Supporting Information:

5'-Phosphate and 5'-Phosphonate Ester Derivatives of (N)-Methanocarba Adenosine with in Vivo Cardioprotective Activity

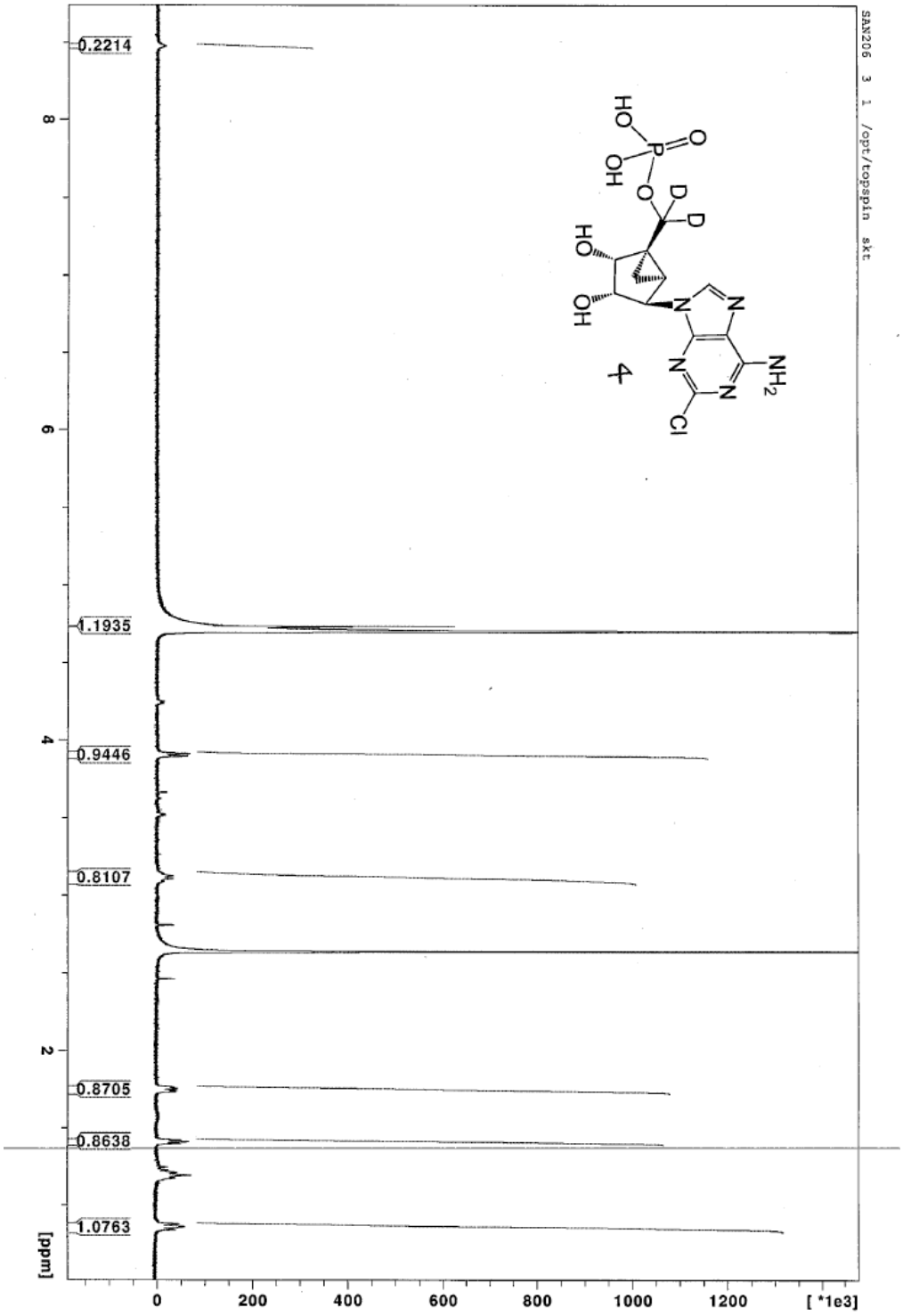
T. Santhosh Kumar, Tiehong Yang, Shilpi Mishra, Chunxia Cronin,
Saibal Charkaborty, Bruce T. Liang, Kenneth A. Jacobson

Contents: **page**

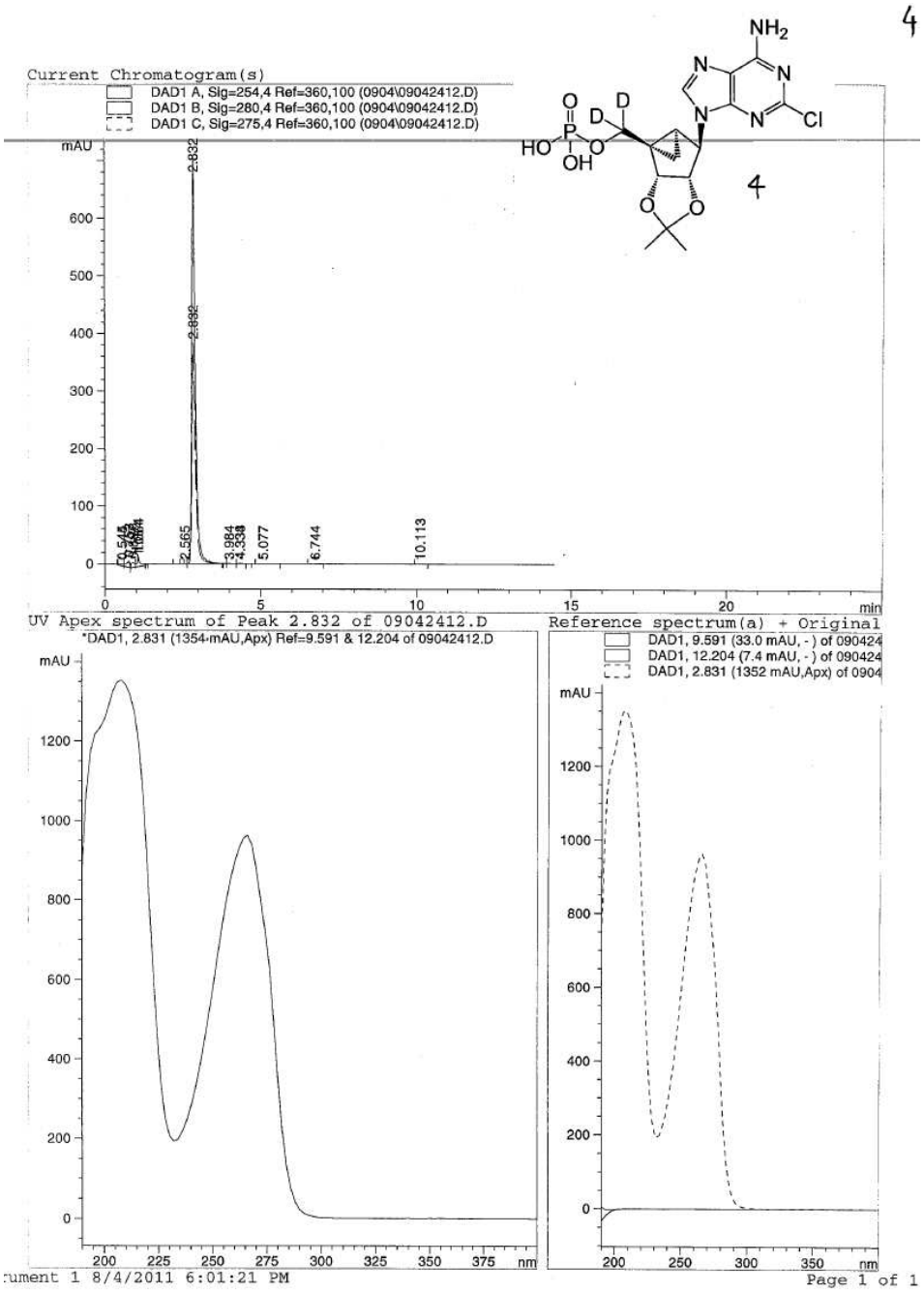
Abbreviations: CSQ, calsequestrin; FS, fractional shortening; LV, left ventricular.

Spectroscopic and HPLC characterization of selected nucleotide derivatives.

