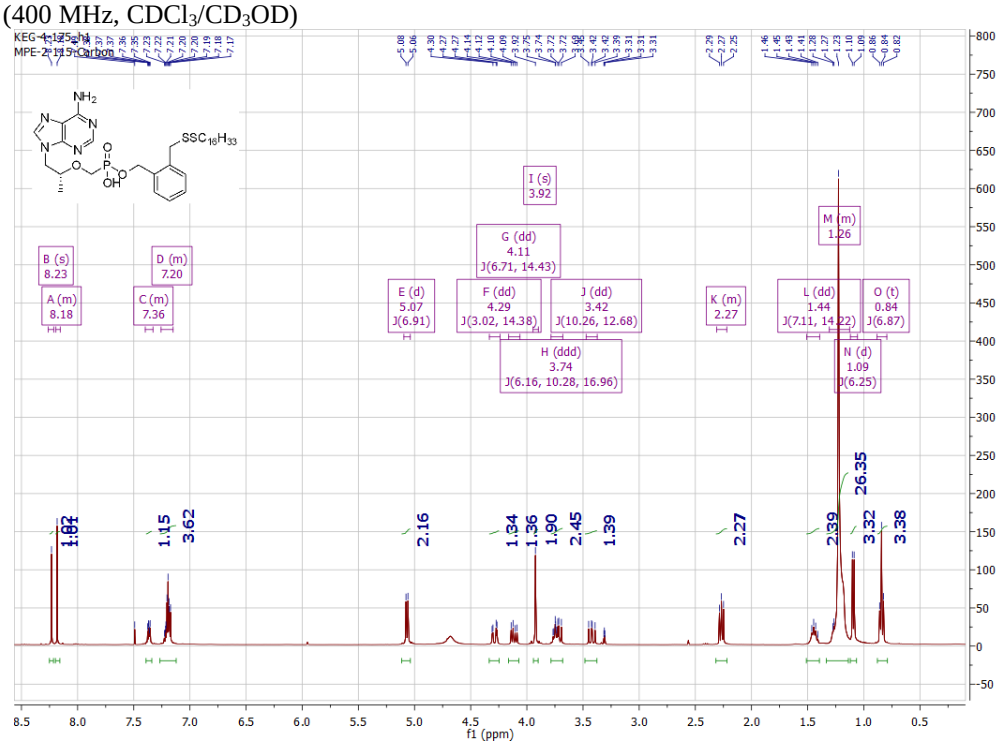


^{13}C NMR (400 MHz, $CDCl_3$)