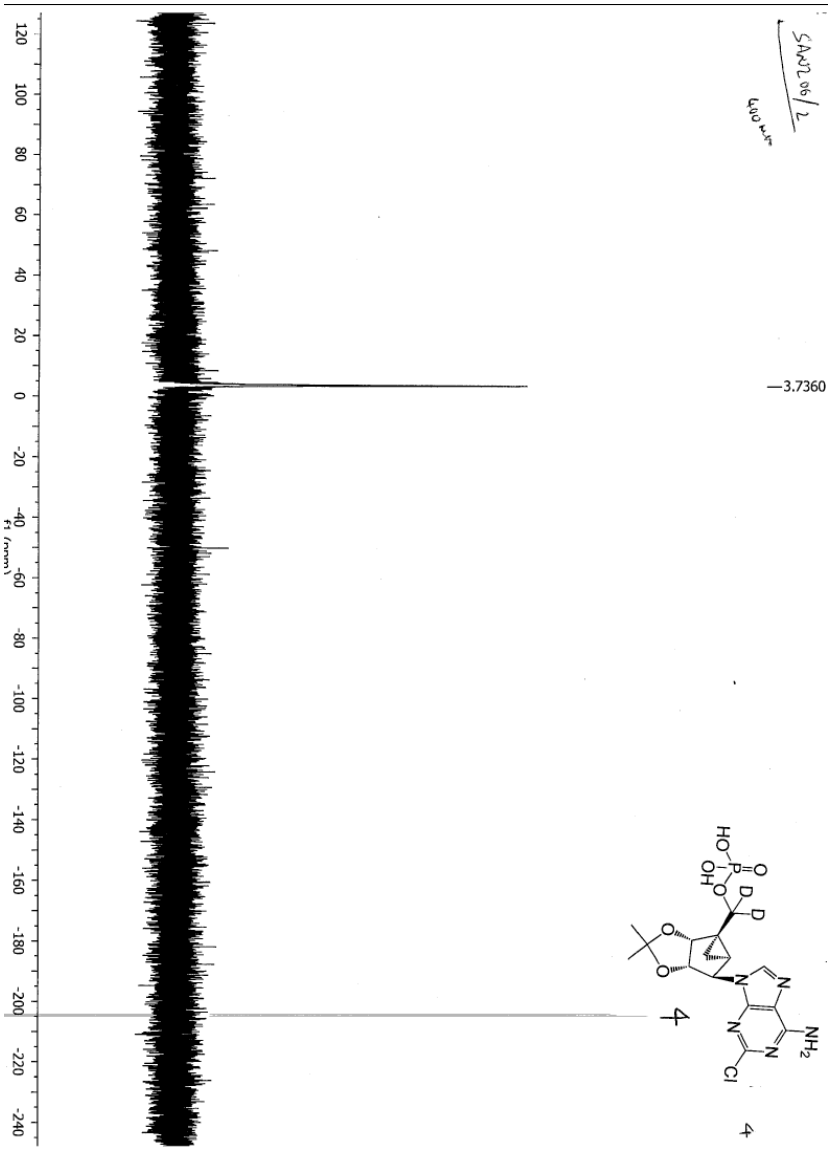
Compound **4**, ^{31}P NMR



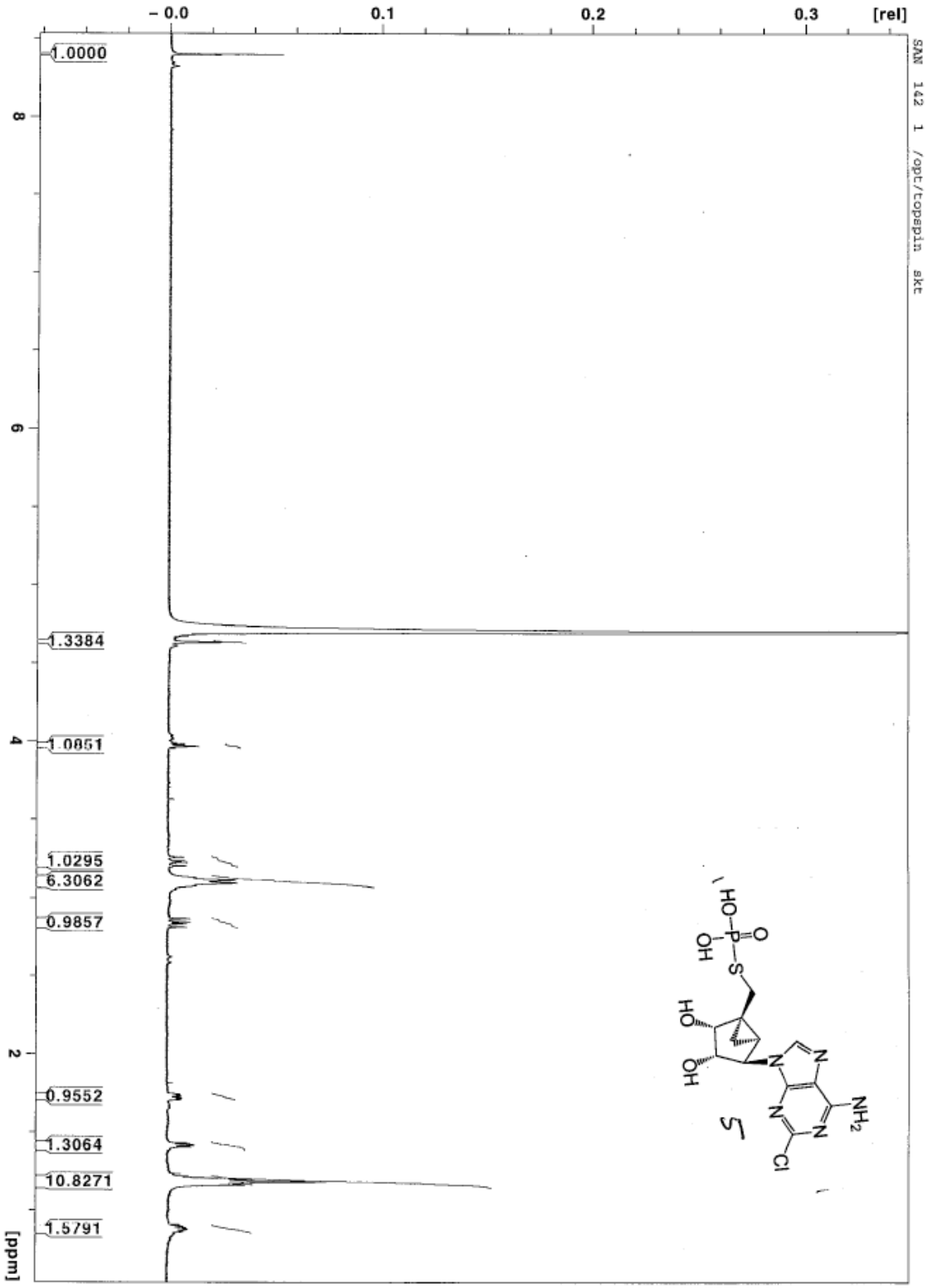
Compound **4**, HPLC



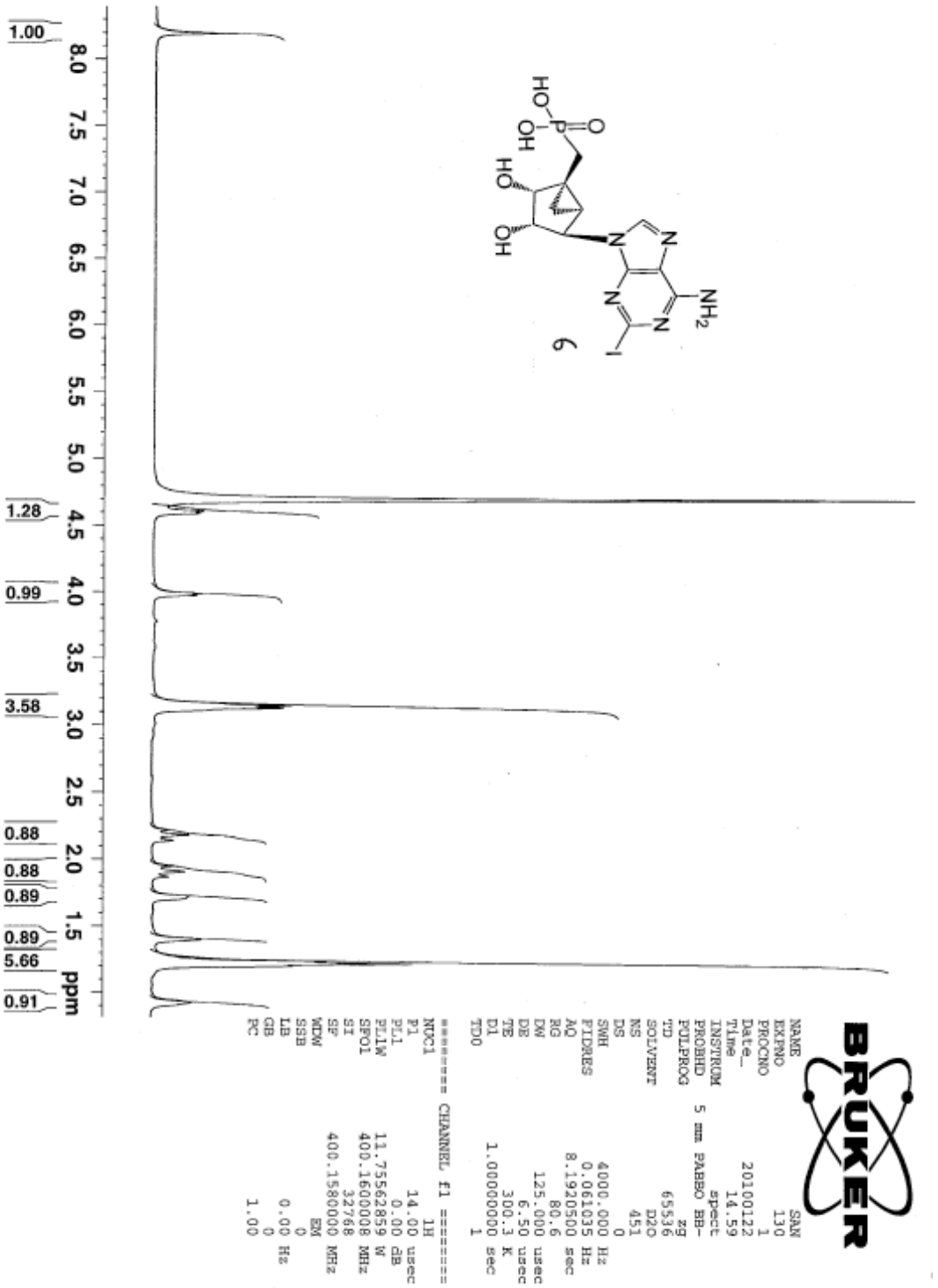
Compound **4**, ^{31}P NMR



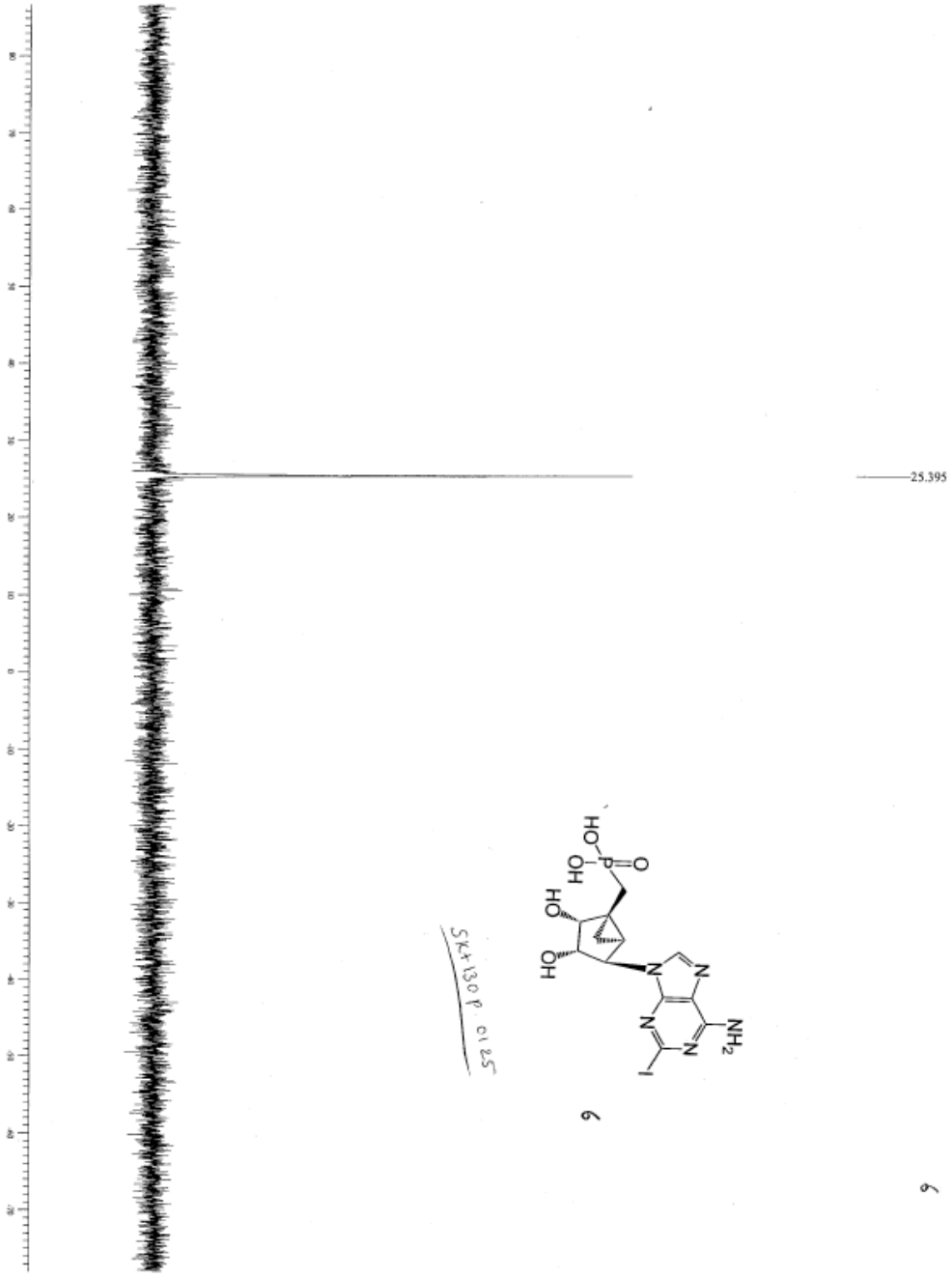
Compound **5**, ^{1}H NMR



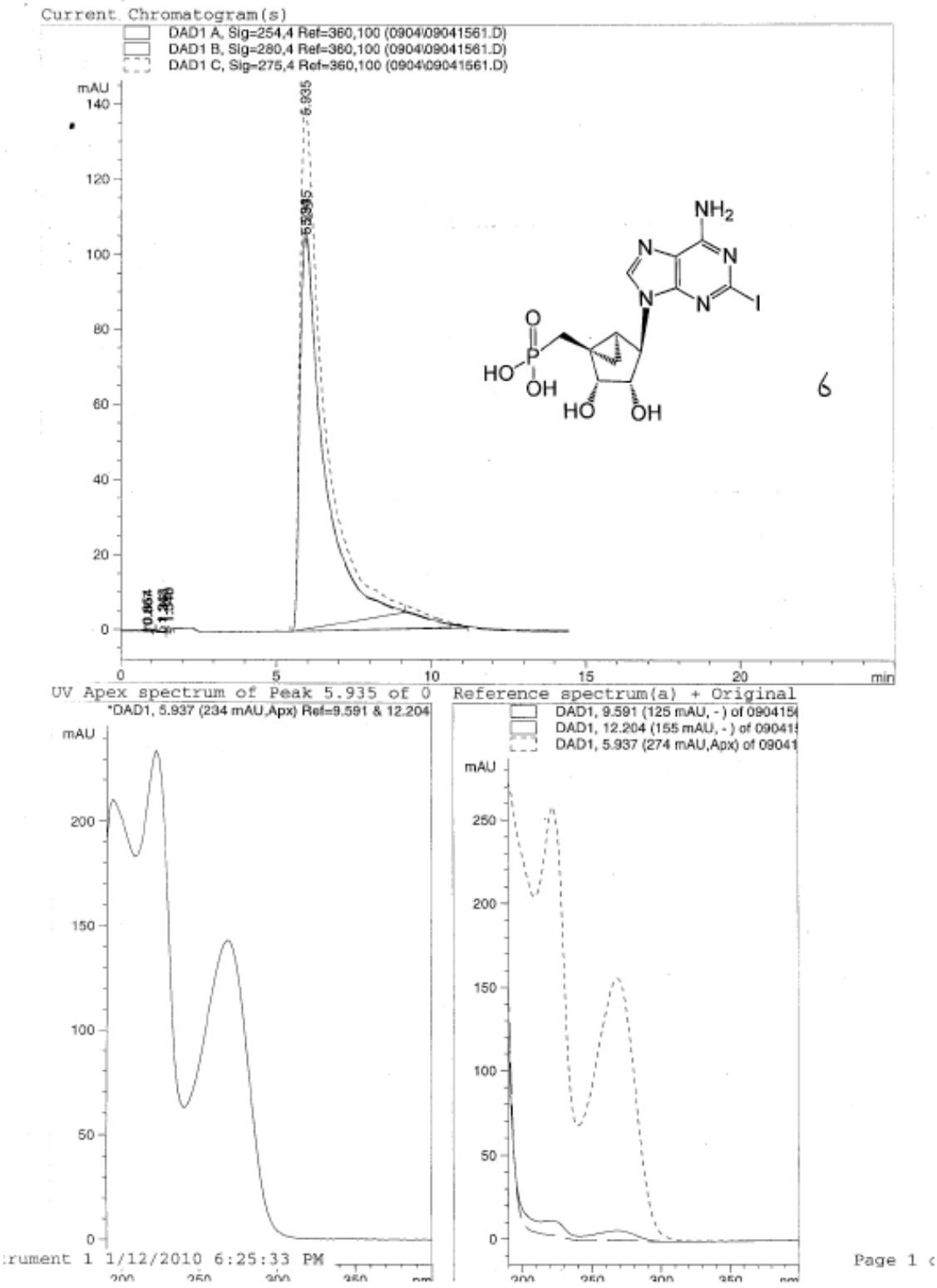
Compound **6**, ^{1}H NMR



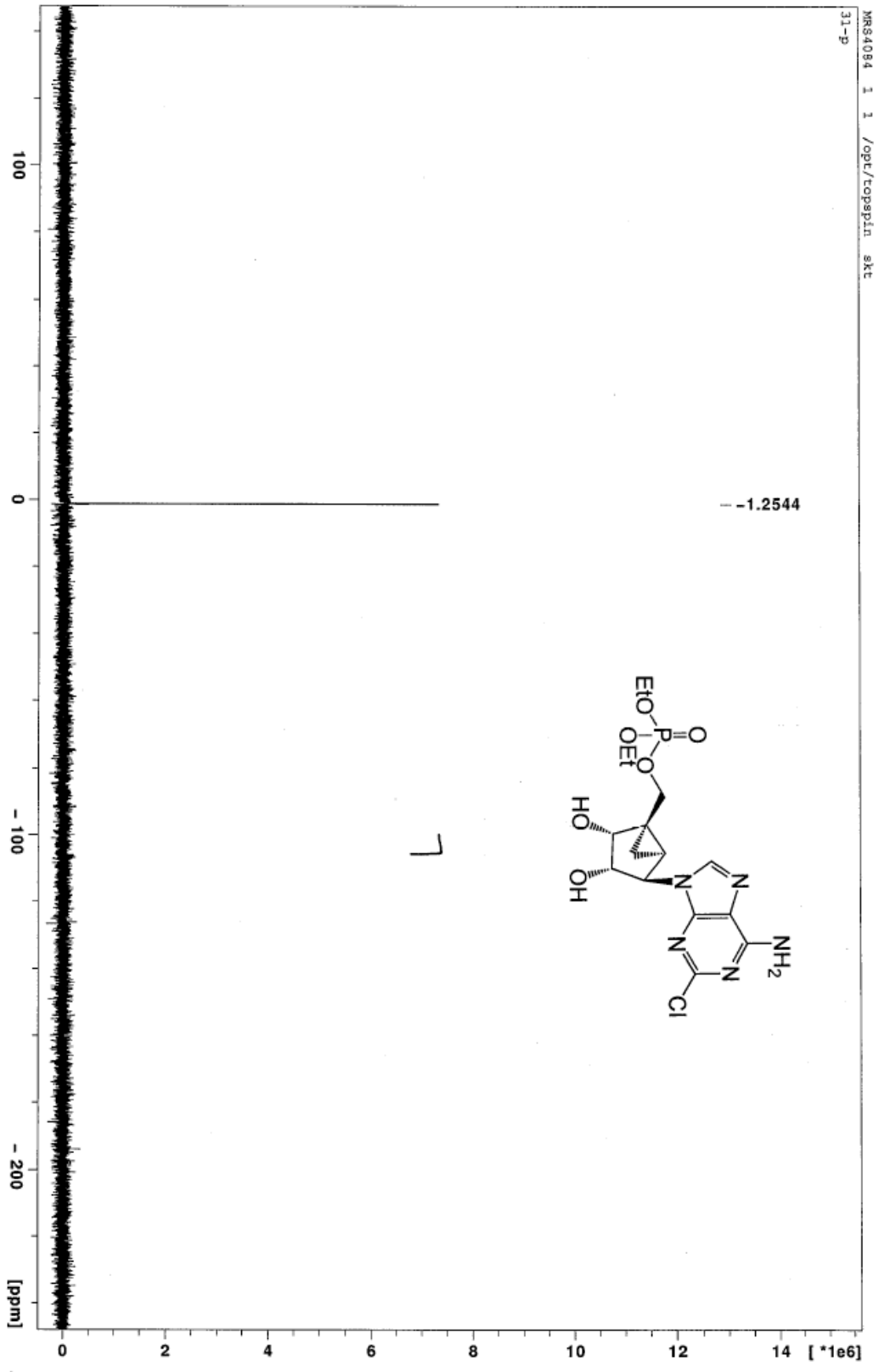
Compound **6**, ^{31}P NMR



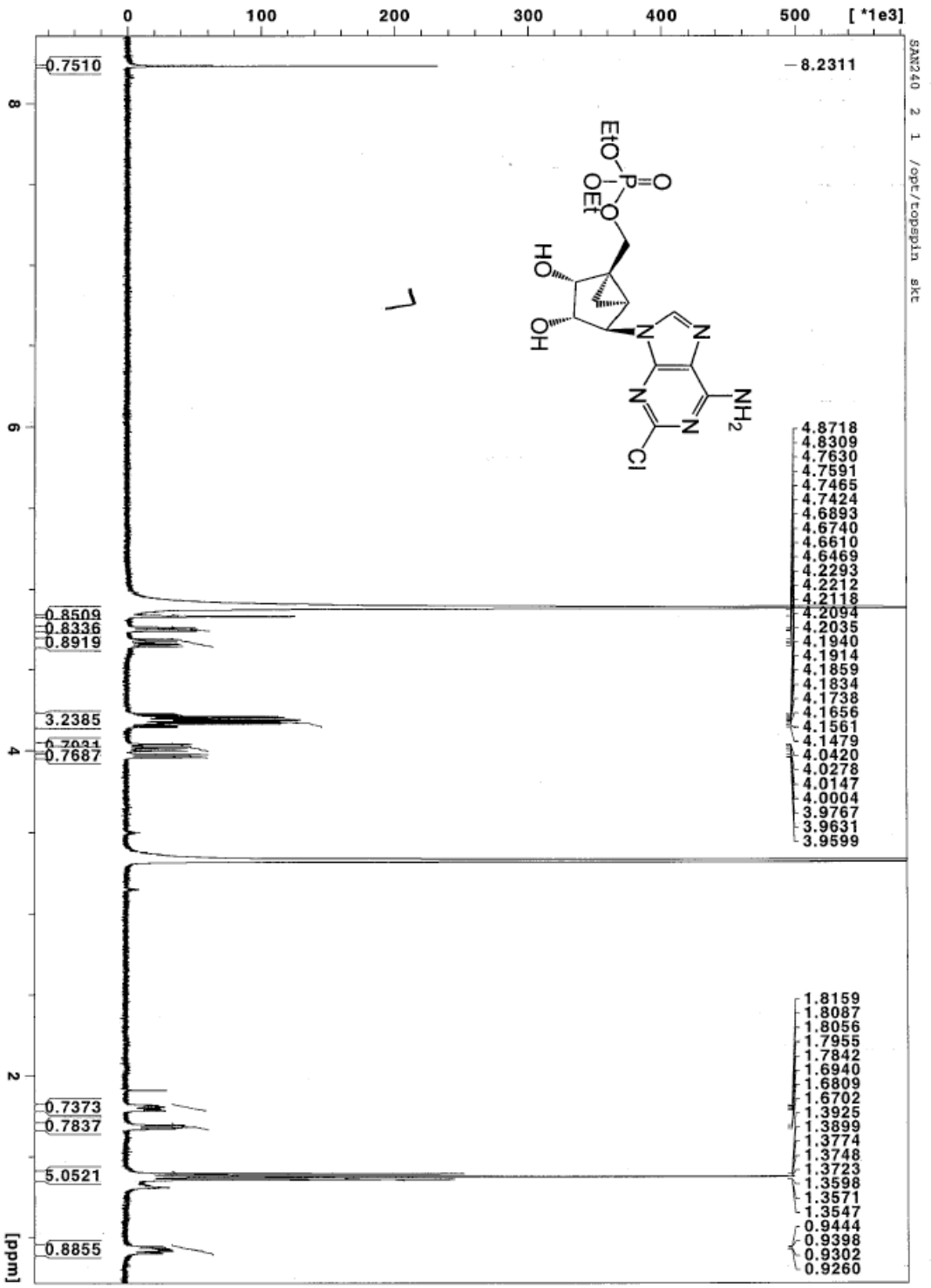
Compound **6**, HPLC



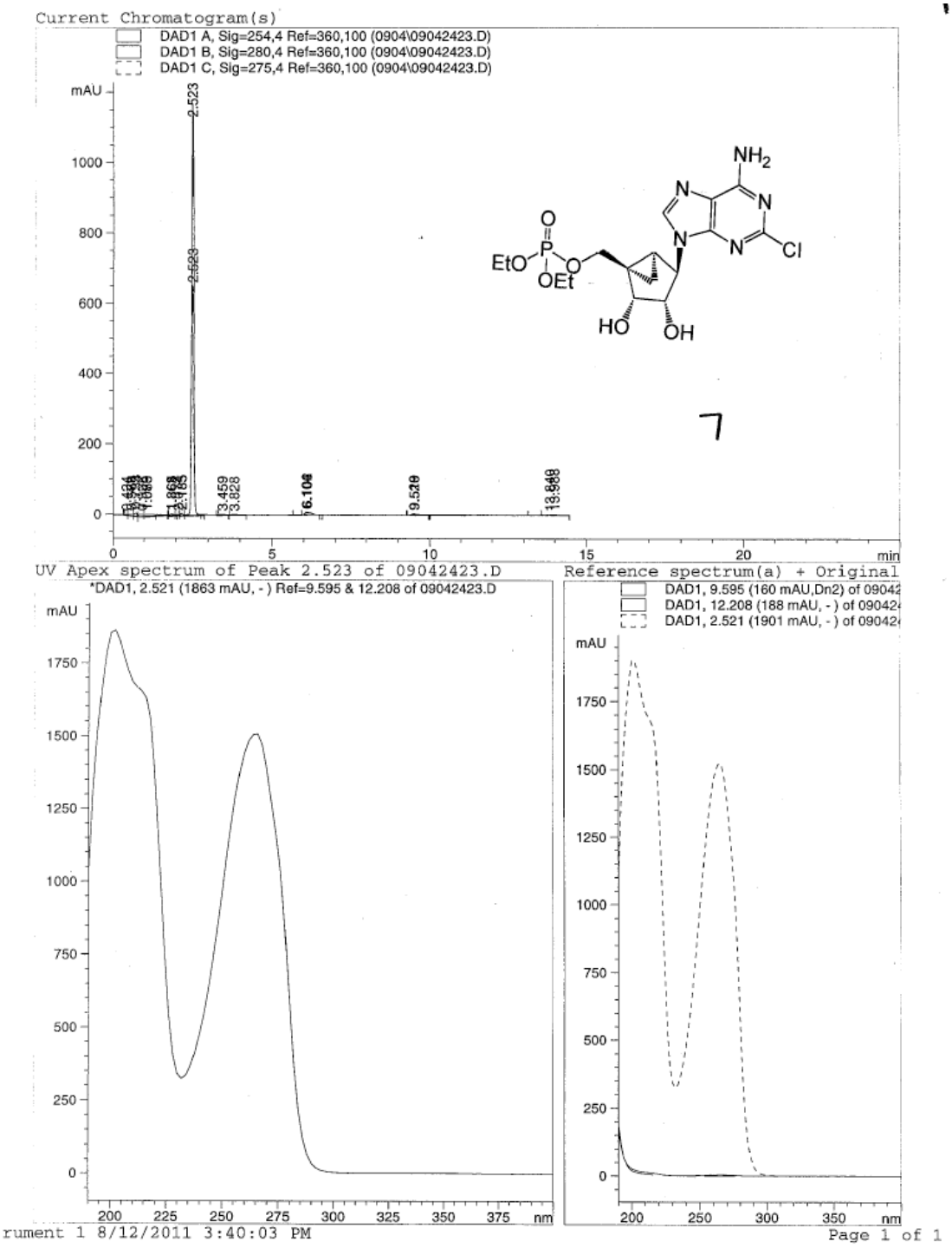
Compound **7**, ^{31}P NMR



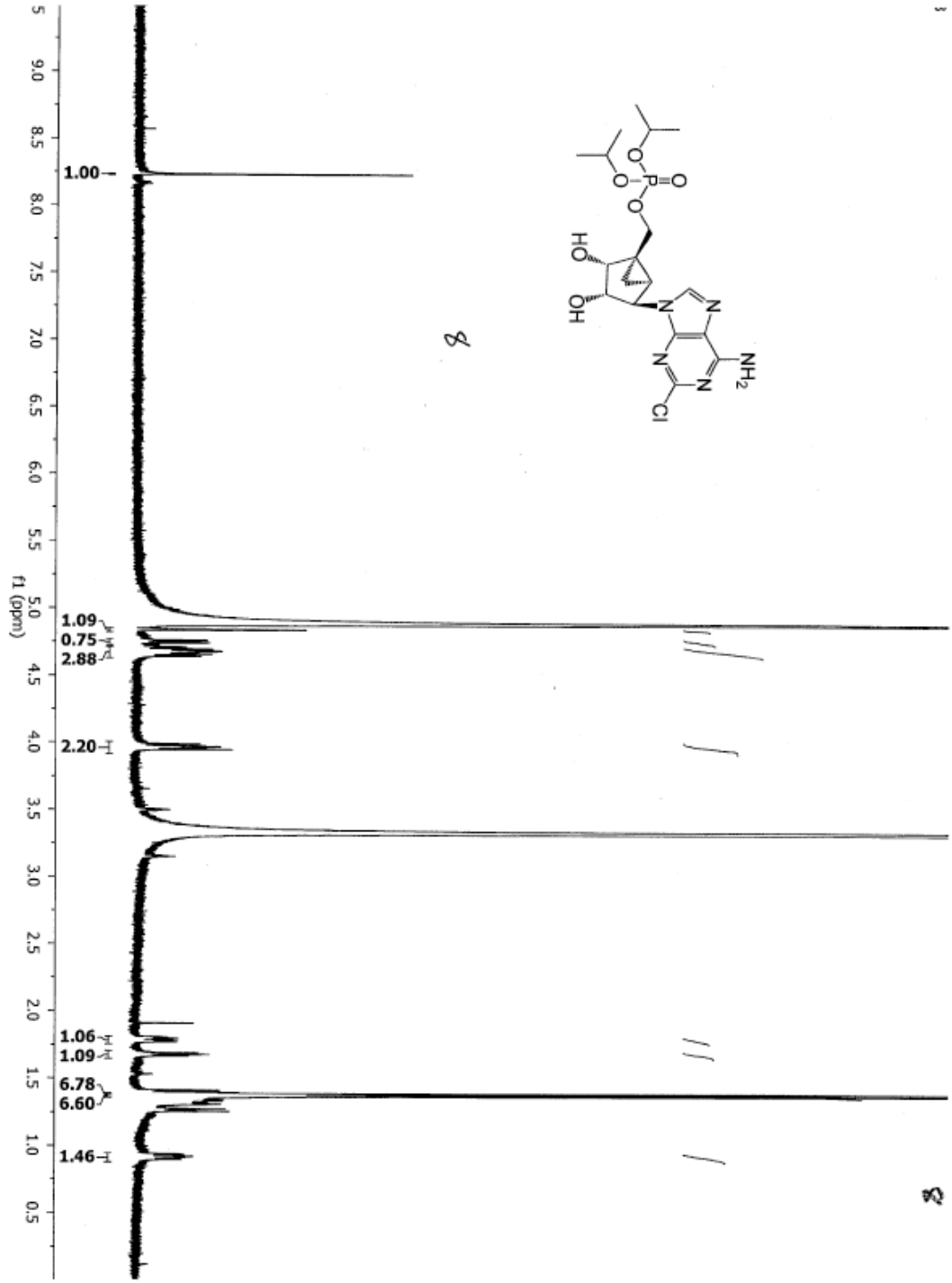
Compound **7**, ^{1}H NMR



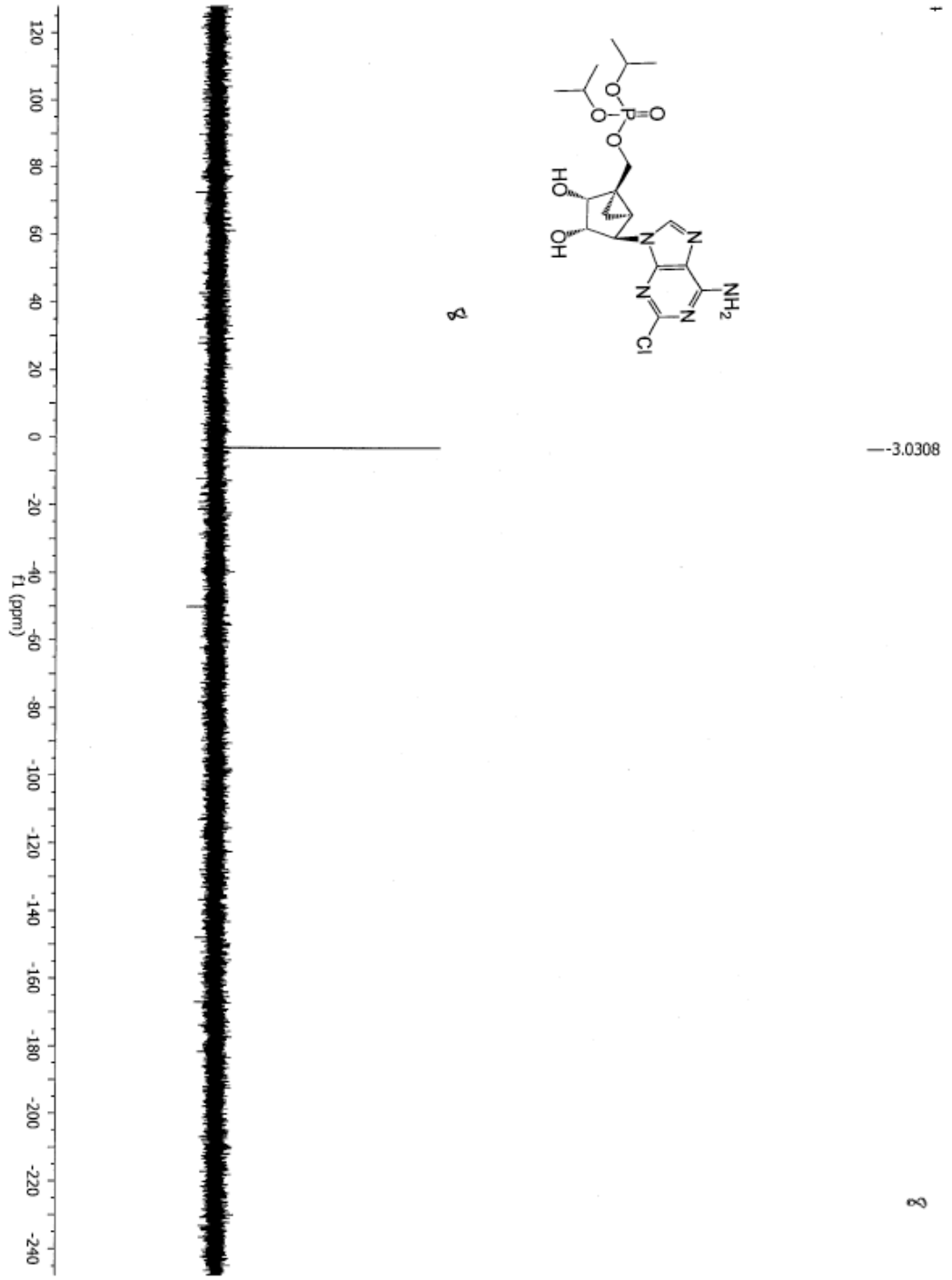
Compound **7**, HPLC



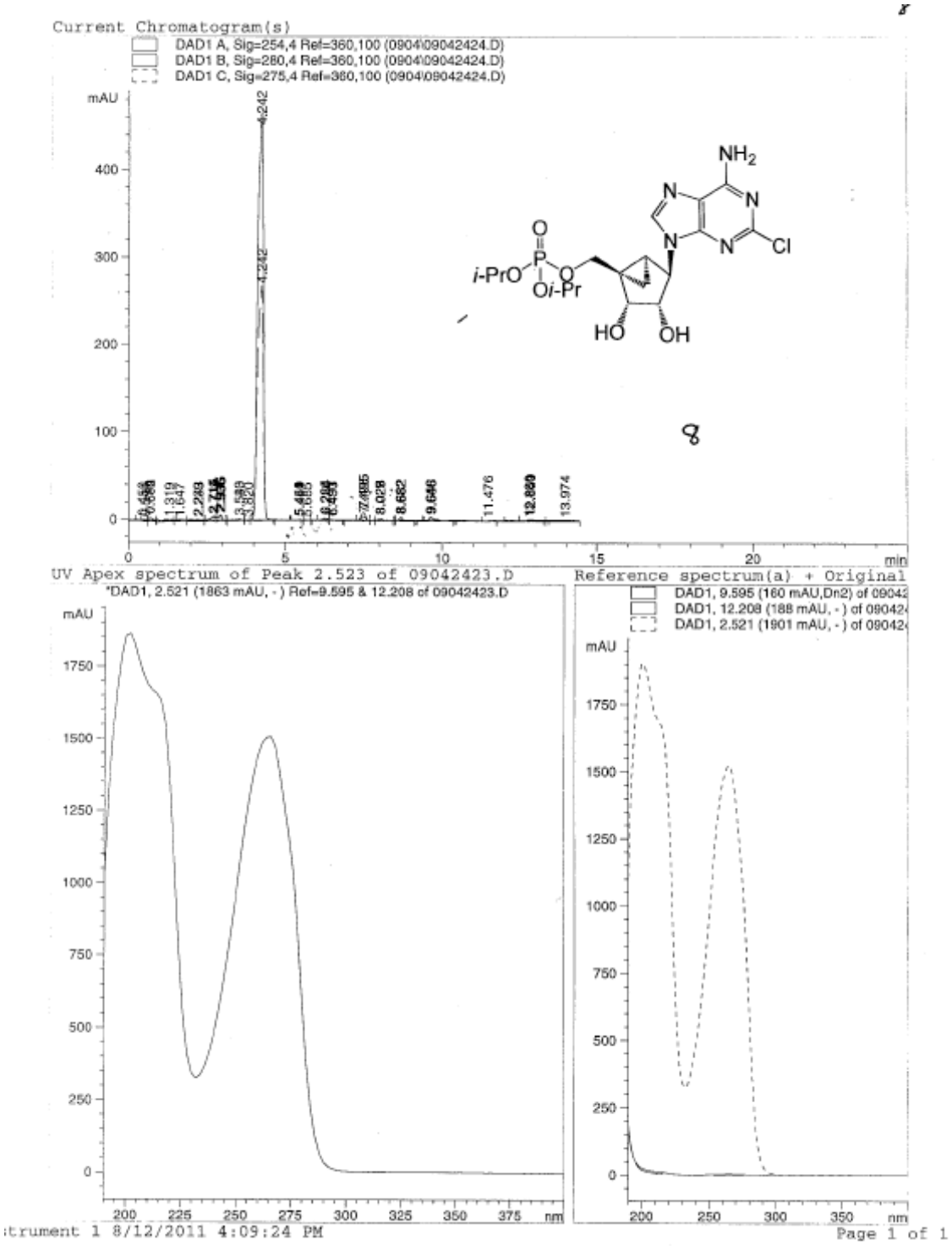
Compound **8**, ^{1}H NMR



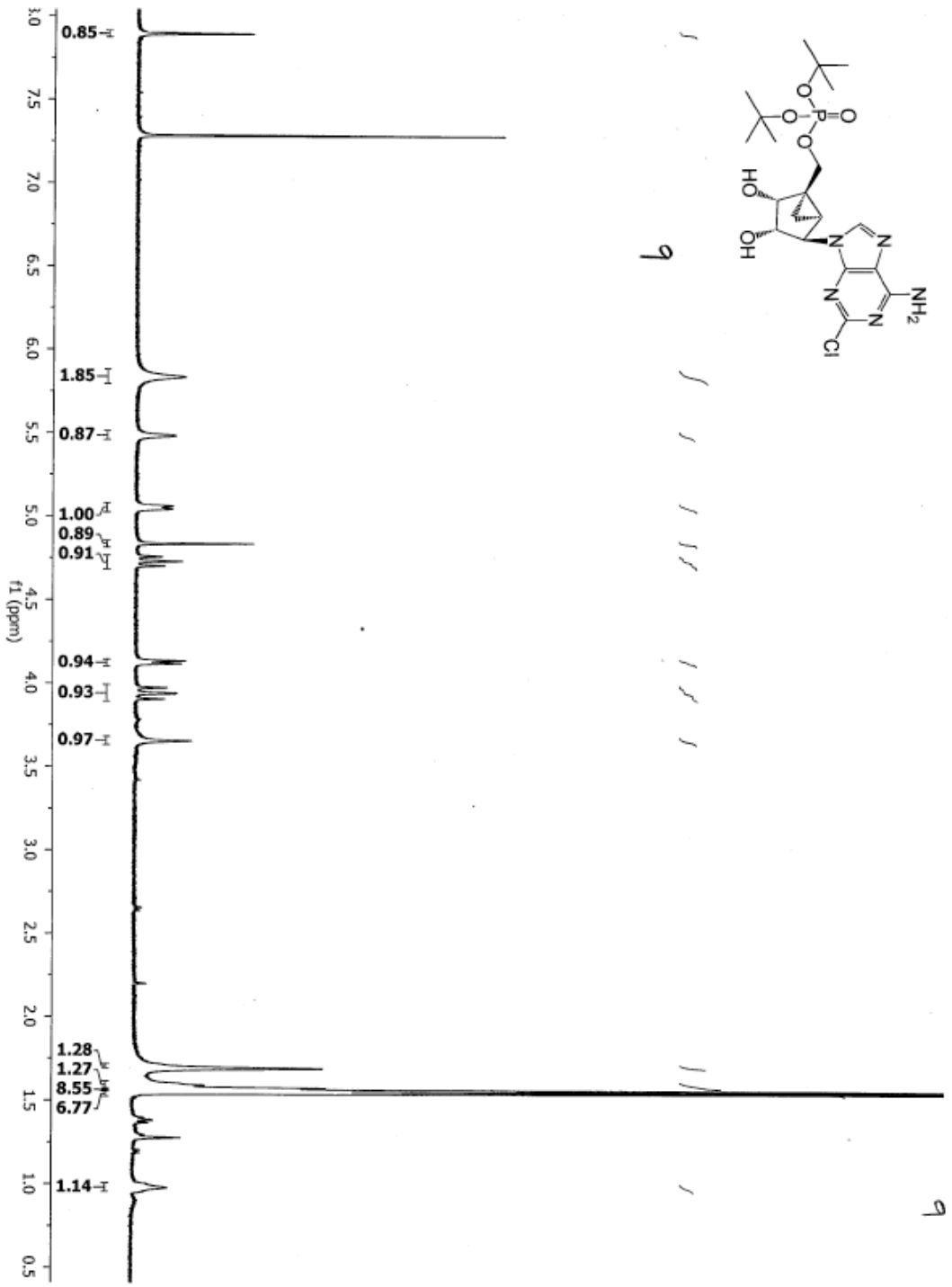
Compound **8**, ^{31}P NMR



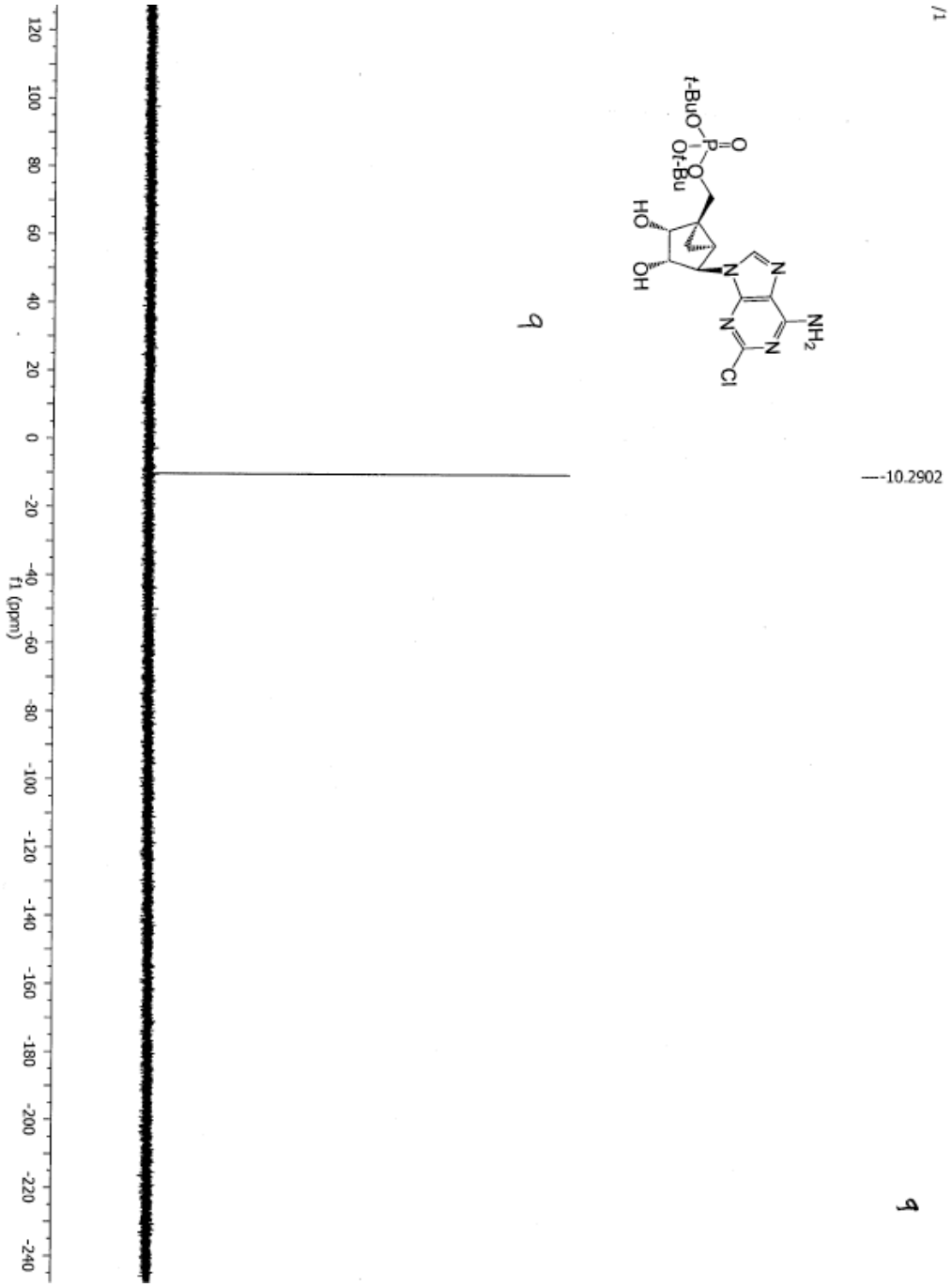
Compound **8**, HPLC



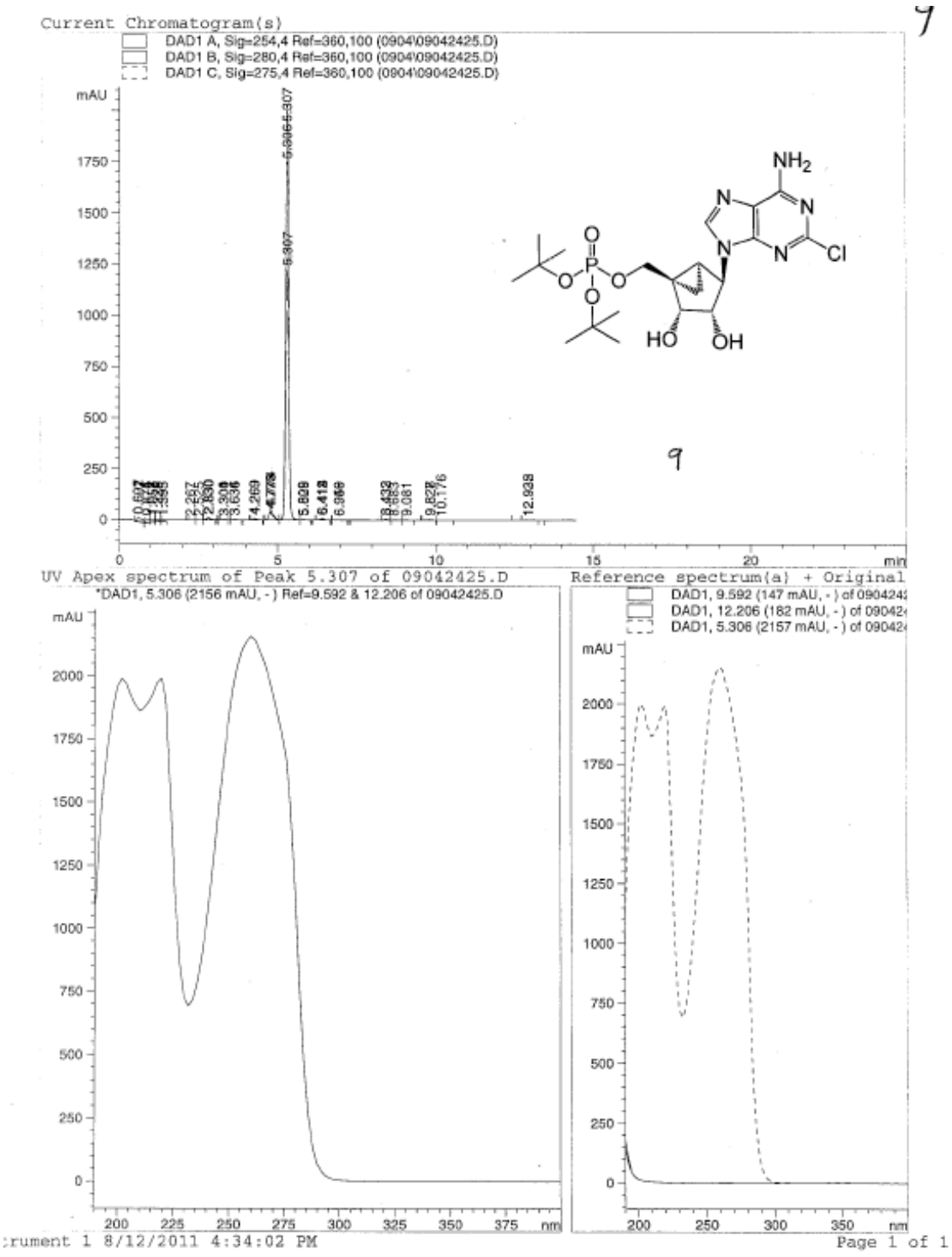
Compound **9**, ^{1}H NMR



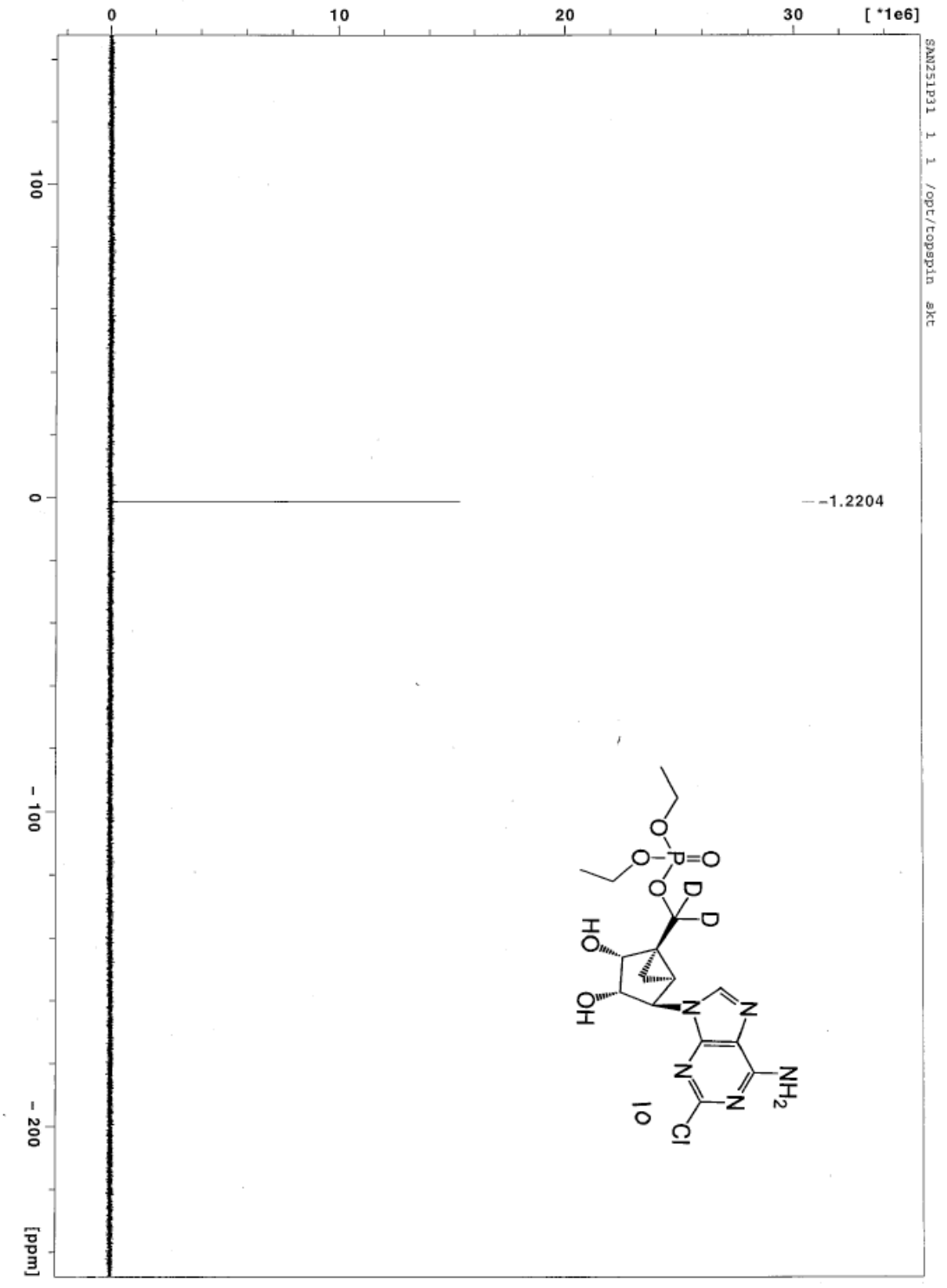
Compound **9**, ^{31}P NMR