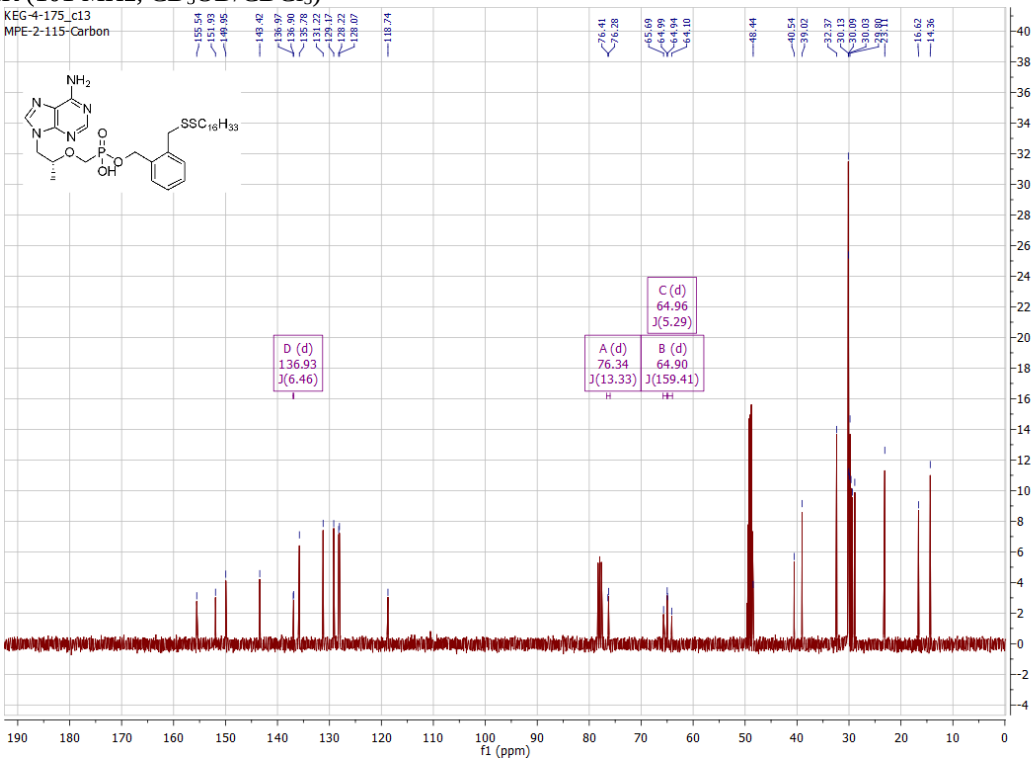


(R)-2-((Hexadecyldisulfanyl)methyl)benzyl (((1-(6-amino-9H-purin-9-yl)propan-2-yl)oxy)methyl)phosphonate (4b)

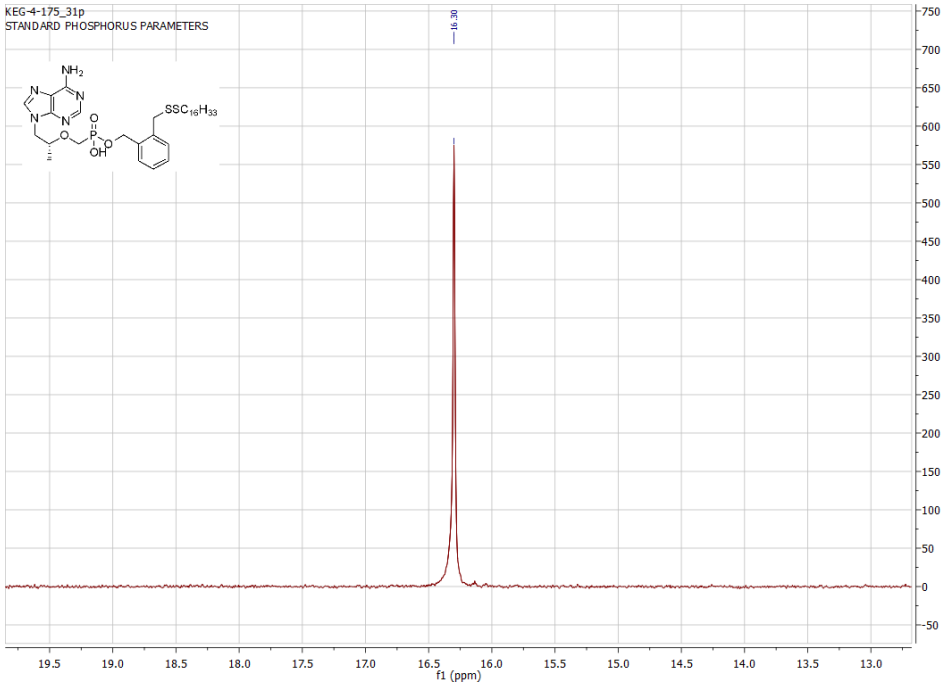
^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD)



^{13}C NMR (101 MHz, $CD_3OD/CDCl_3$)