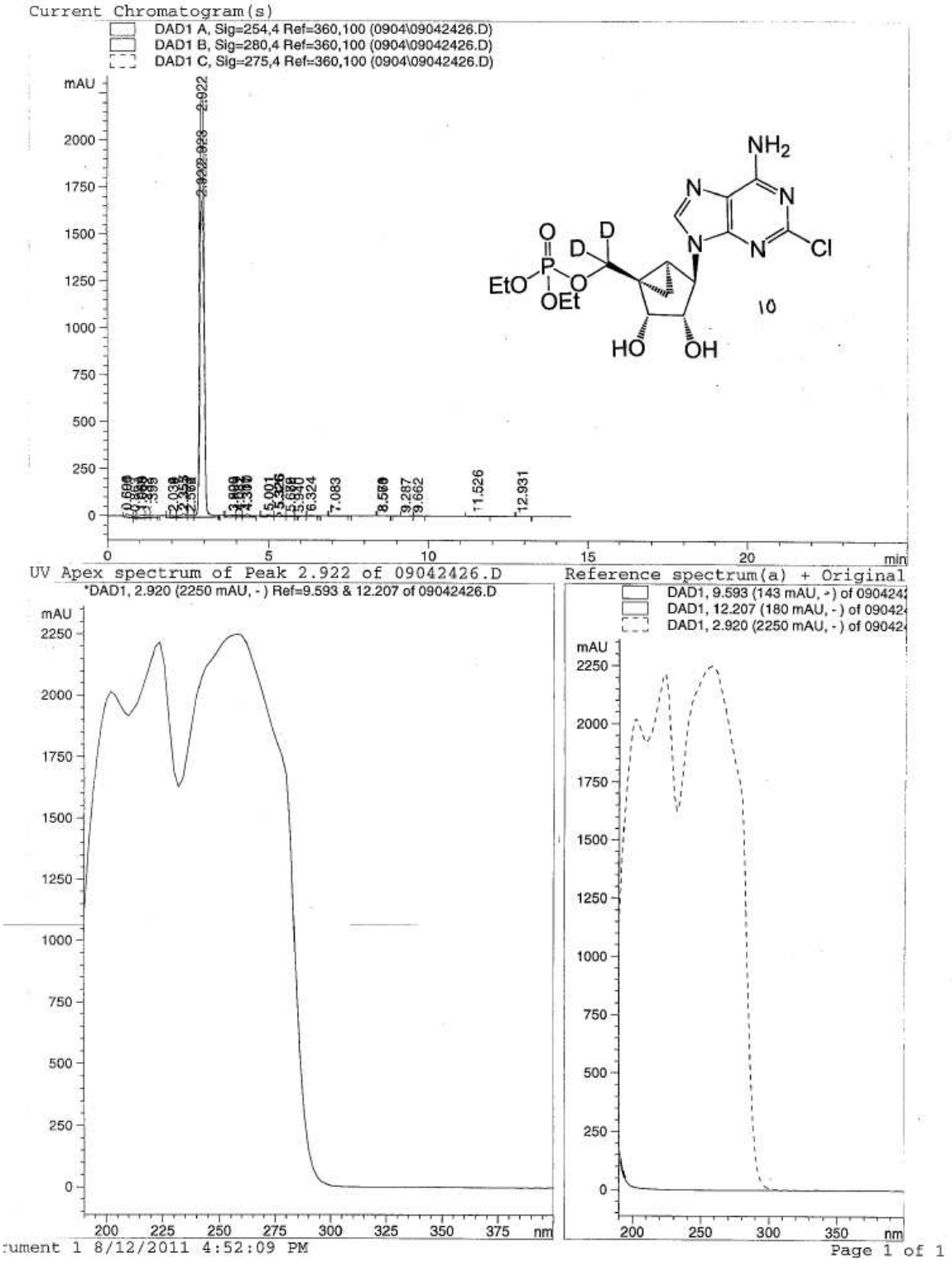
Compound **9**, HPLC



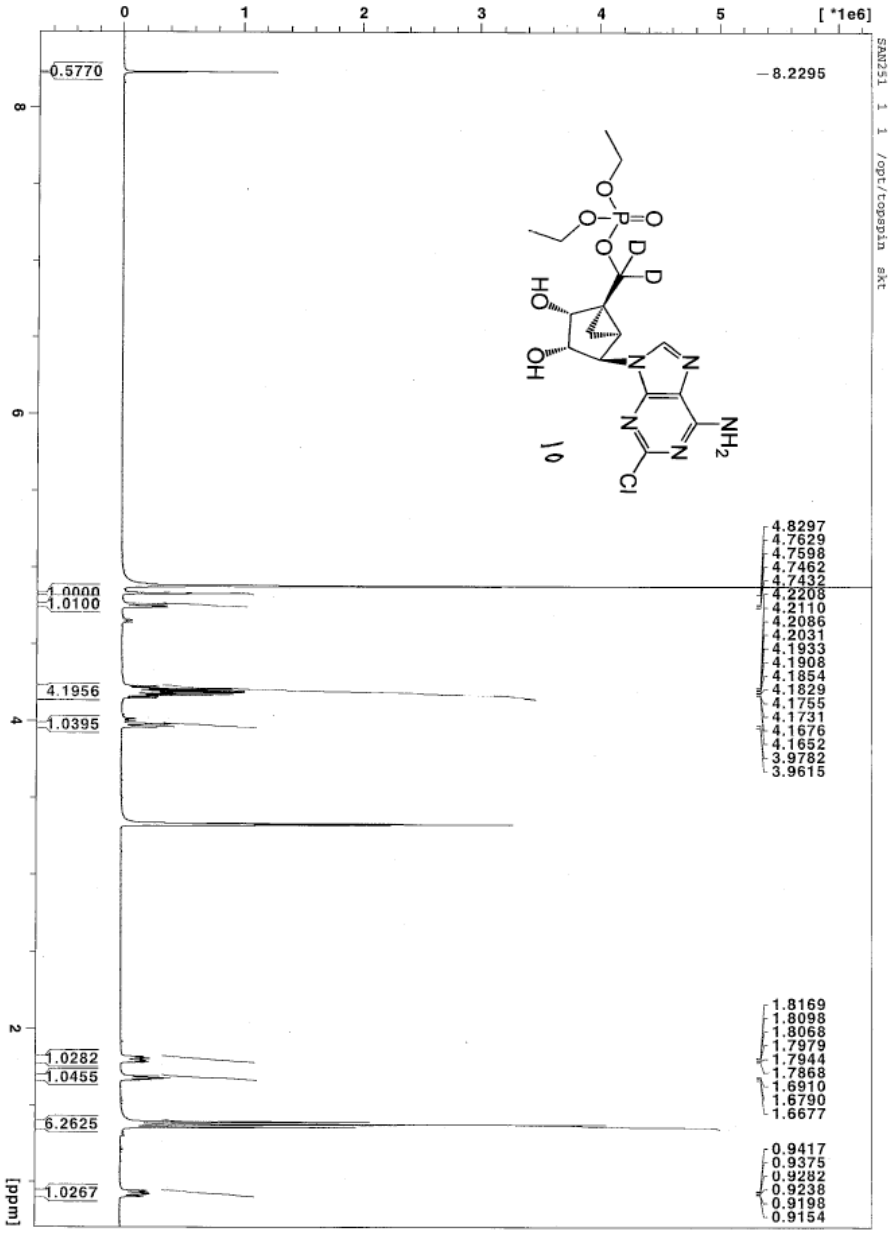
Compound **10**, ^{31}P NMR



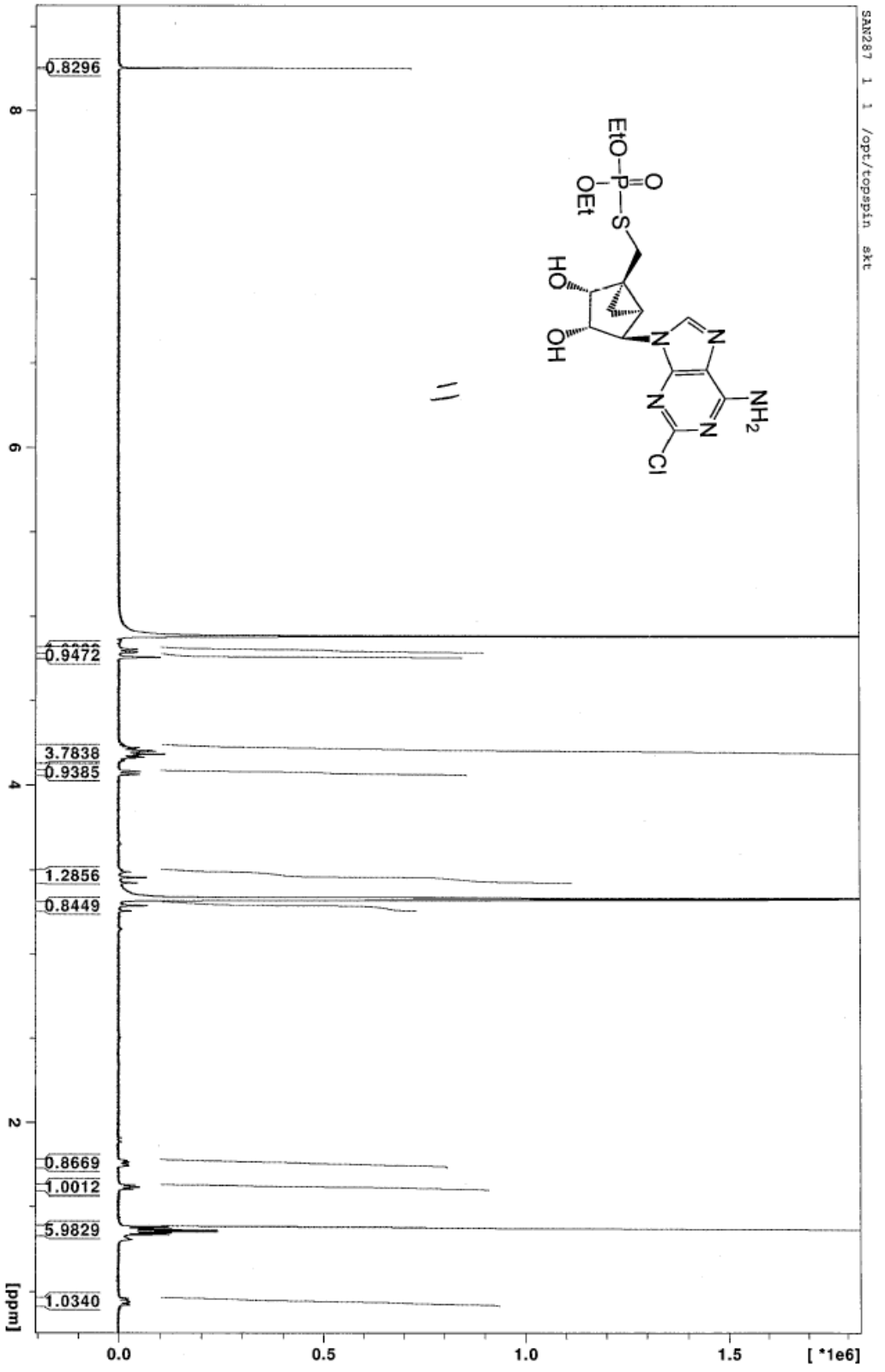
Compound **10**, HPLC



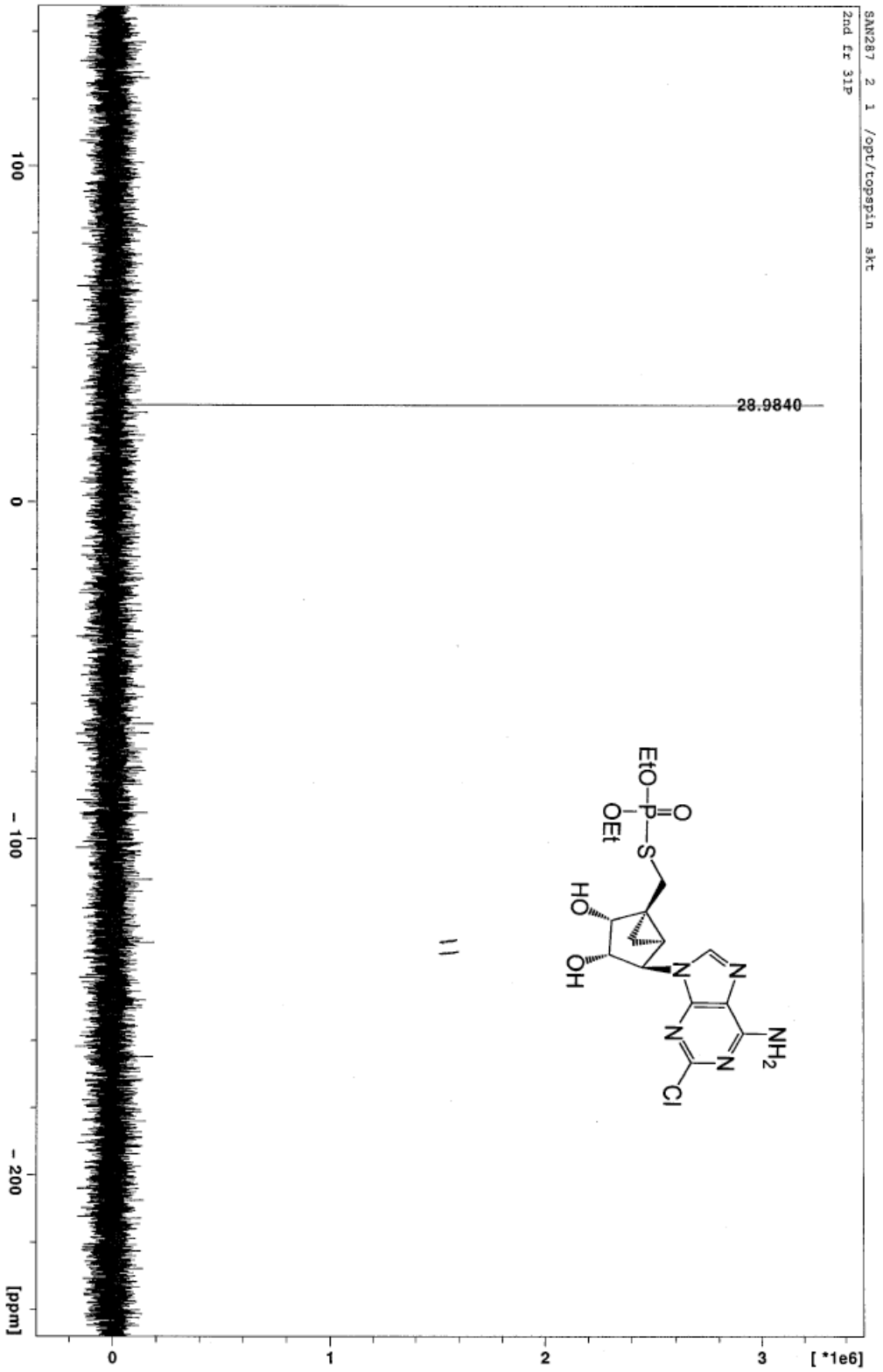
Compound **10**, ^{1}H NMR



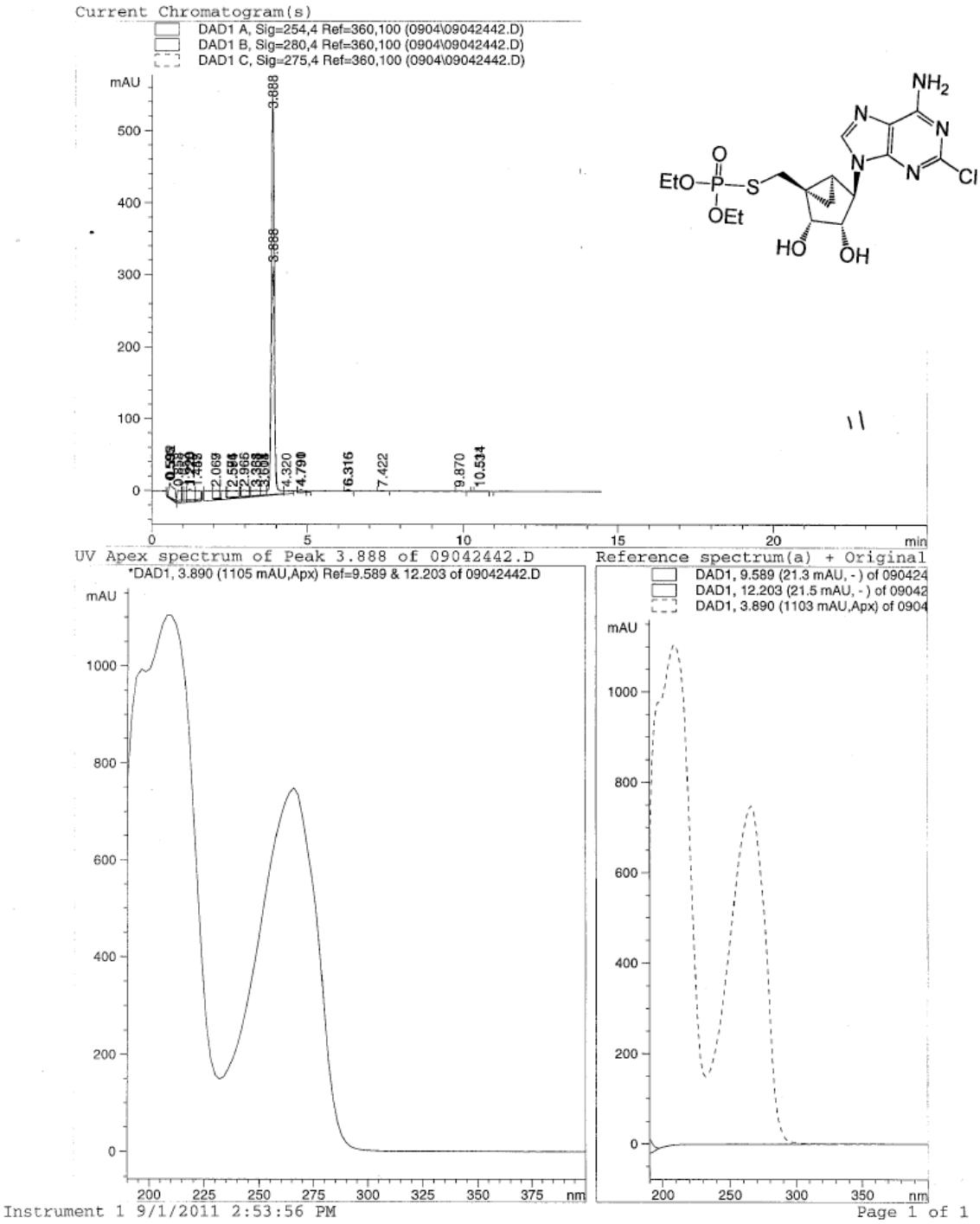
Compound **11**


SAN287 1 1 /opt/topspin skt
0.8296
0.9472
3.7838
0.9385
1.2856
0.8449
0.8669
1.0012
5.9829
1.0340
8
6
4
2
[ppm]
0.0
0.5
1.0
1.5
[*1e6]
EtO
OEt
O
P
S
HO
OH
N
N
N
N
NH2
Cl

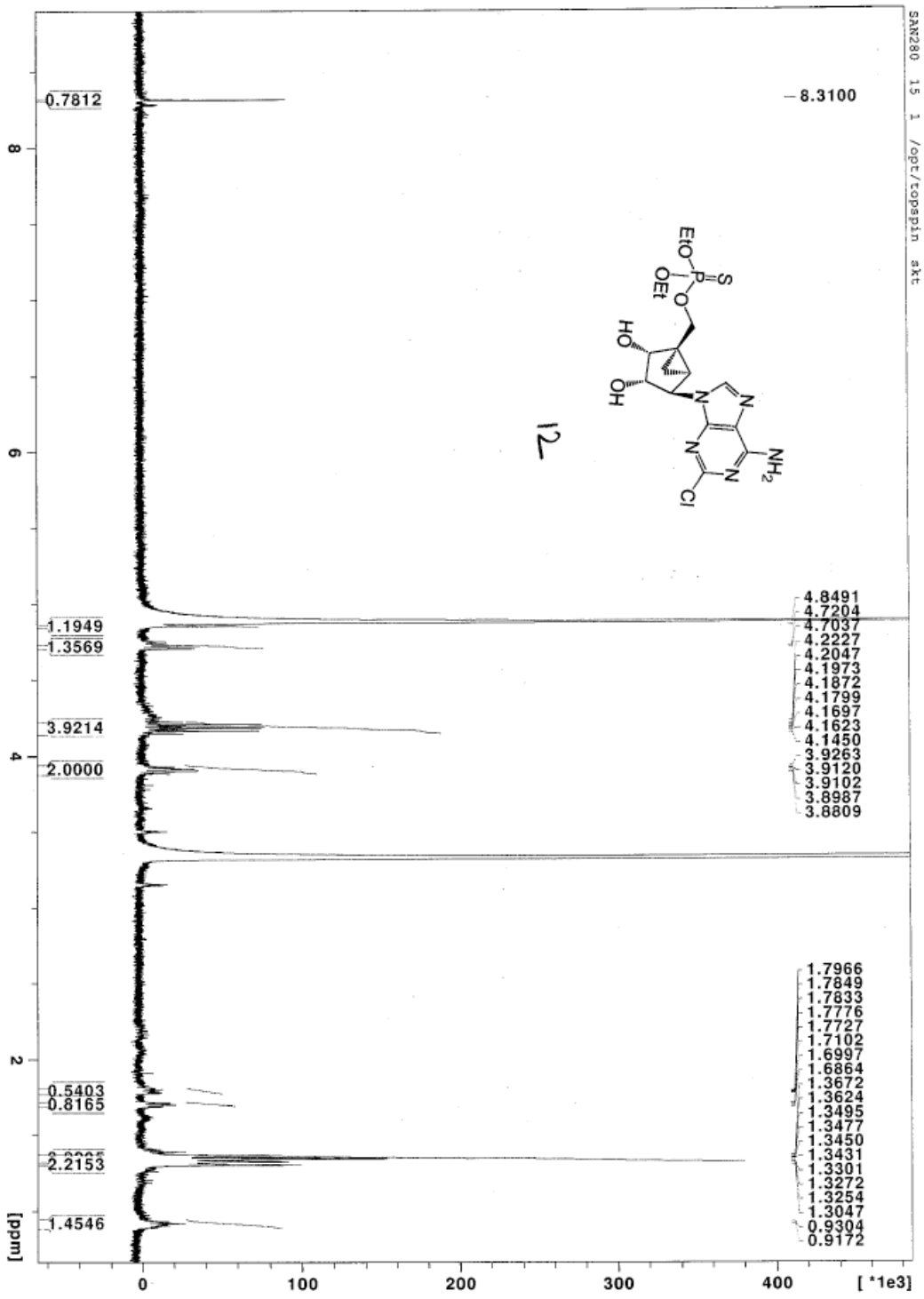
Compound **11**, ^{31}P NMR



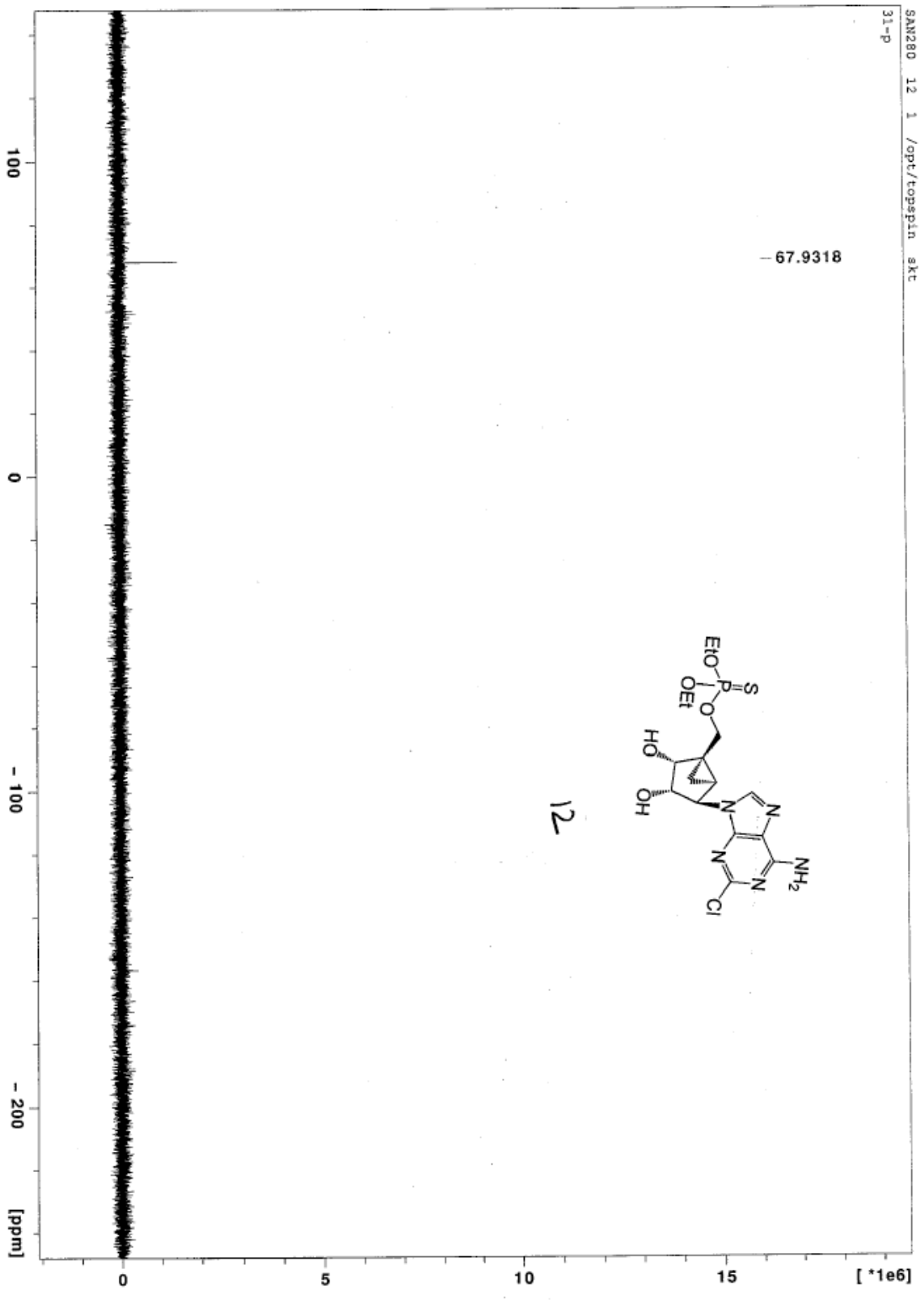
Compound **11**, HPLC



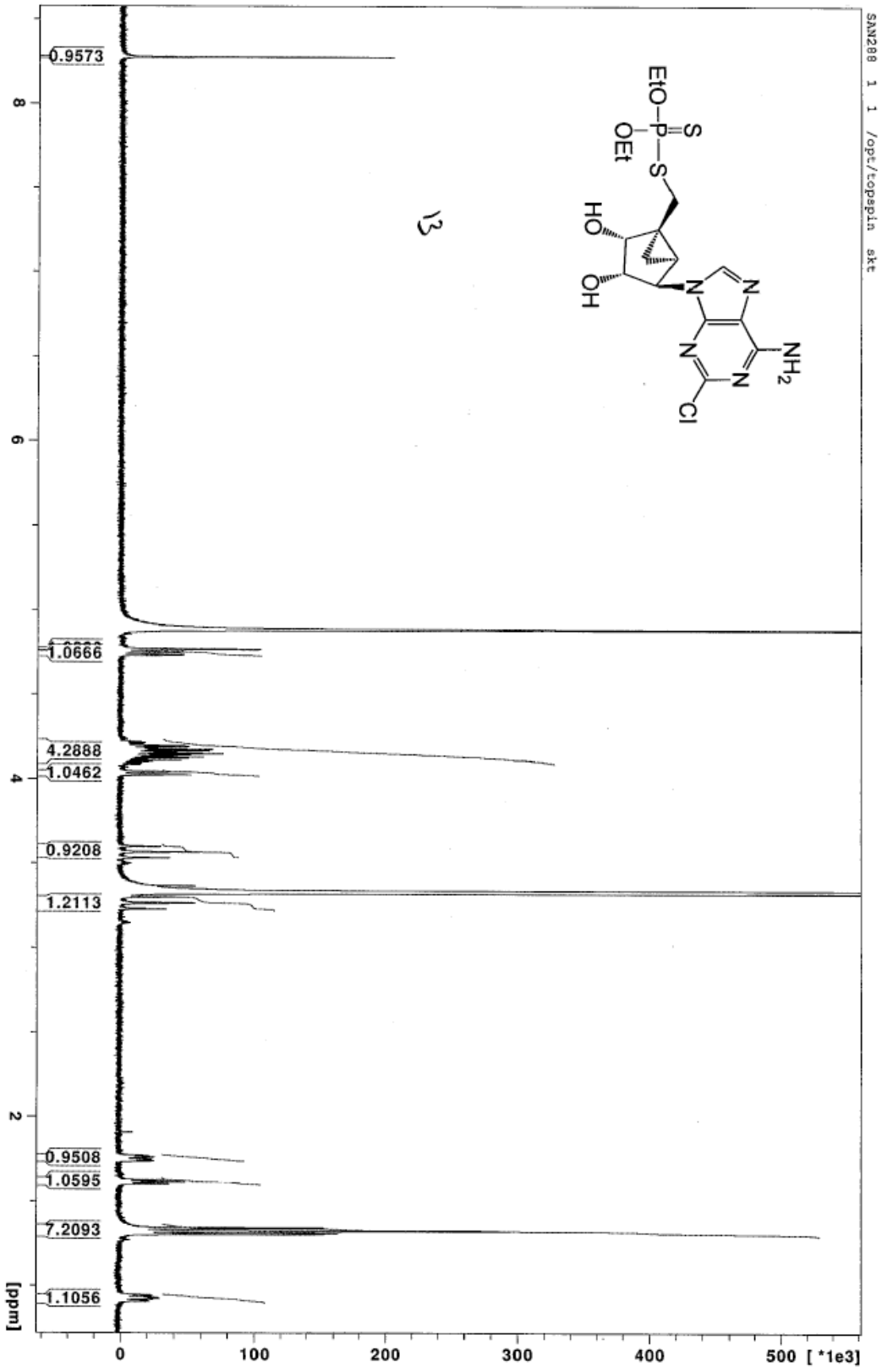
Compound **12**, ^{1}H NMR



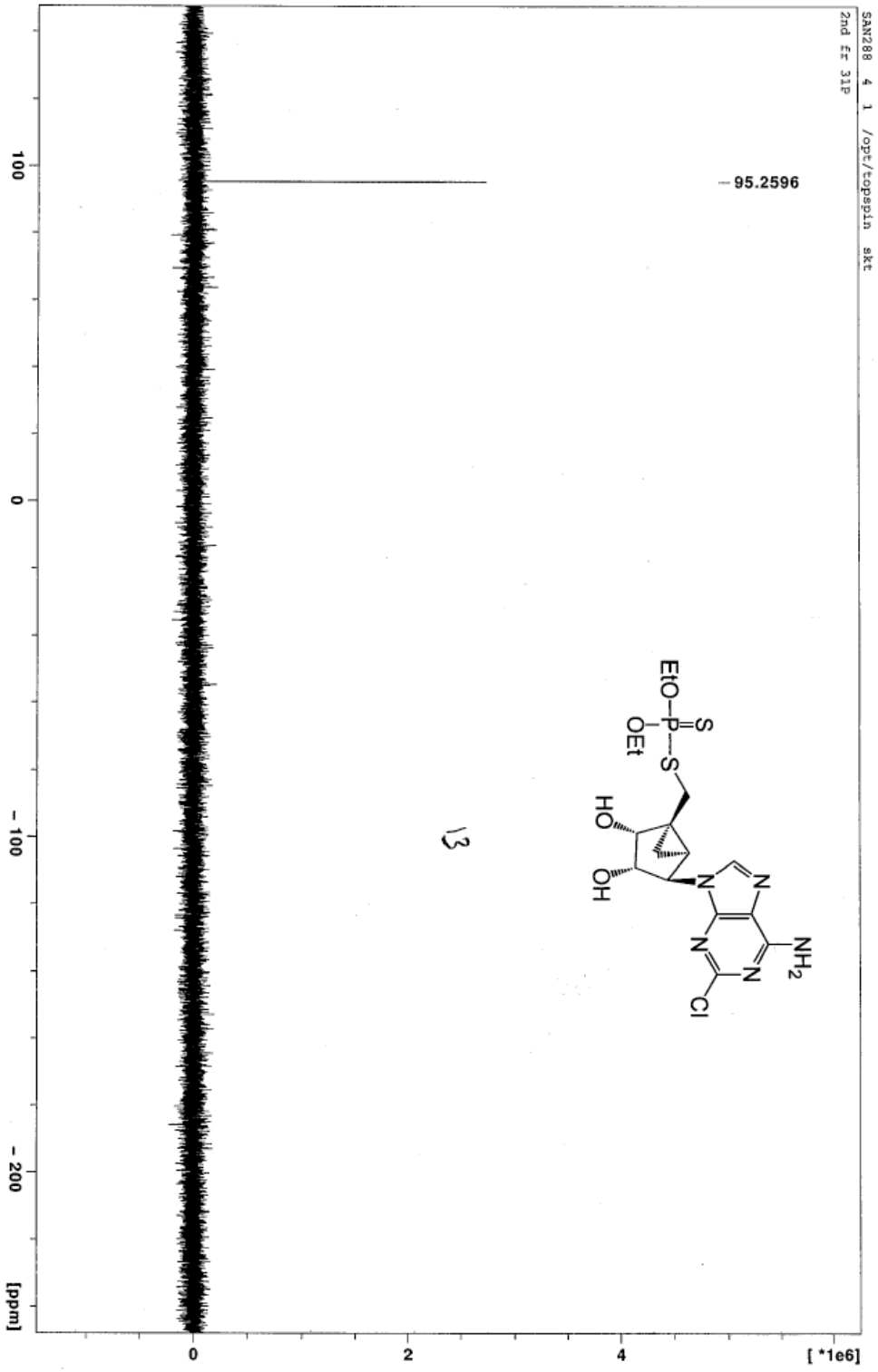
Compound **12**, ^{31}P NMR



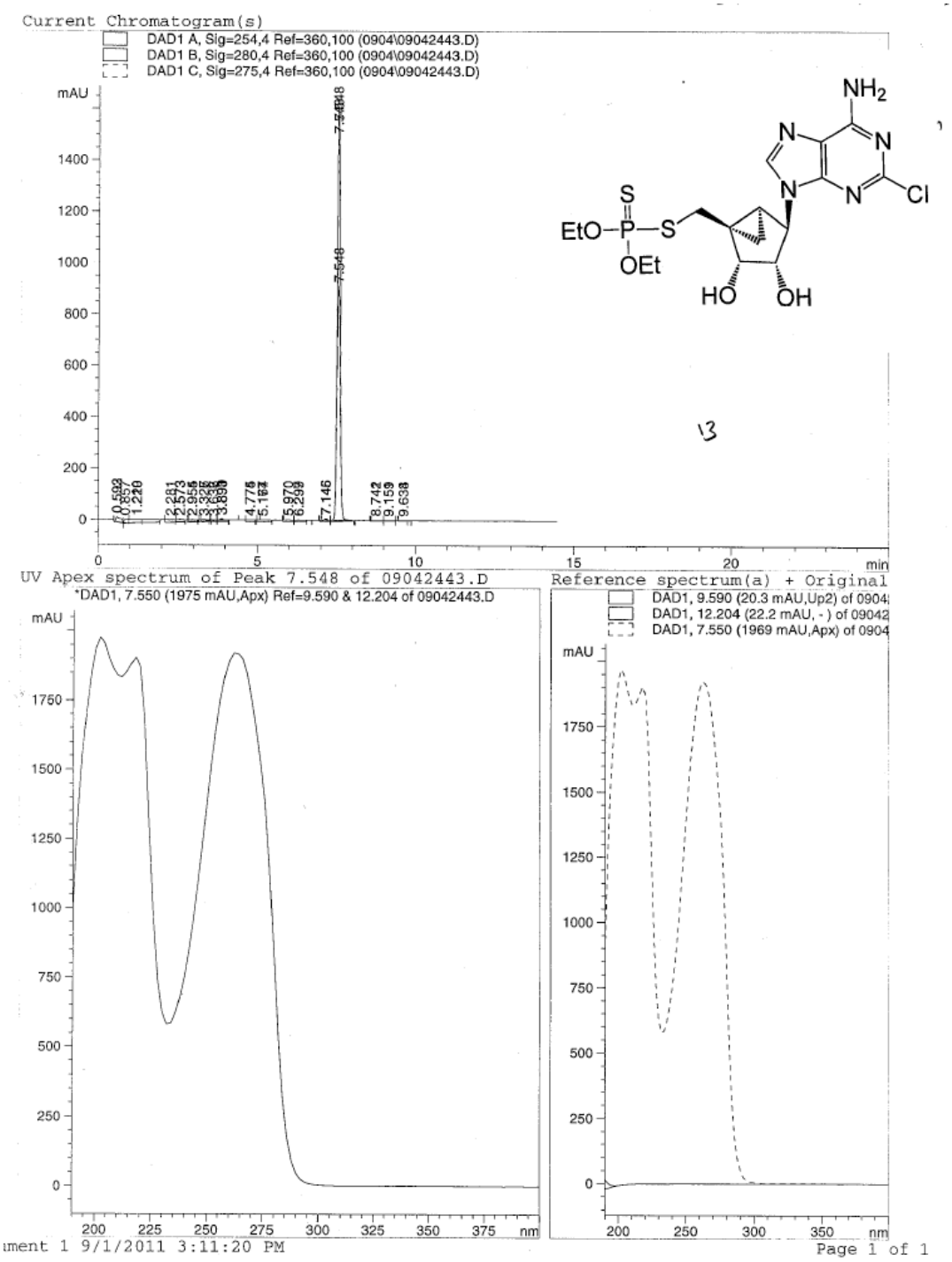
Compound **13**, ^{1}H NMR



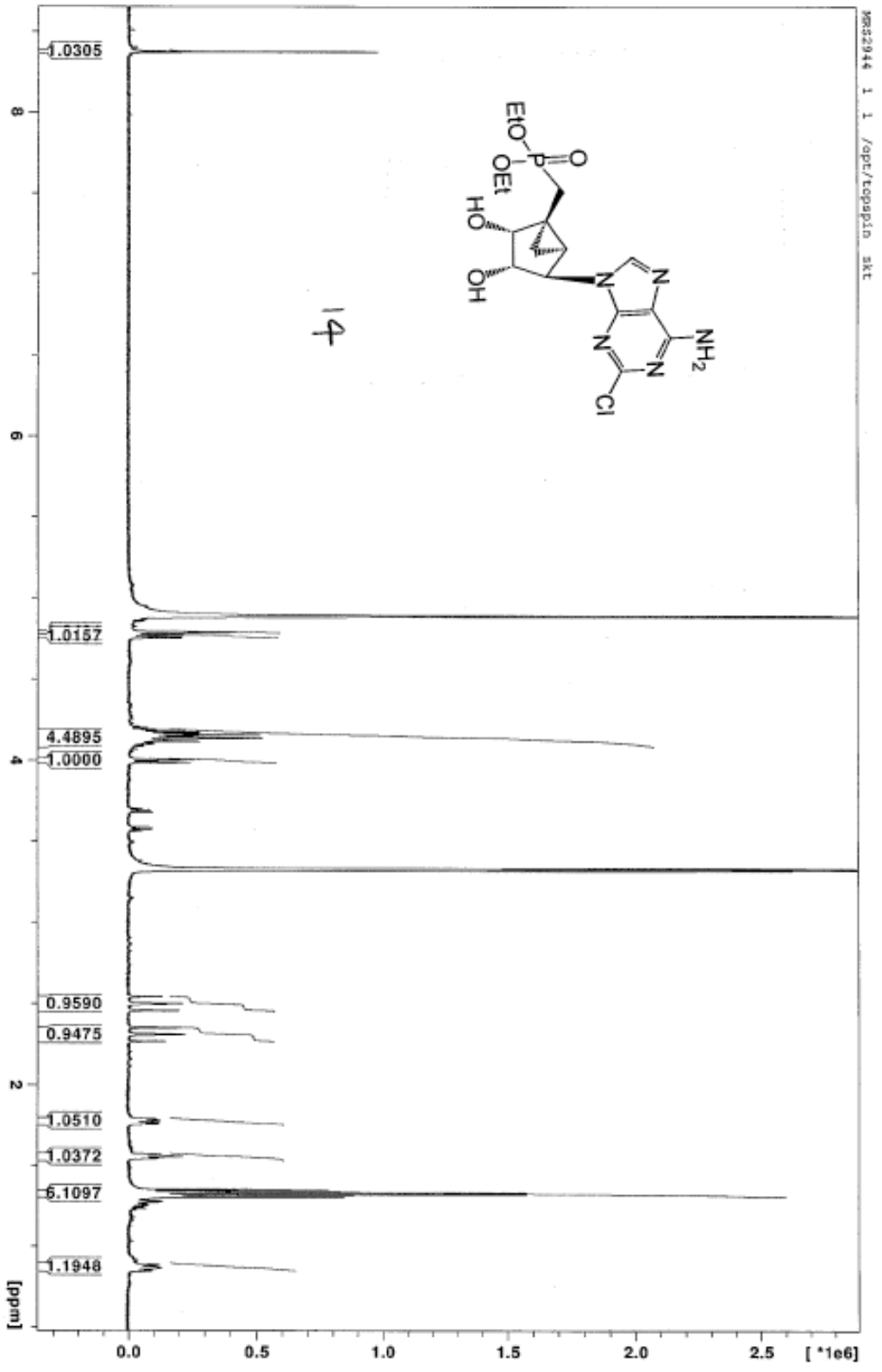
Compound **13**, ^{31}P NMR



Compound **13**, HPLC



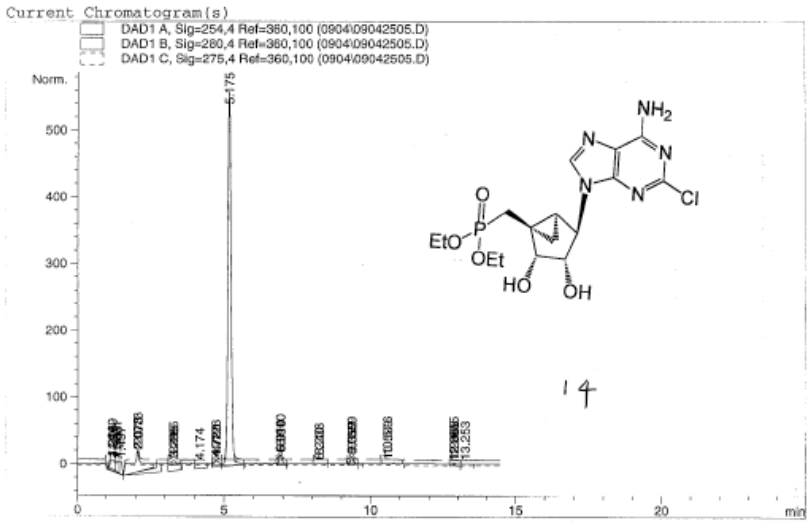
Compound **14**, ^{1}H NMR

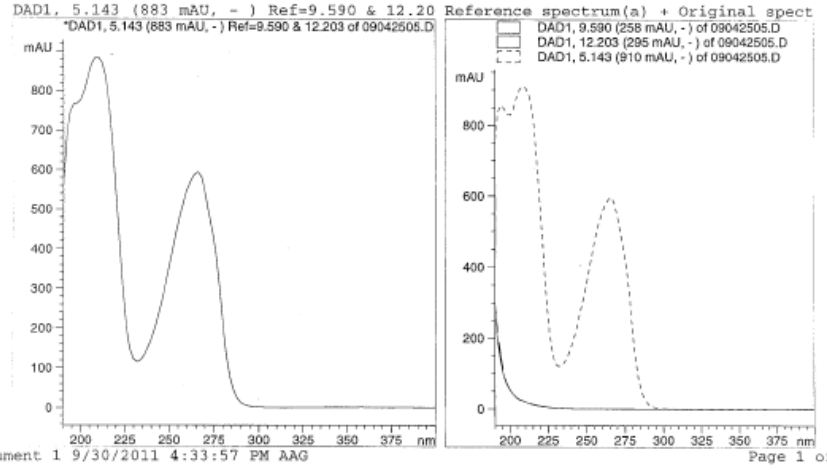


Compound **14**, HPLC

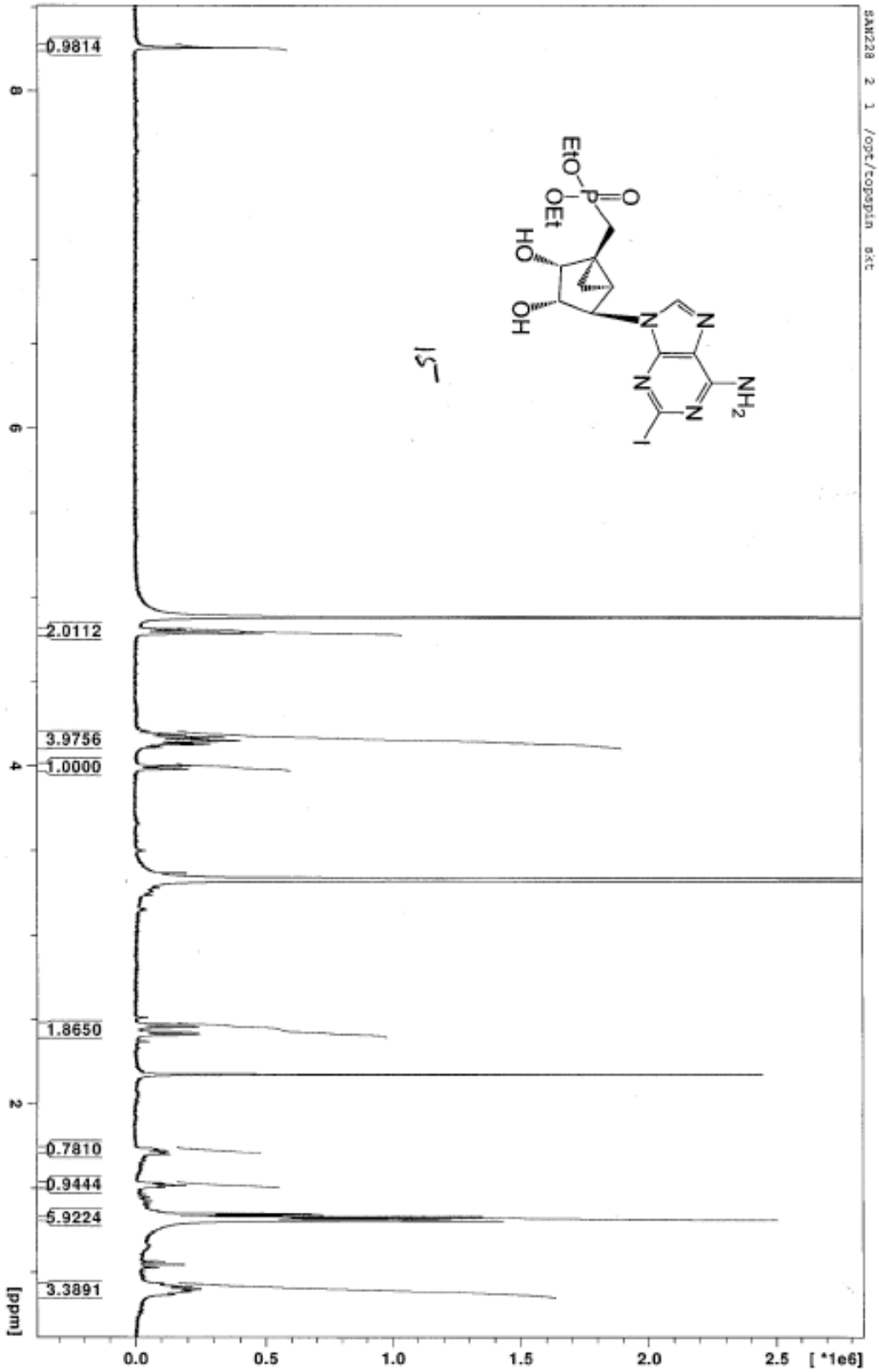
```
Print of all graphic windows
#50 extr EtOAc 5/5 1g NaCl dil 1/33.3 H2O 2

================================================================================
Injection Date  : 9/30/2011 4:10:07 PM
Sample Name     : 50 ext 1/33.3 2                Location : Vial 1
Acq. Operator   : AAG
Method          : C:\HPCHEM\1\METHODS\METHOD1.M
Last changed    : 9/30/2011 4:09:16 PM by AAG
                  (modified after loading)
```