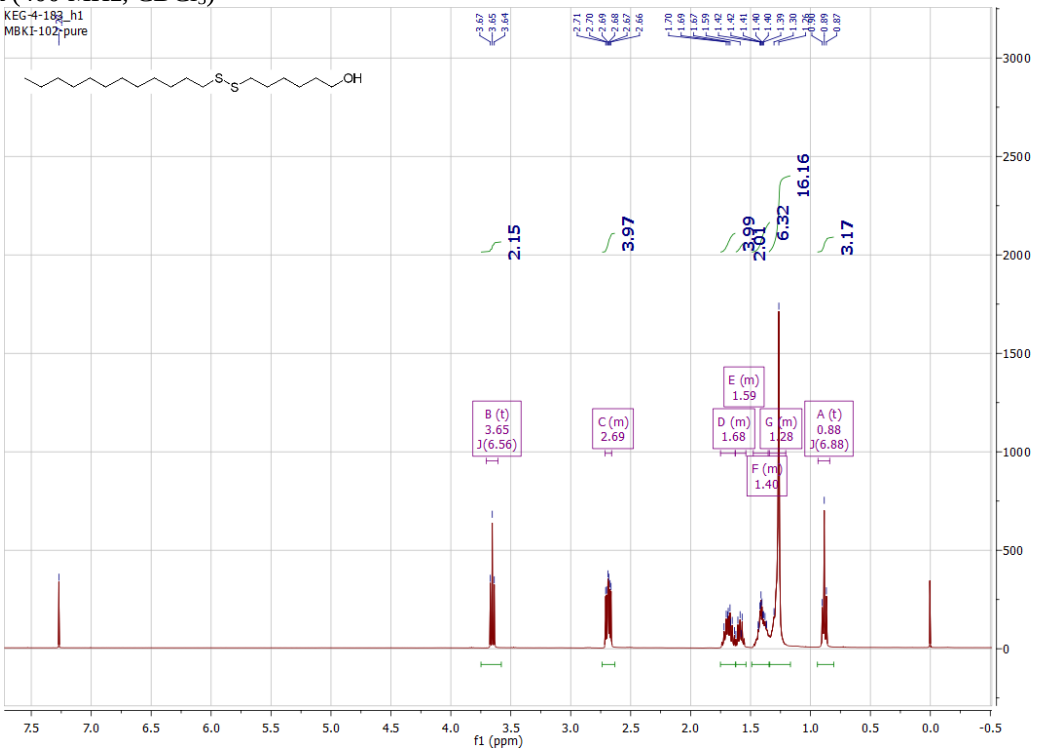


^{31}P NMR (162 MHz, $CDCl_3/CD_3OD$)