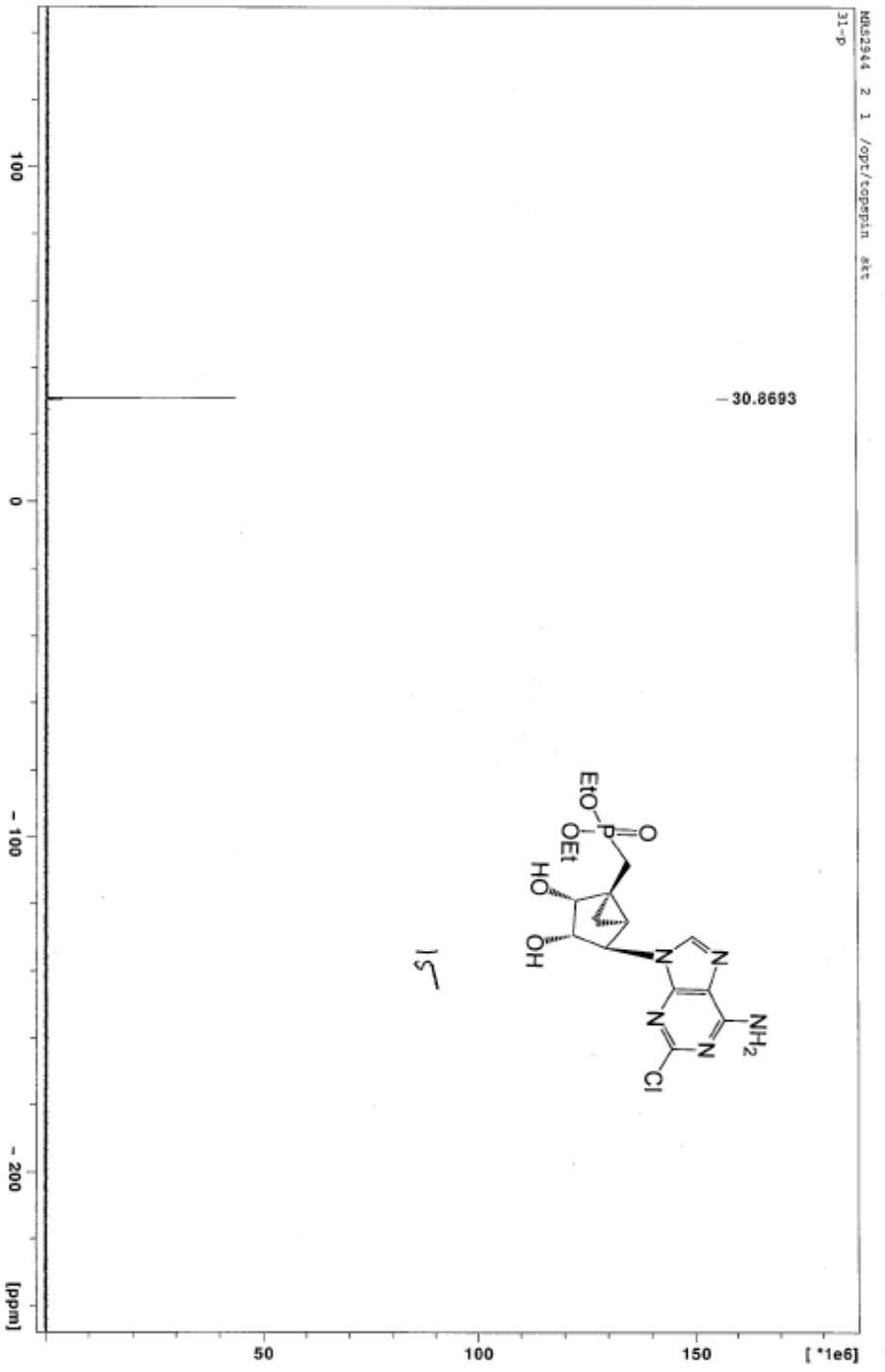




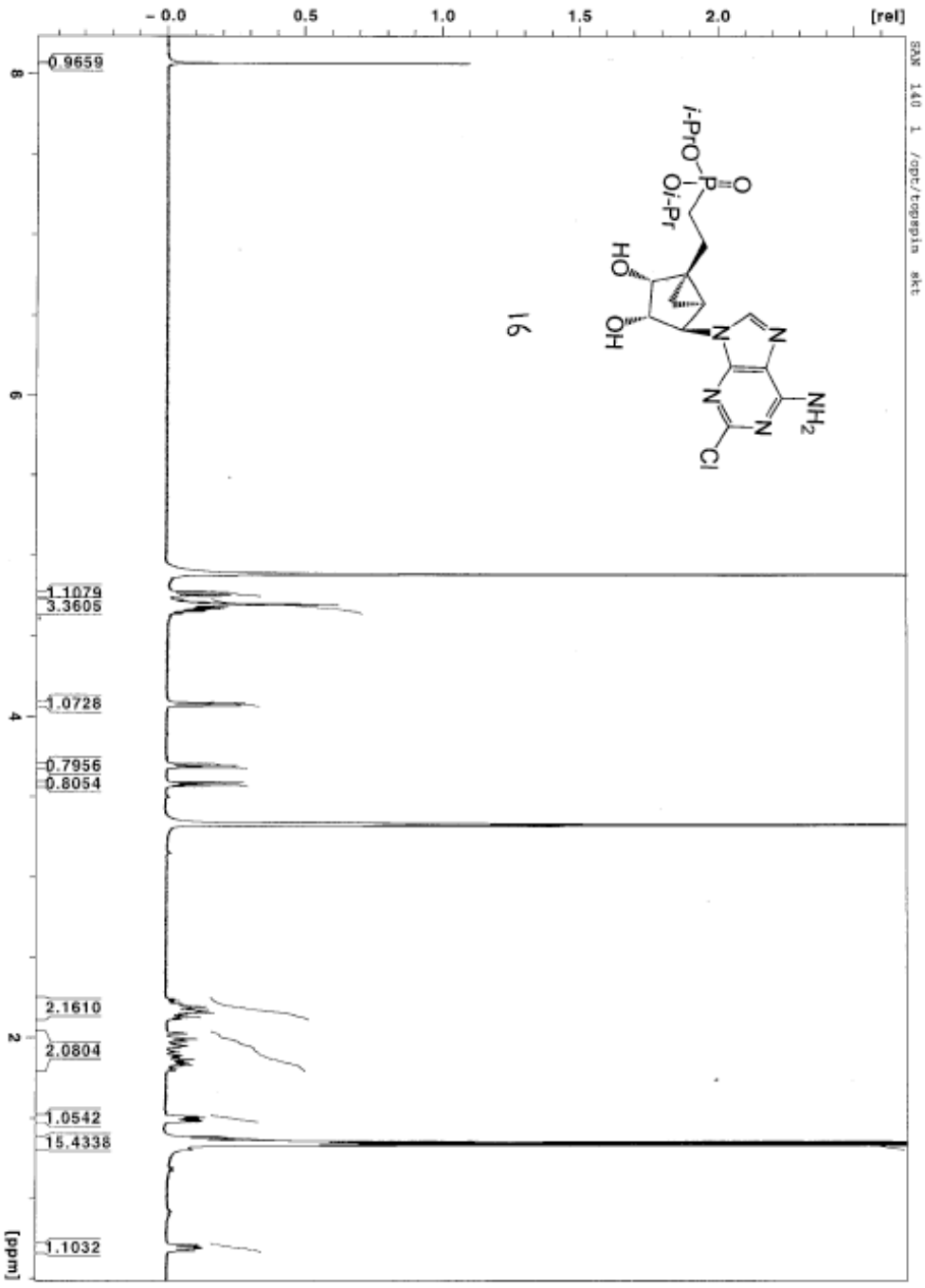
Compound **15**, ^{1}H NMR



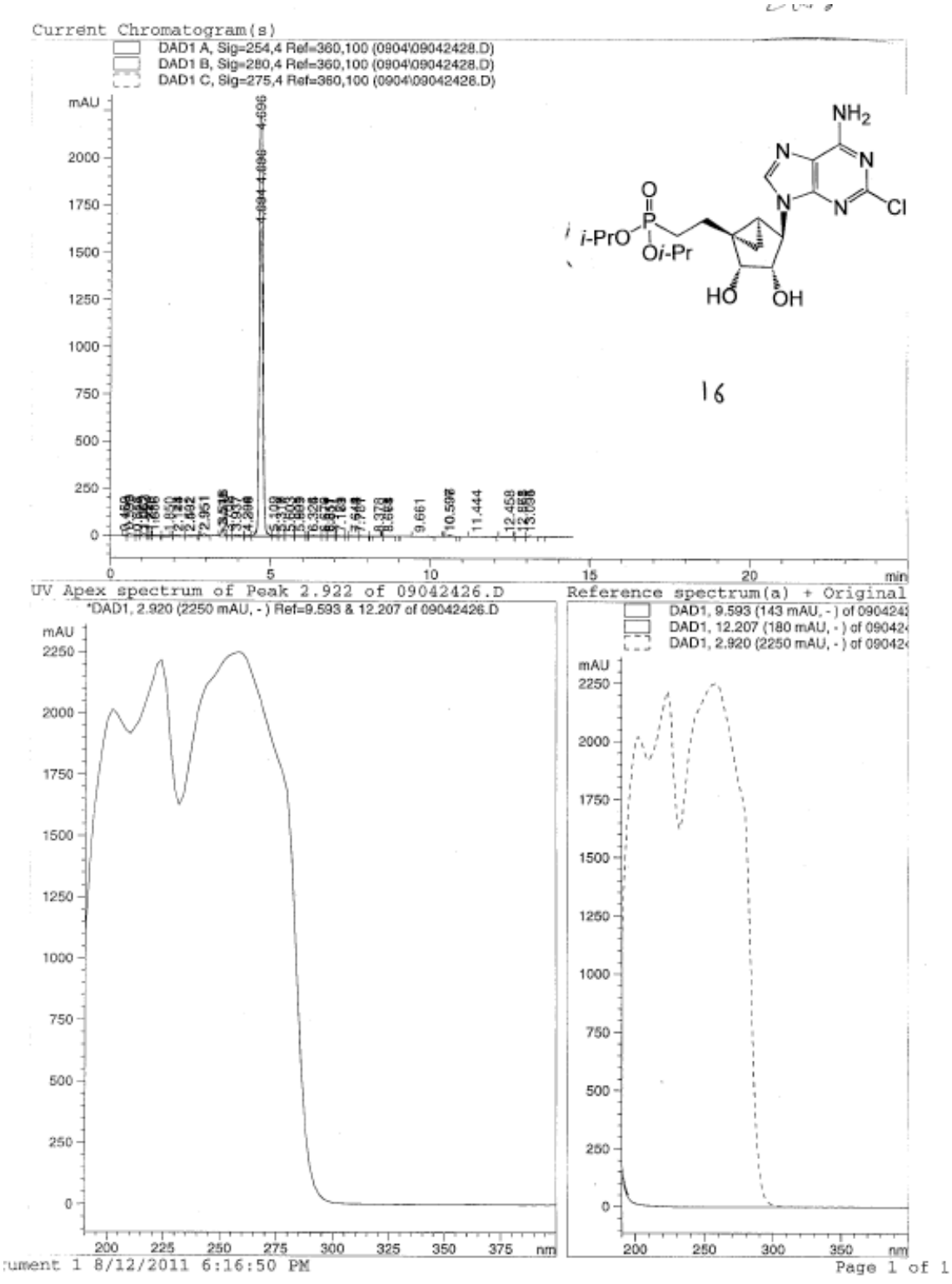
Compound **15**, ^{31}P NMR



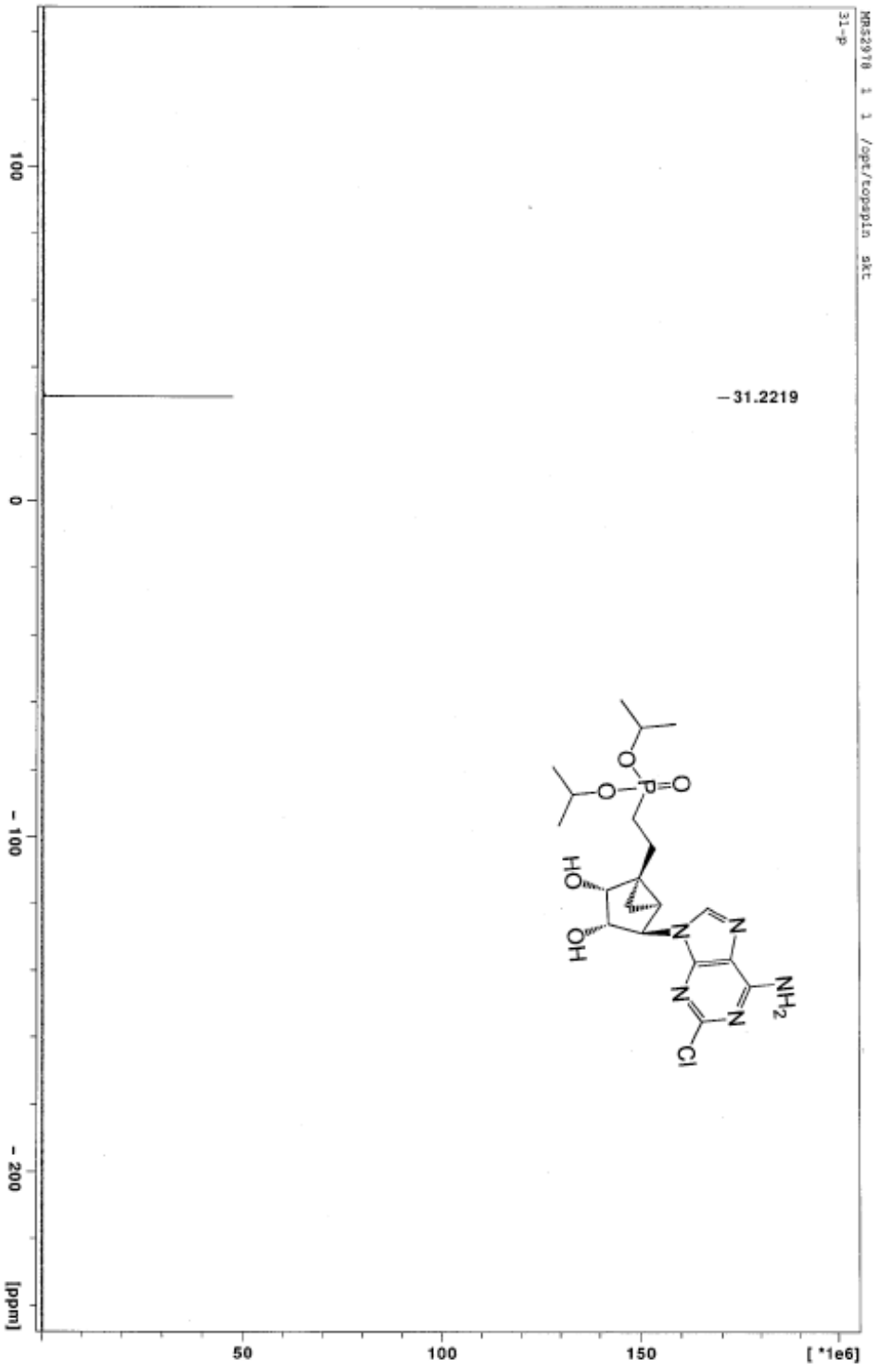
Compound **16**, ^{1}H NMR



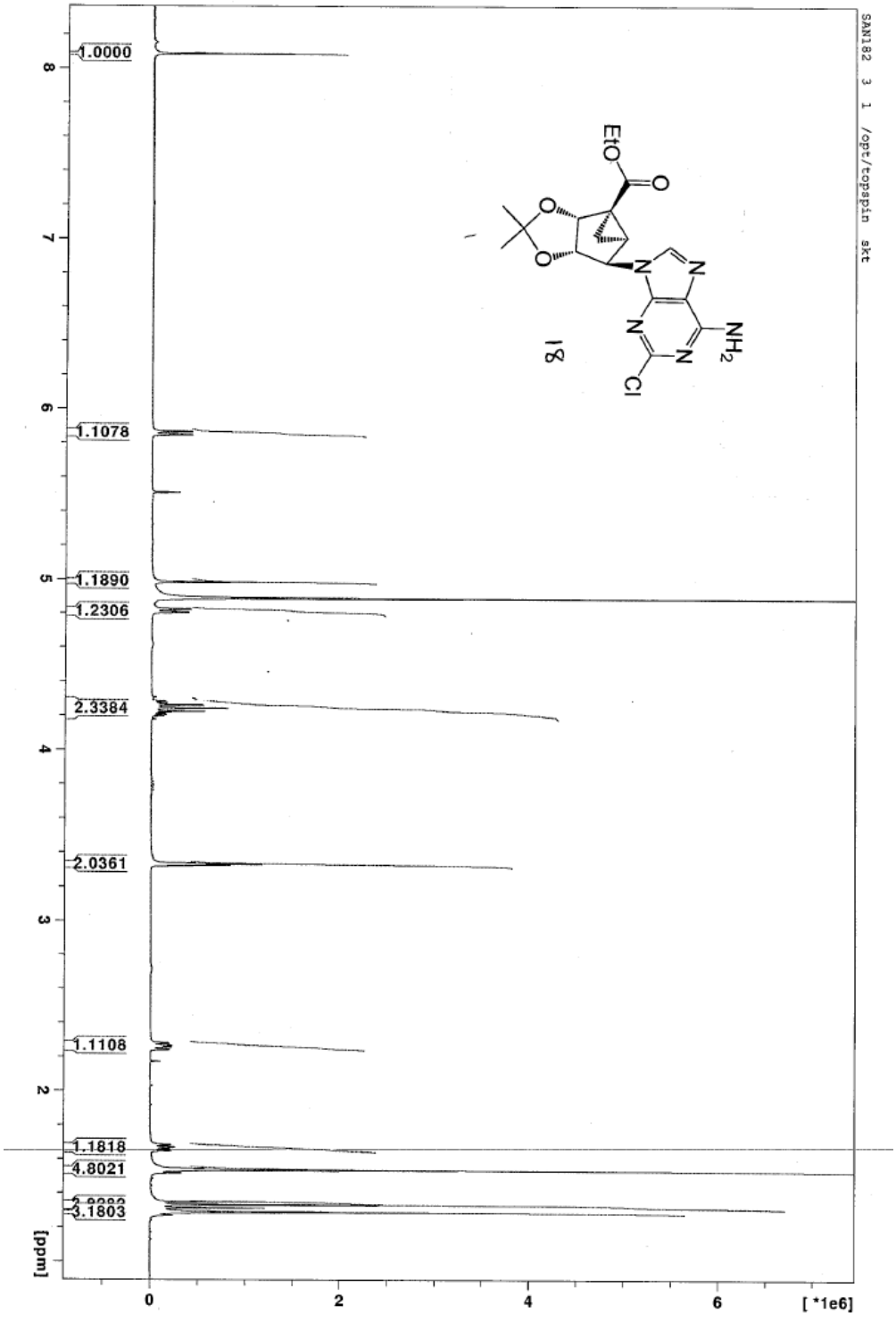
Compound **16**, HPLC



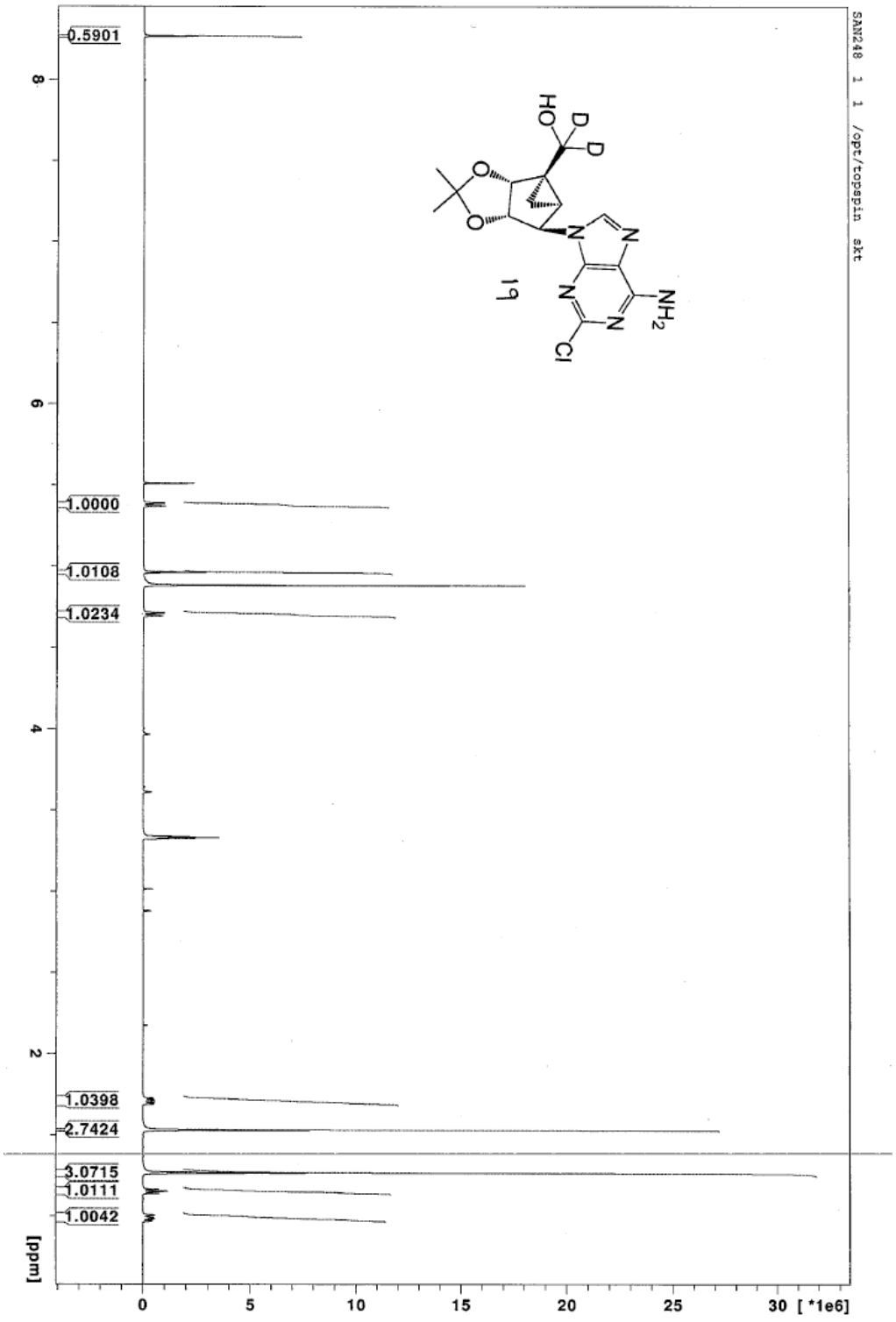
Compound **16**, ^{31}P NMR



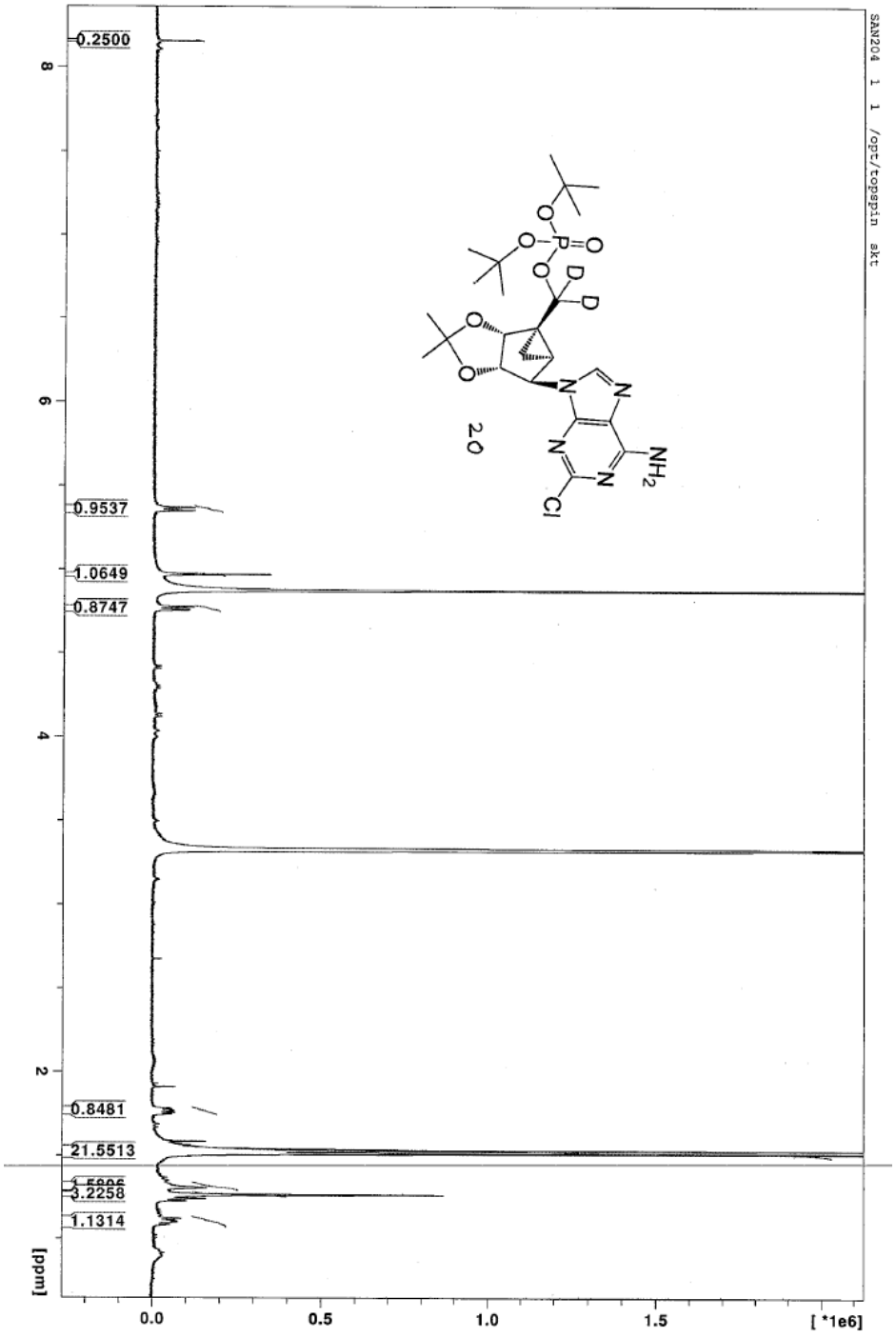
Compound **18**, ^{1}H NMR



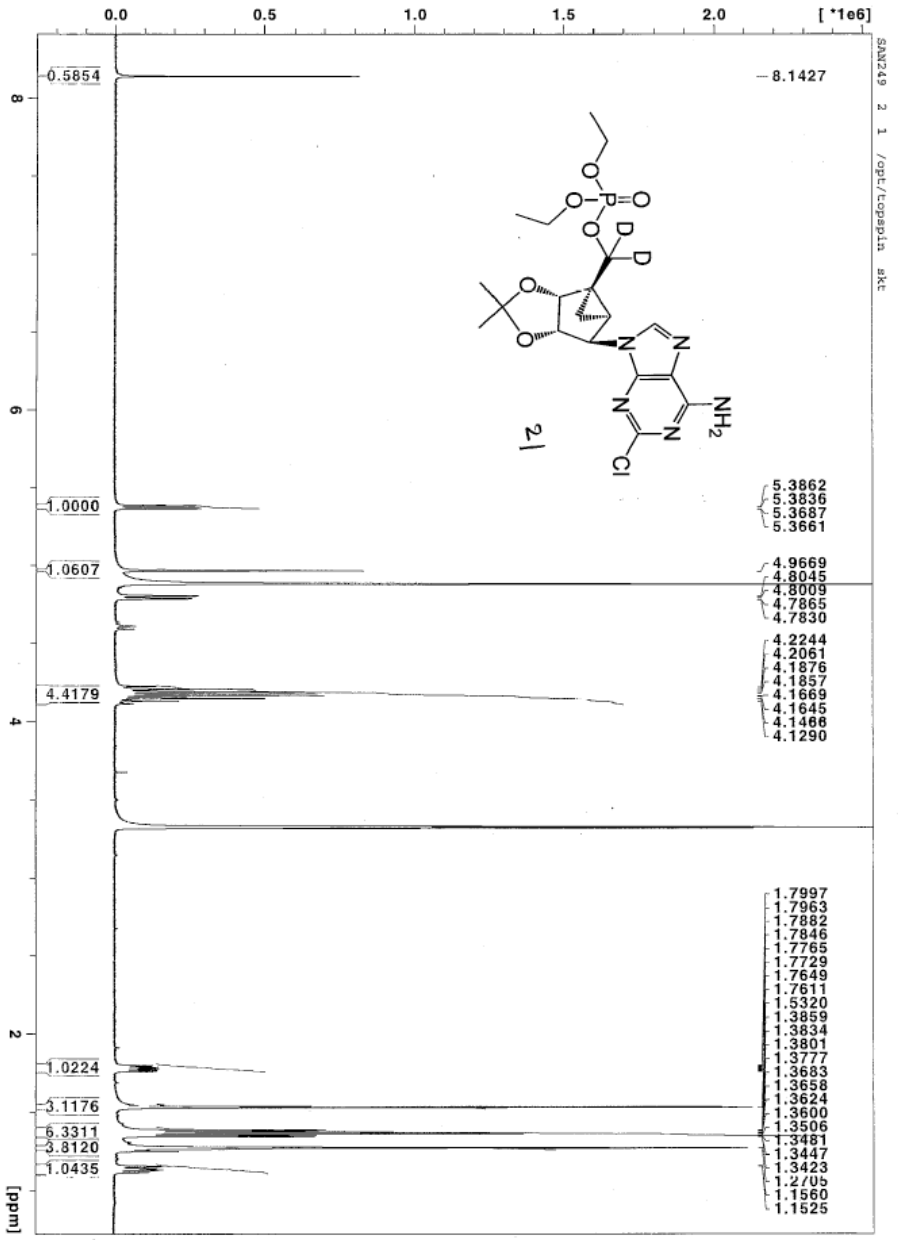
Compound **19**, ^{1}H NMR



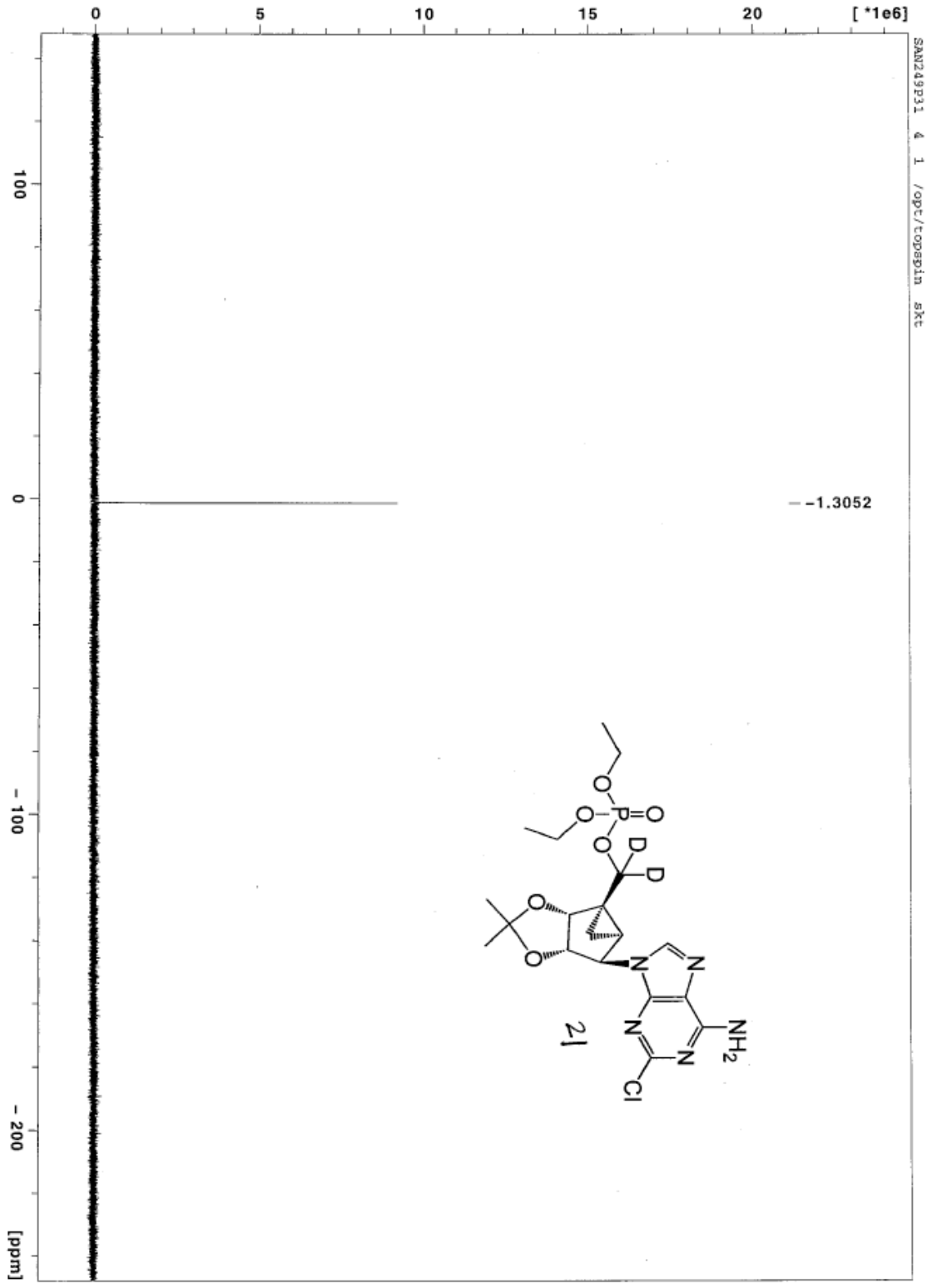
Compound **20**, ^{1}H NMR



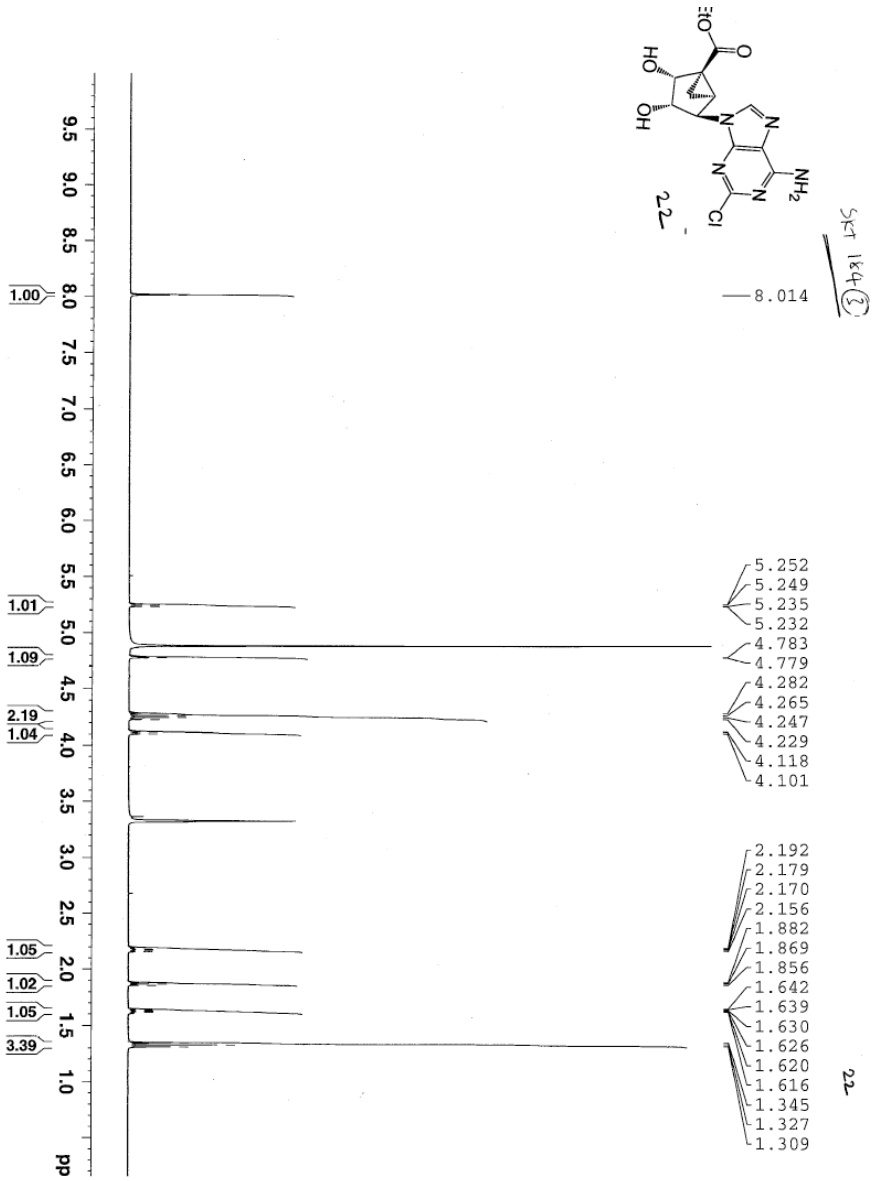
Compound **21**, ^{1}H NMR



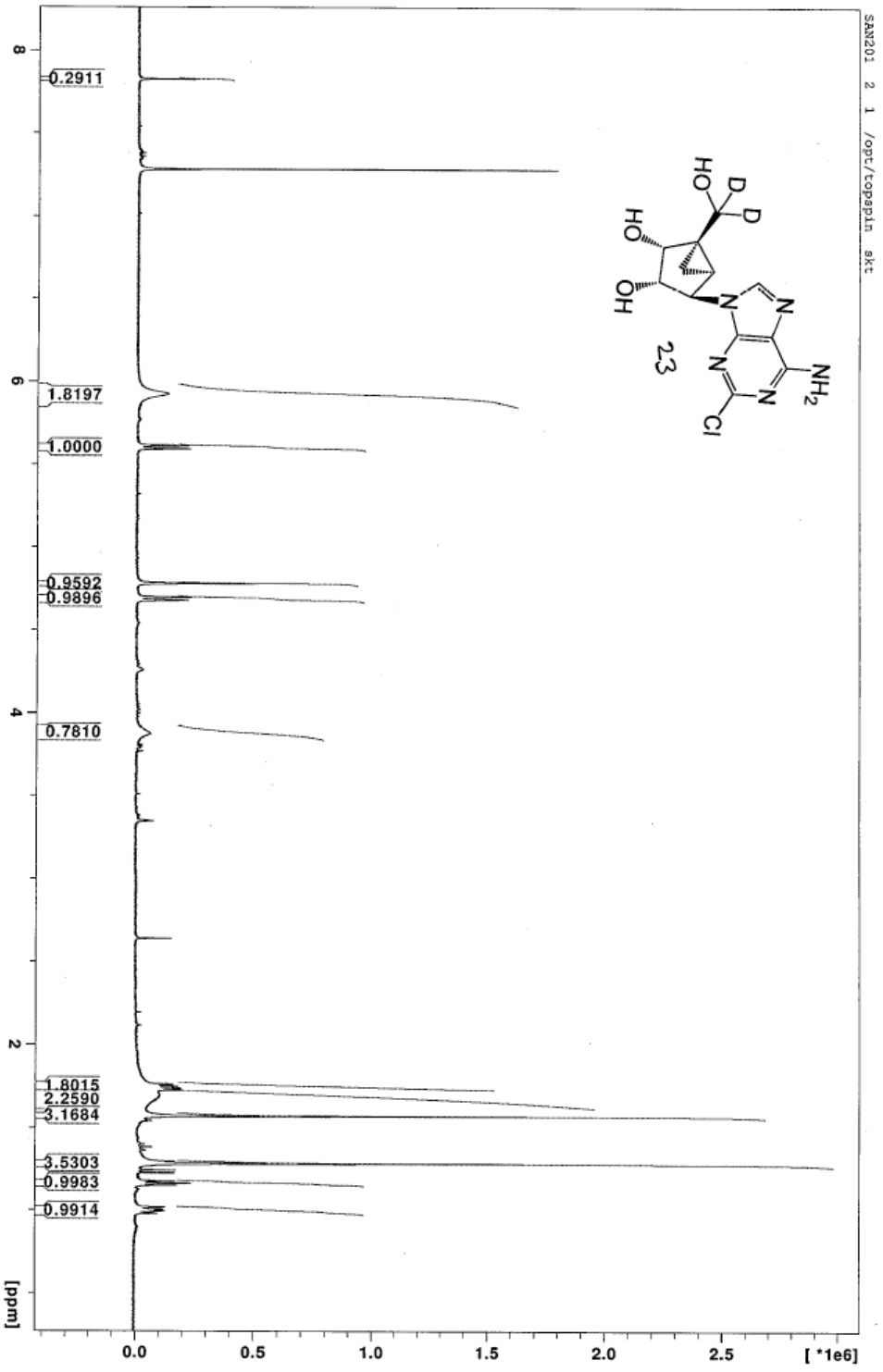
Compound **21**, ^{31}P NMR



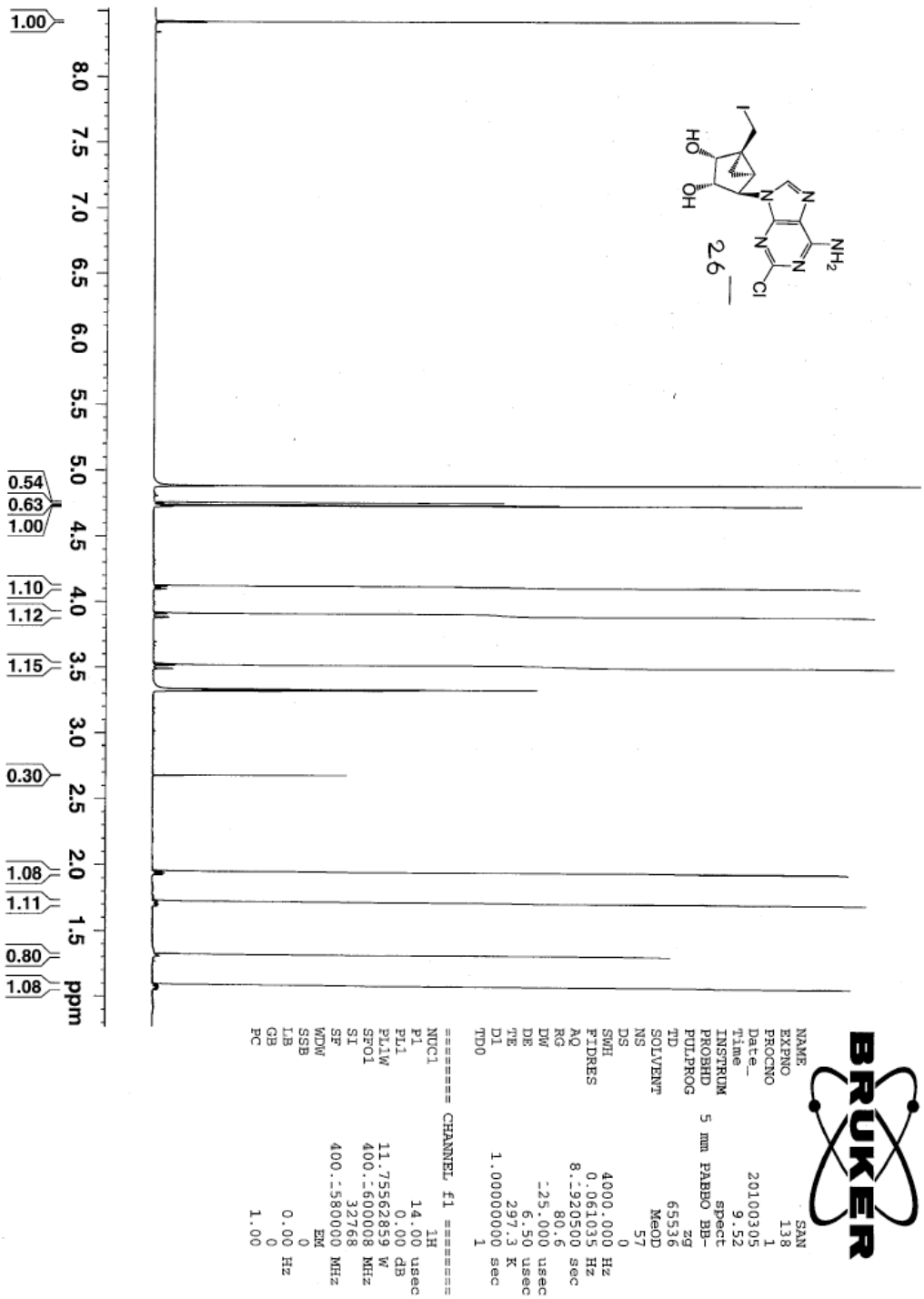
Compound **22**, ^{1}H NMR



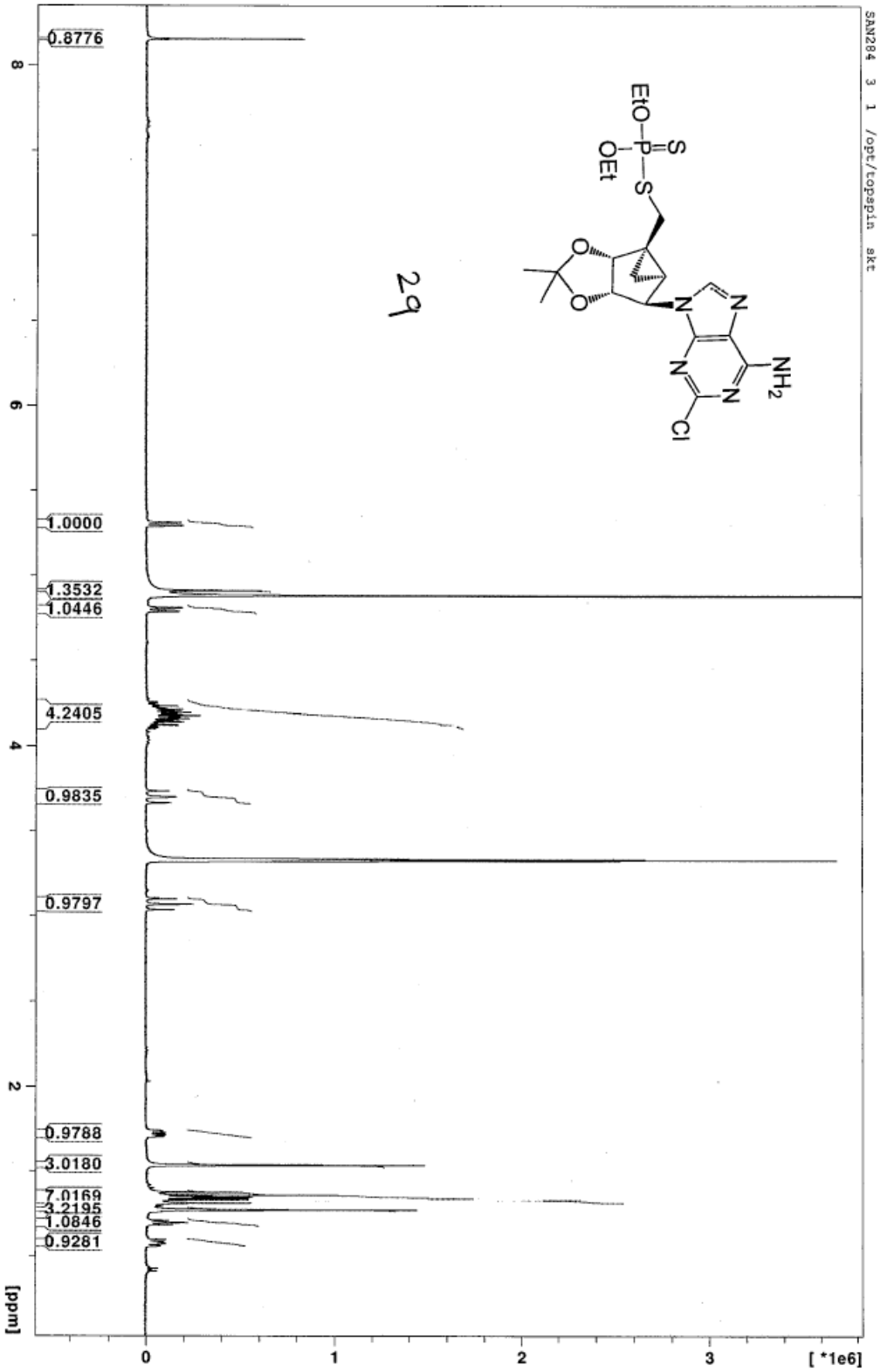
Compound **23**, ^{1}H NMR



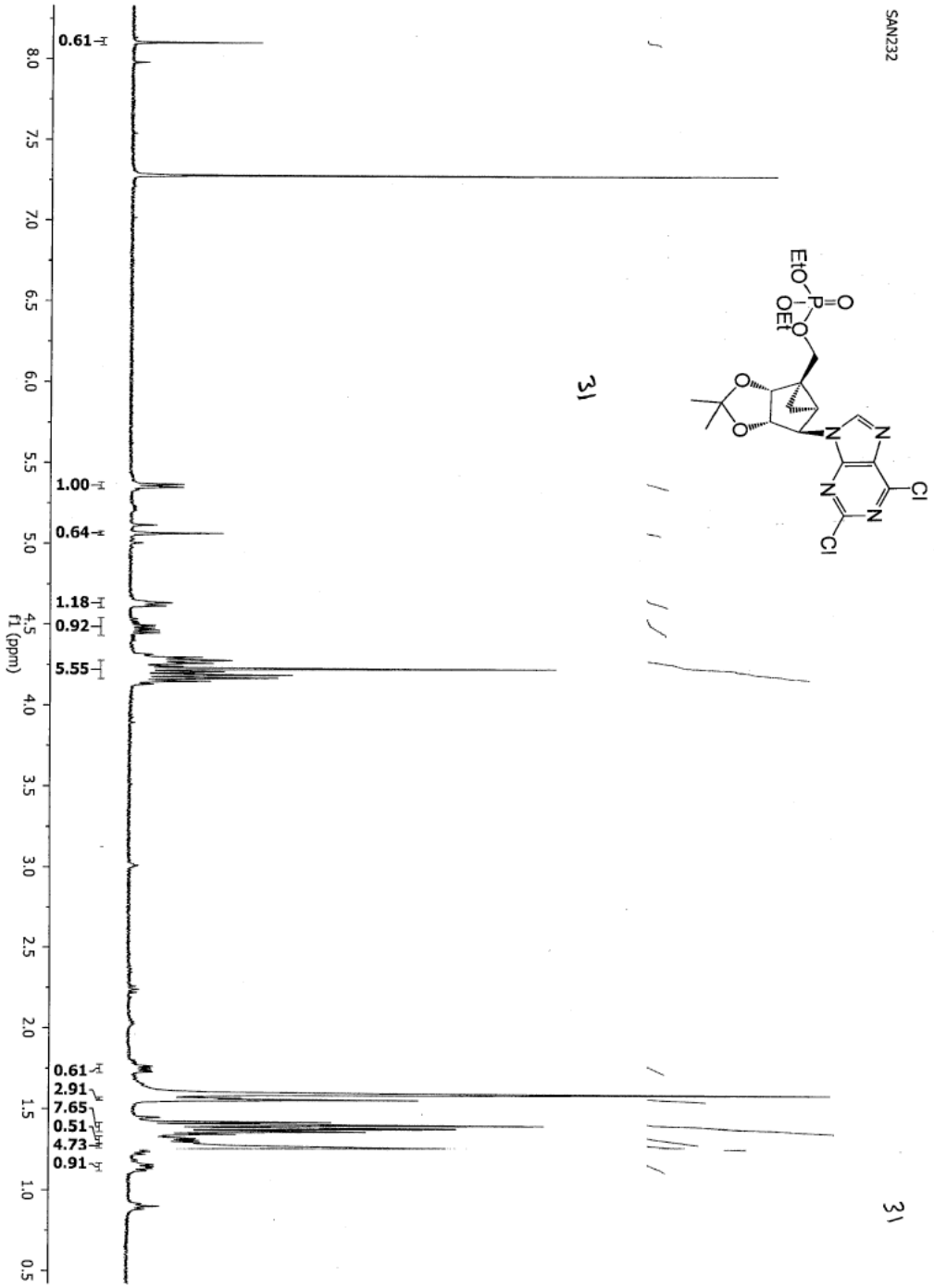
Compound **26**, ^{1}H NMR



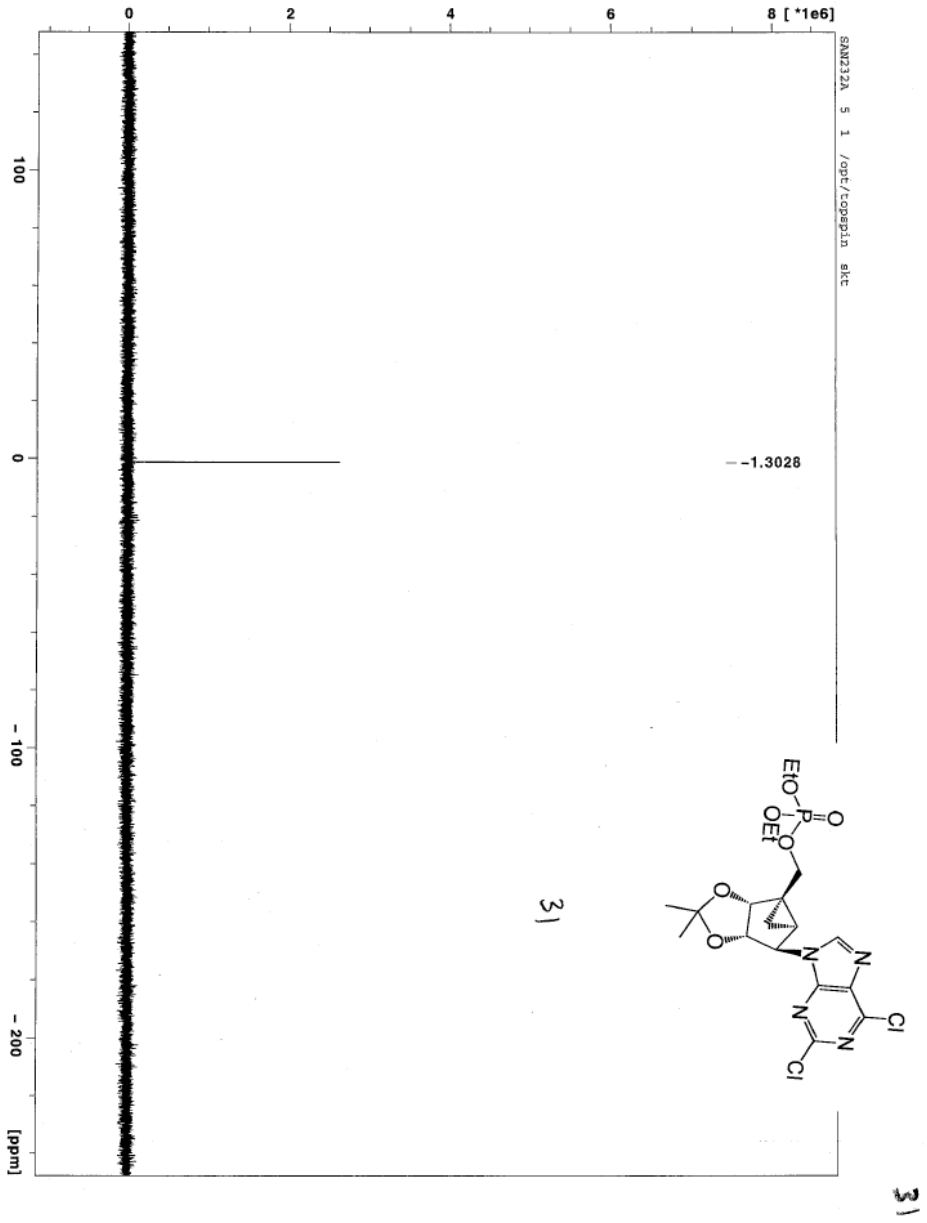
Compound **29**, ^{1}H NMR



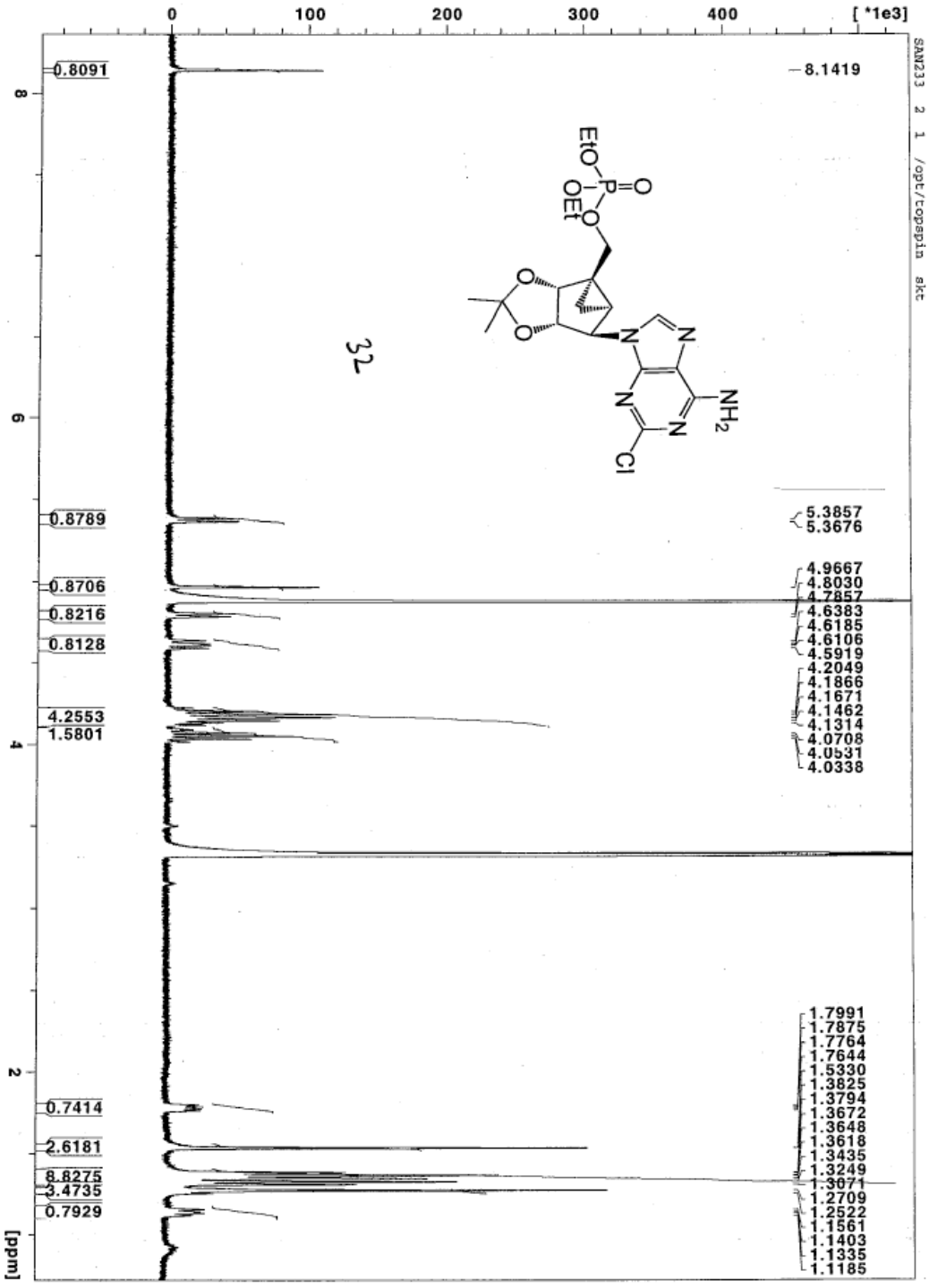
Compound **31**, ^{1}H NMR



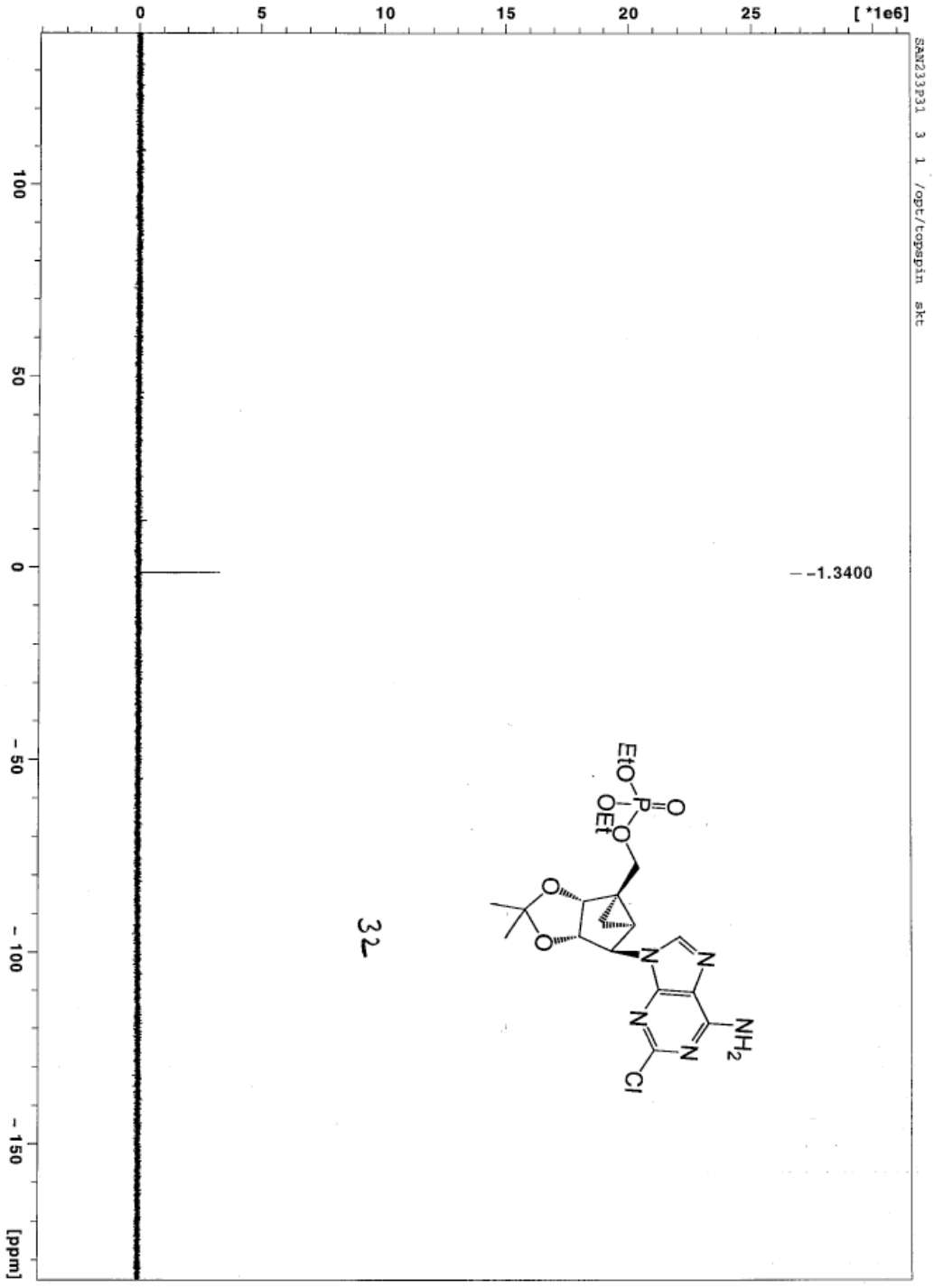
Compound **31**, ^{31}P NMR



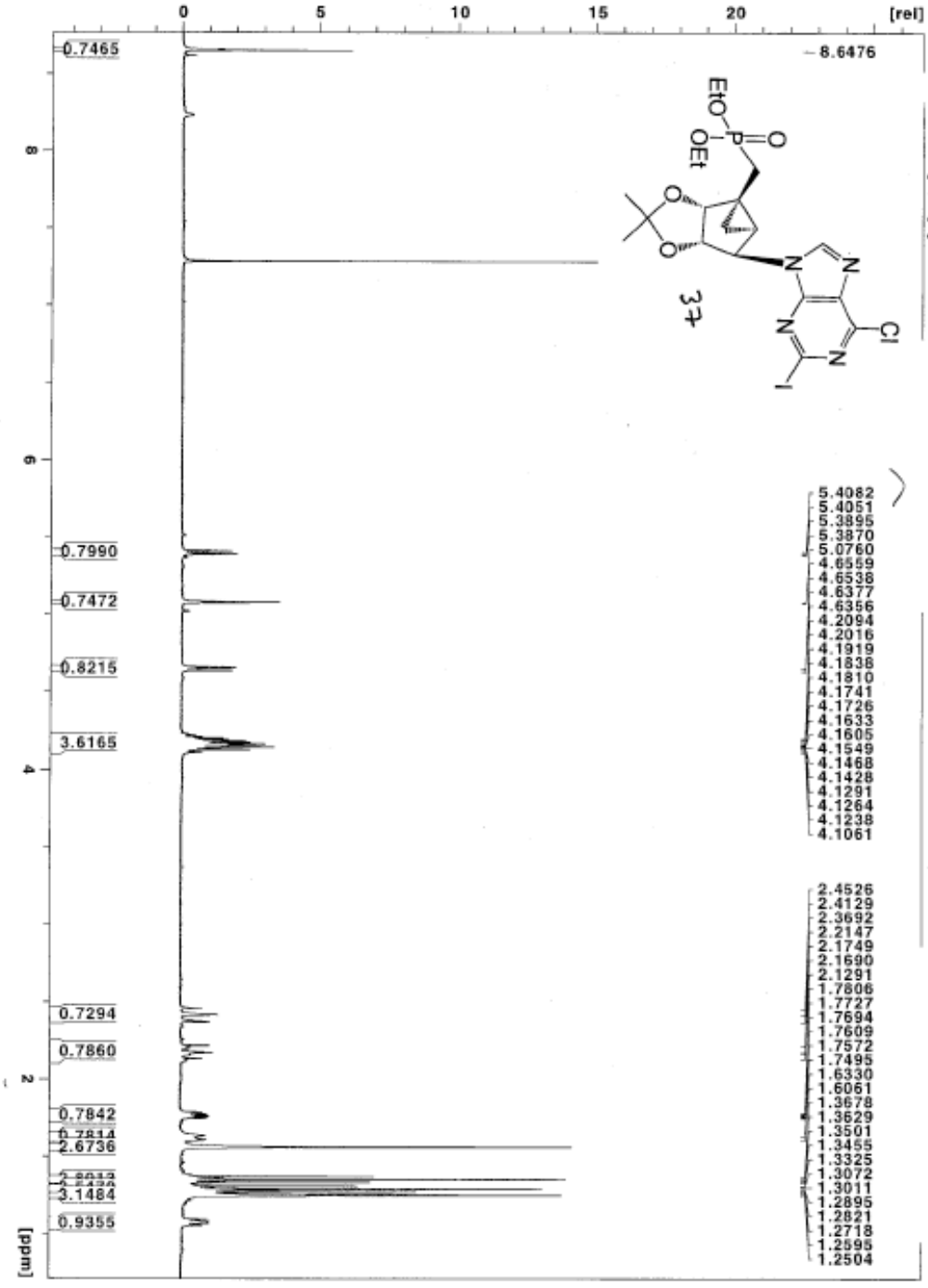
Compound **32**, ^{1}H NMR



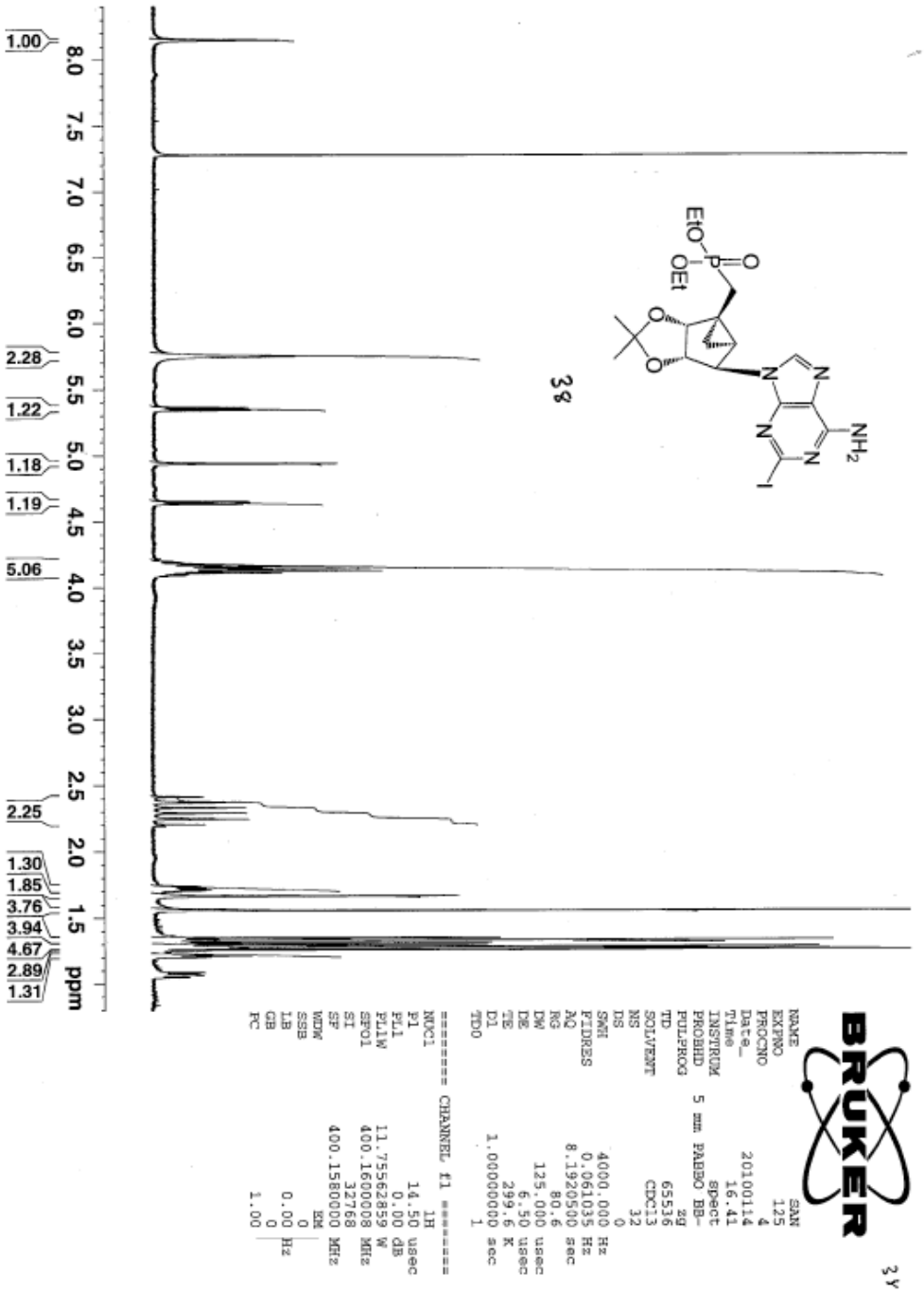
Compound **32**, ^{31}P NMR



Compound **37**, ^{1}H NMR



Compound **38**, ^{1}H NMR



In vivo assay procedures (CSQ mouse model)

Although the principal screen used to evaluate the compounds was the LAD ligation model (see main text), selected phosphonate ester prodrugs were compared for cardioprotective effects in calsequestrin-overexpressing mice (genetic model of heart failure). Administered chronically via a mini-osmotic pump (Alzet), some analogues produced significantly increased intact heart contractile function compared to vehicle-infused mice.

CSQ-overexpressing mice as a model of systolic heart failure and compound administration.

Mice displaying the CSQ model of severe cardiomyopathy and heart failure were bred and maintained according to a previously described method.[5] The CSQ transgenic (TG) mice were originally provided by Dr. Larry Jones[1,2] and developed hypertrophy followed by a lethal heart failure phenotype with death near the age of 3 months.

Compound **1** and its analogues were dissolved in phosphate-buffered saline, pH=7.4 at 3.3 or 10 μM (200 μL total volume), filtered for sterility for *in vivo* administration at 6 μ L per d for 14 d via a mini-osmotic pump (Alzet) in the CSQ mice. Intact heart function *in vivo* was assessed by echocardiography following infusion of nucleotide or vehicle.

Mouse echocardiography

Transthoracic echocardiography was performed using a linear 30-MHz transducer according to manufacturer's instructions (Vevo 660 High Resolution Imaging System from VisualSonics, Toronto, Canada) similar to previously described methods.[3,4] Two dimensional-targeted M-mode echocardiographic measurements were carried out at mid-papillary muscle level. Mice were anesthetized with 1% isoflurane using a vaporizer as previously described.[5] Left ventricular end-diastolic (LVEDD) and end-systolic (LVESD) diameters, and FS (defined as LVEDD-LVESD/LVEDD) were measured. Parameters were measured digitally on the M-mode tracings and were averaged from more than 3 cardiac cycles.