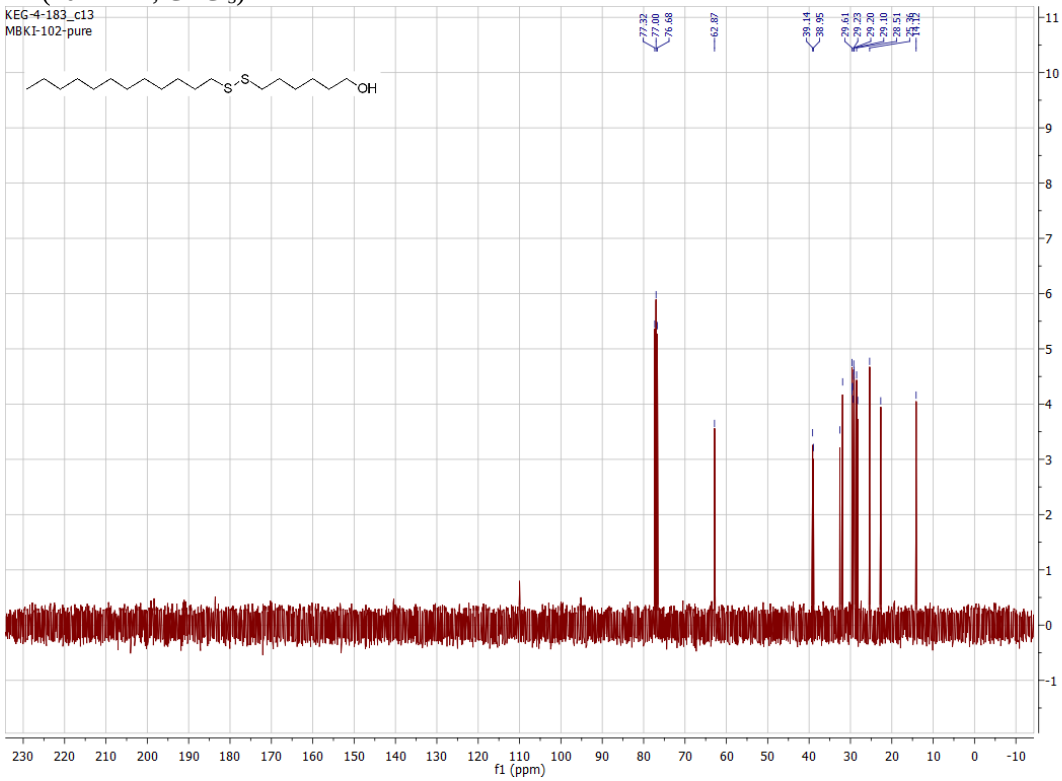


6-(Dodecyldisulfanyl)hexan-1-ol (5a)

^{1}H NMR (400 MHz, $CDCl_3$)

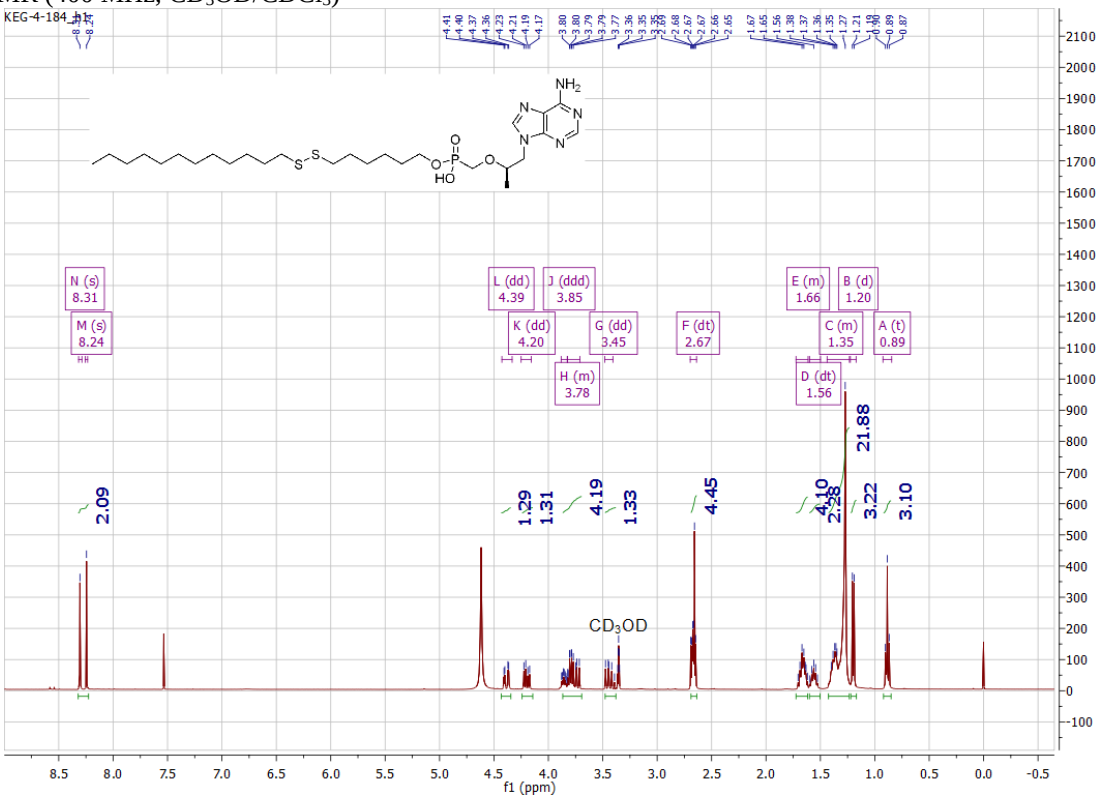


^{13}C NMR (101 MHz, $CDCl_3$)

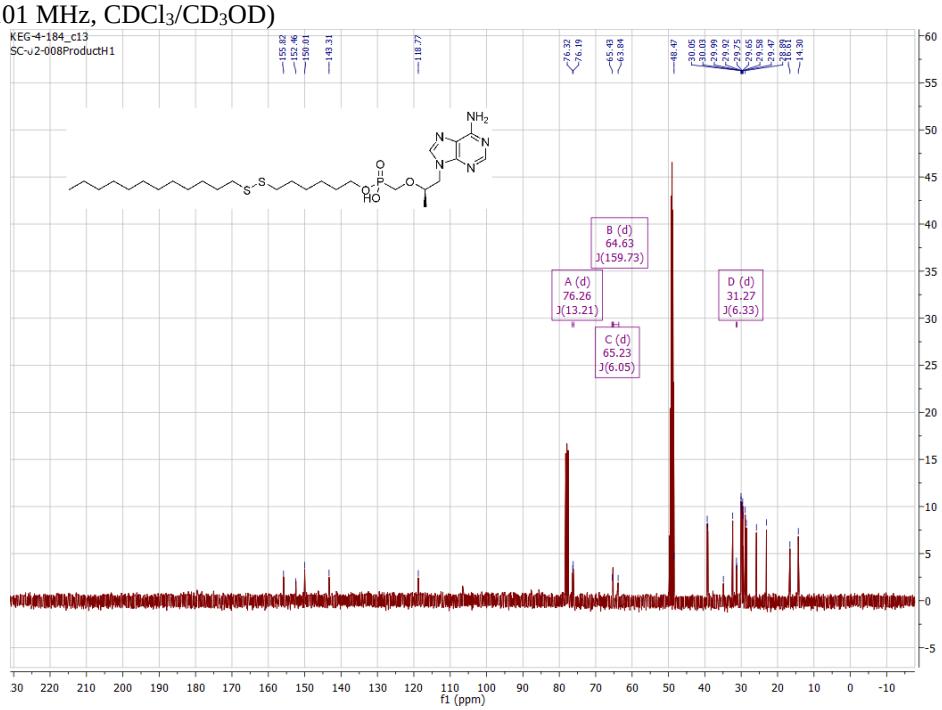


6-(Dodecyldisulfanyl)hexyl hydrogen ((((R)-1-(6-amino-9H-purin-9-yl)propan-2-yl)oxy)methyl)phosphonate (5b)

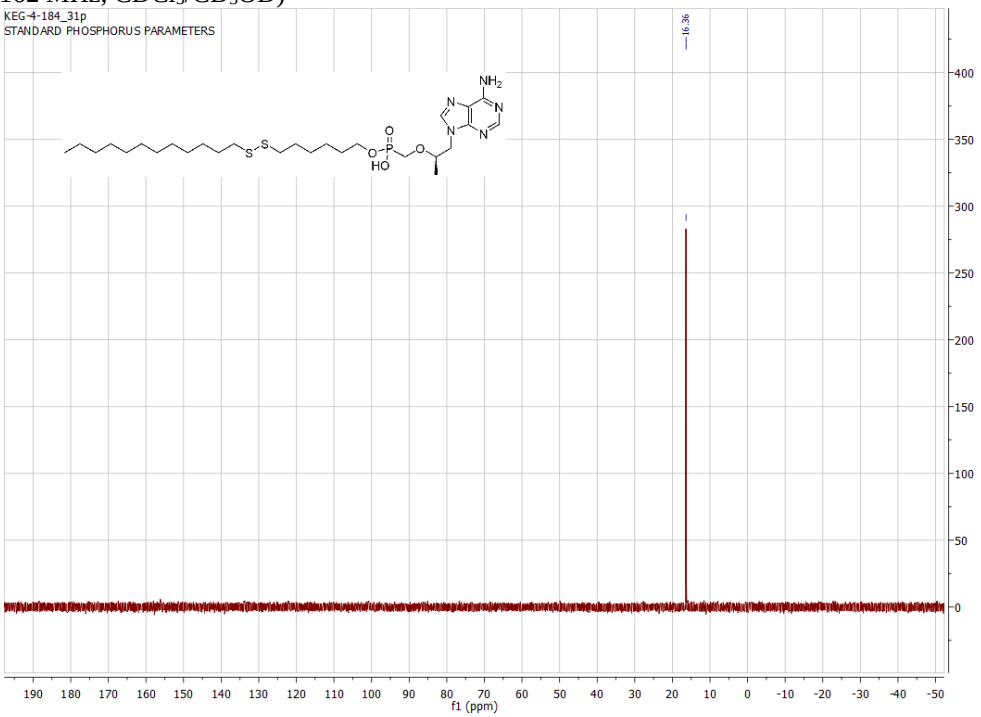
^{1}H NMR (400 MHz, $CD_3OD/CDCl_3$)