Data analysis

Unless otherwise indicated, data were provided as mean ± standard error of the mean. For analysis of multiple groups, one-way ANOVA and posttest comparison were used. Student's t-test for paired or unpaired samples was used to evaluate the effects of experimental interventions; $P<0.05$ was taken as statistically significant.

Results

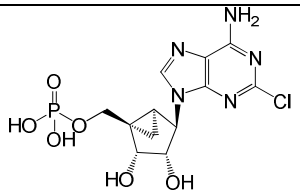
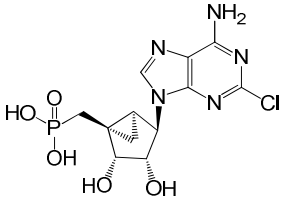
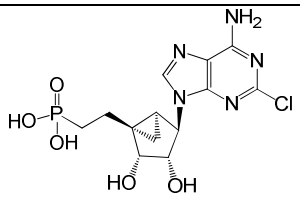
Cardioprotection by the charged nucleotide analogues **1** – **3** in the CSQ mouse models was reported earlier.[5] However, some of the other protected ester and free phosph(on)ate derivatives lost protective activity.

A two-week infusion of the 2-iodo phosphonate derivative 6 (n=5 mice) did not improve FS or prevent LV wall thinning in CSQ-overexpressing mice with heart failure (data not shown). Thus, 2-Cl substitution of the adenine moiety as in phosphonate **2** was essential for activity; substitution with iodo in **6** abolished protection. Several new phosphate and phosphonate analogues, such as thio derivatives, were compared. A 2-week infusion of thiophosphate **5** (n=5), containing a 5'-thioester, could protect the CSQ

mice with a better preservation of LV septal (0.492 ± 0.012 mm) and posterior (0.493 ± 0.016 mm) wall thickness as compared those obtained in NS-infused (both septal and posterior: 0.450 ± 0.007 mm) CSQ mice ($P<0.05$, data not shown). Thus, substitution of oxygen with sulfur was tolerated.

The activity of masked (uncharged) nucleotide analogues was explored using the same experimental model. Only some of the ester prodrug derivatives of the previously characterized cardioprotective agents **1**–**3** were shown to act *in vivo*. These findings implied that an *in vivo* cleavage step to liberate the charged nucleotide active drug was necessary. Among the prodrug derivatives, diisopropyl ester **16** of phosphonate (1'S,2'R,3'S,4'R,5'S)-4'-(6-amino-2-chloropurin-9-yl)-2',3'-(dihydroxy)-1'-(phosphonoethylene)-bicyclo[3.1.0]hexane **3** was highly efficacious. This phosphonate diester resulted in an improved FS as compared to vehicle (Figure S1, Table S1). In mice infused with **16**, the LV posterior wall thickness and septal thickness during systole and the LV posterior wall thickness during diastole were greater than those in NS-infused CSQ mice (Figure S2).

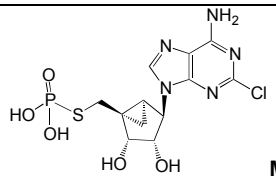
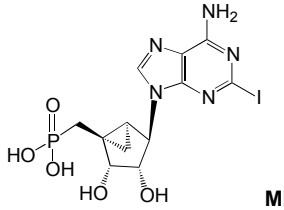
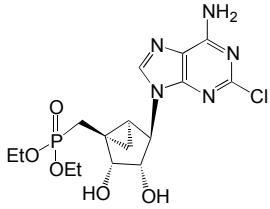
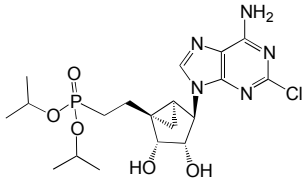
Table S1. Known phosphate and phosphonate analogues: structure and effects on *in vivo* heart function as determined by echocardiography-derived FS in CSQ heart failure mice (data from reference 5).