^{13}C NMR (101 MHz, $CDCl_3/CD_3OD$)

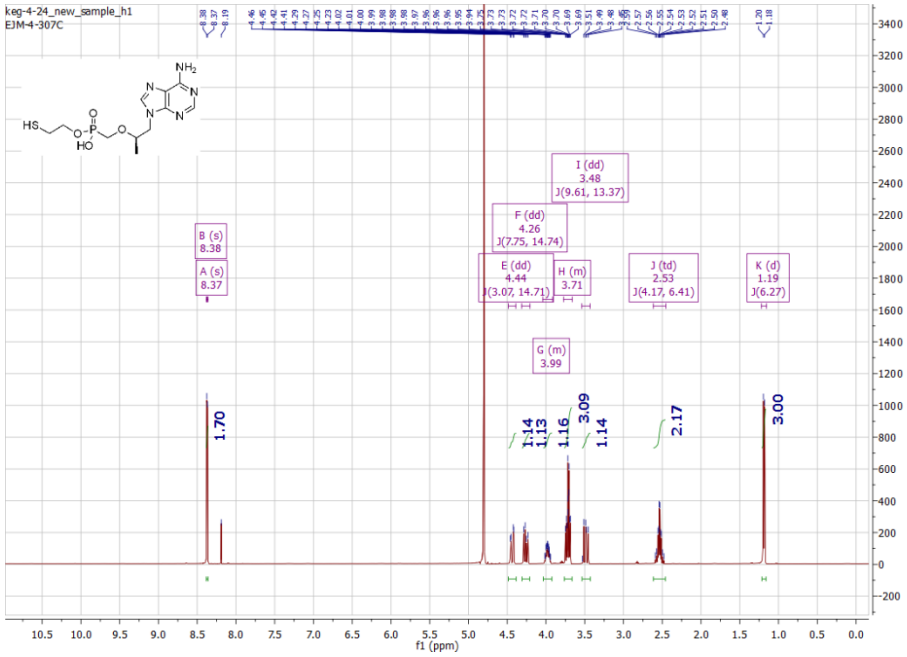


^{31}P NMR (162 MHz, $CDCl_3$/CD_3OD)



2-Mercaptoethyl hydrogen ((((*(R)*-1-(6-amino-9H-purin-9-yl)propan-2yl)oxy)methyl)phosphonate (6)

^{1}H NMR (400 MHz in D_2O)



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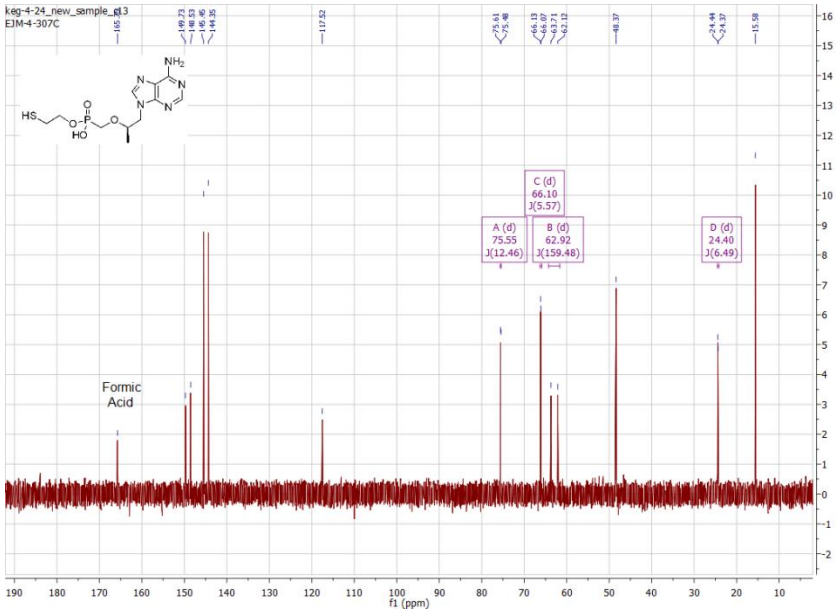
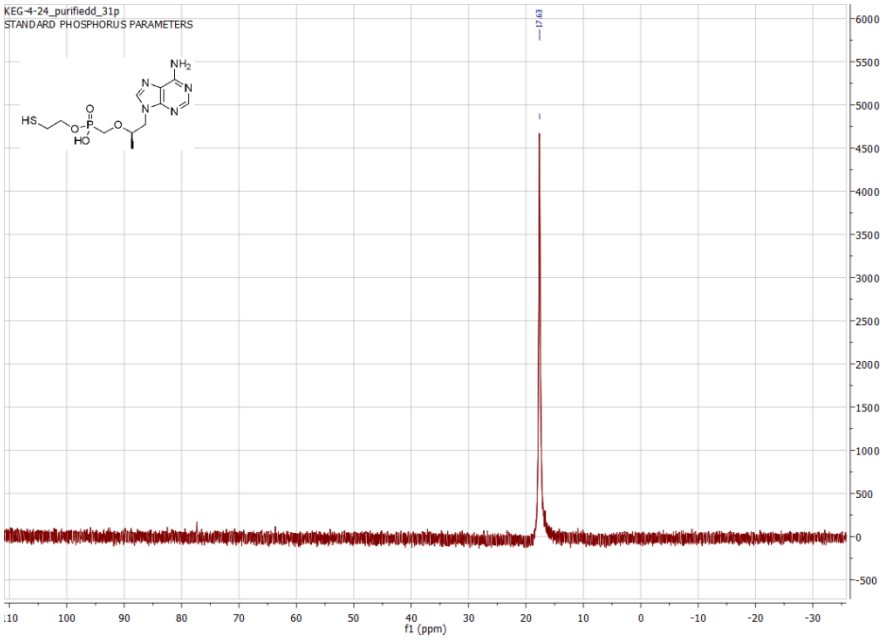
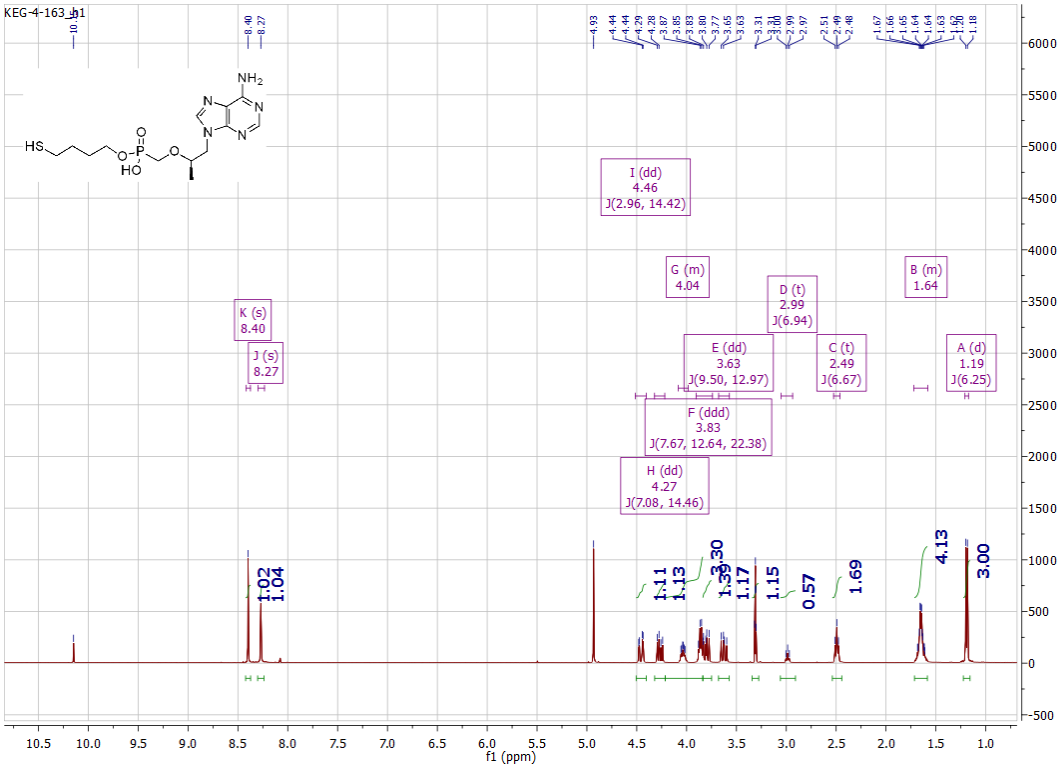


^{31}P NMR (162 MHz in D_2O)

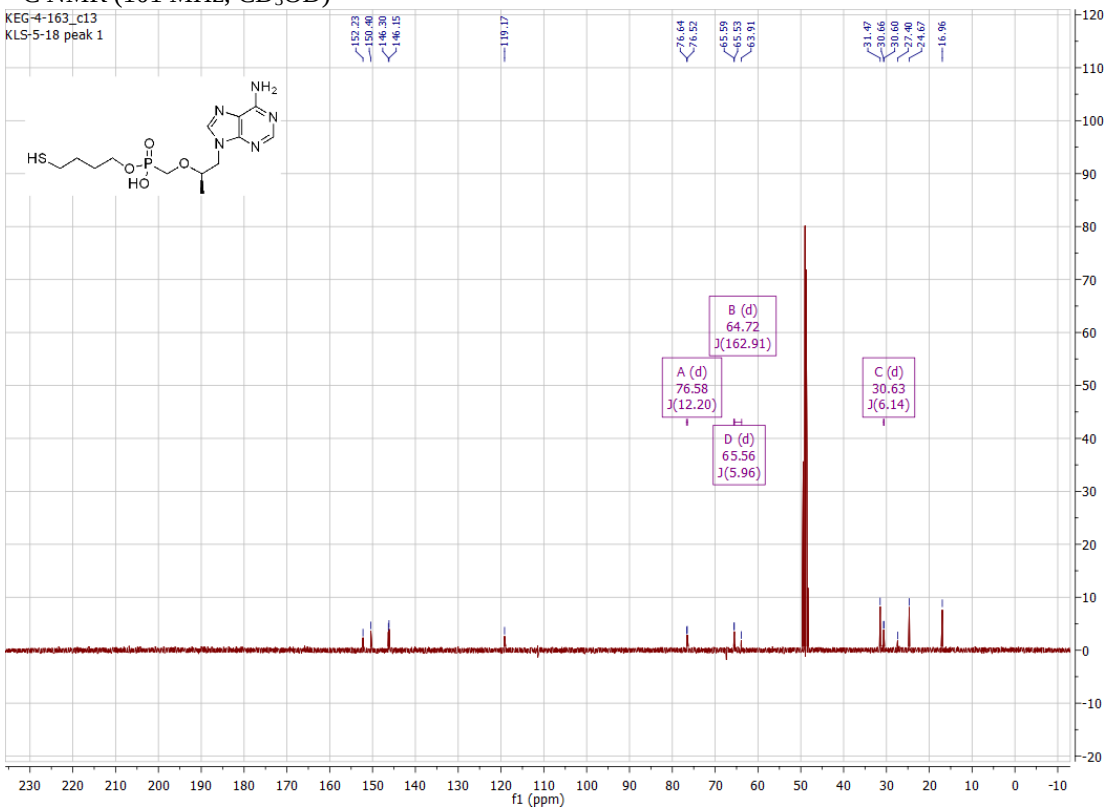


4-Mercaptobutyl hydrogen ((((*R*)-1-(6-amino-9H-purin-9-yl)propan-2-yl)oxy)methyl)phosphonate (7)

^{1}H NMR (400 MHz, CD_3OD)



^{13}C NMR (101 MHz, CD_3OD)



^{31}P NMR (162 MHz, CD_3OD)