No	Structure	FS in % in CSQ Mice[a]	n =
1	 MRS 2339	15.47 ± 1.15	10
2	 MRS2775	20.25 ±1.19*	8
3	 MRS2925	19.26 ± 1.23*	16

[a] at 3.3 μM. The vehicle control mice displayed a %FS of 13.78 ± 1.19% (n=16).[2]

*P<0.05 vs. vehicle

Table S2. Structures of novel phosphate and phosphonate analogues synthesized as potential cardioprotective agents. Selected analogues were tested for their effects on *in vivo* heart function as determined by echocardiography-derived FS in CSQ heart failure mice.

No	Structure	FS in % in CSQ Mice[a]	n=
Charged nucleotides			
5	 **MRS2977**	14.33 ± 3.77	4
6	 **MRS2972**	10.33 ± 1.77	5
Masked nucleotide			
14	 **MRS2944**	8.58 ± 2.09	4
16	 **MRS2978**	12.94 ± 0.98*	6

[a] at 10 μM. The vehicle control mice displayed a %FS of 7.13 ± 1.49% (n= 4). *P<0.05 vs. vehicle. The CSQ mice used here were phenotypically more severe than the mice used for data in Table 1.[5]

Fig. S1. Chronic infusion of **16** (MRS2978) caused an increased LV contractile fractional shortening in calsequestrin (CSQ)-overexpressing model of heart failure. Two-dimensional directed M-mode echocardiography was carried out as described.[x] Following 14 d of infusion of **16** (n=6 mice) or normal saline (NS, n=4) in CSQ mice, LV fractional shortening (FS) was compared between the two groups. Treatment with **16** resulted in improved *in vivo* LV contractile function in animals with heart failure. Data are mean and standard error.

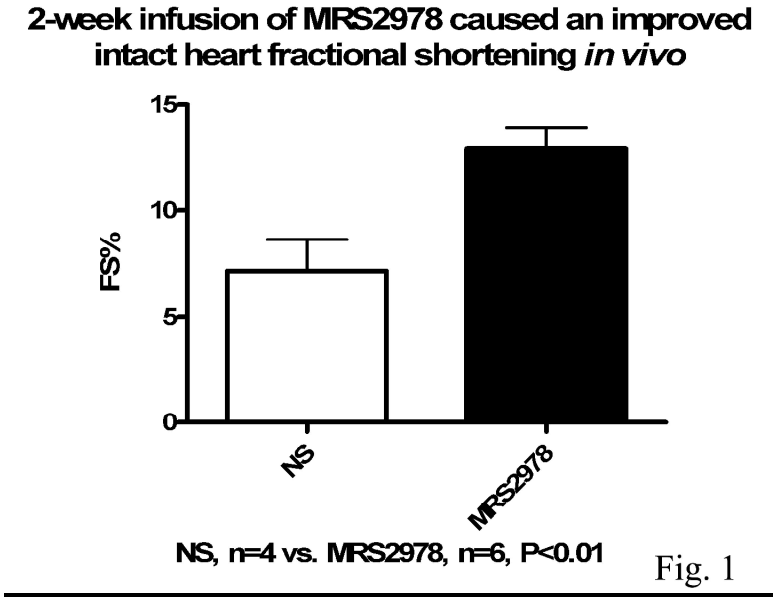


Fig. 1

Fig. S2. Chronic infusion of **16** (MRS2978) resulted in preservation of LV wall thickness in CSQ-overexpressing heart failure mice. The *in vivo* cardiac wall thickening was assessed by echocardiography following 14 d of infusion of **16** or NS in CSQ mice. **a:** In **16**-infused animals, the LV posterior wall thickness during systole (LVPW@S) was greater (*P<0.05*) than that in NS-infused CSQ mice (*P<0.01)*. **b:** Similar data were obtained when septal thickness during systole (IVS@S) was compared between **16**-treated and NS-treated CSQ mice, *P*< MRS *0.01*. **c**: LV posterior wall thickness during diastole was also greater in **16**-infused than in NS-infused CSQ mice, P<0.05. Data are expressed as mean ±SEM. The data suggest that treatment with **16** was able to prevent LV wall thinning in heart failure.

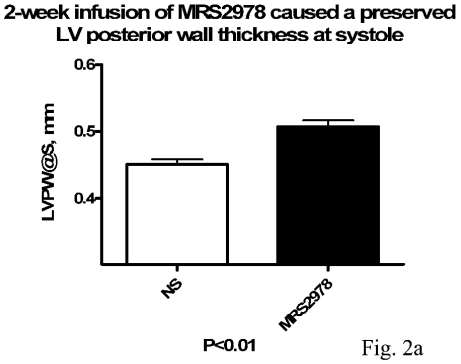


Fig. 2a

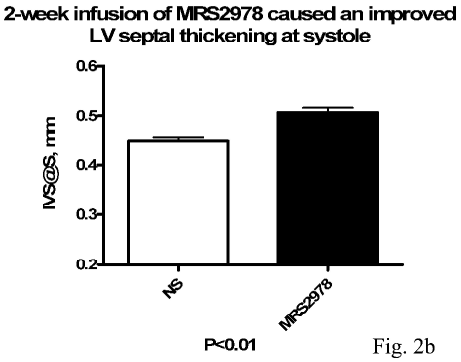


Fig. 2b

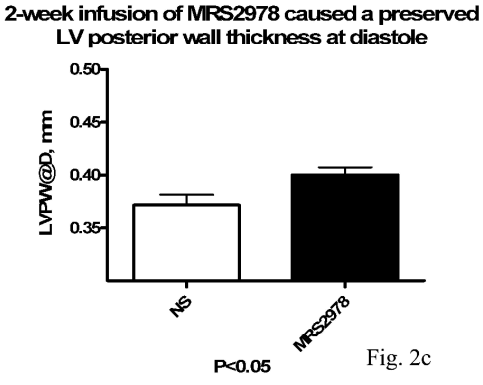


Fig. 2c

Stability studies

Phosphonate diester **16** (MRS2978) was tested for stability when incubated with aqueous medium at pH 1 and pH 11 at 50˚C or in the presence of rat plasma,[6] or mouse or rat liver microsomes.[7,8] Analysis was by HPLC, using an internal standard 1,3-dimethyl-7-(2,3-dihydroxypropyl)xanthine. Phosphonate diester **14** (MRS2944) was tested for stability when incubated in the presence of rat plasma.

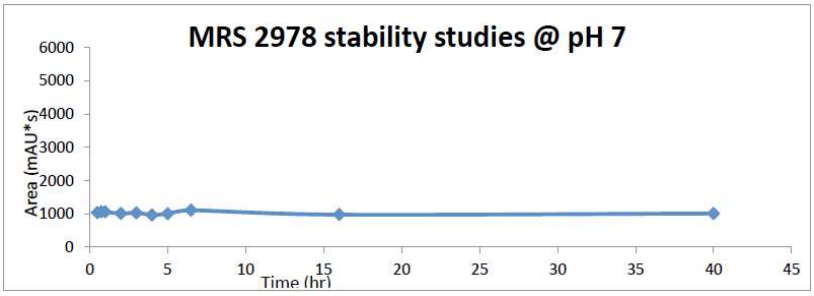
Procedures:

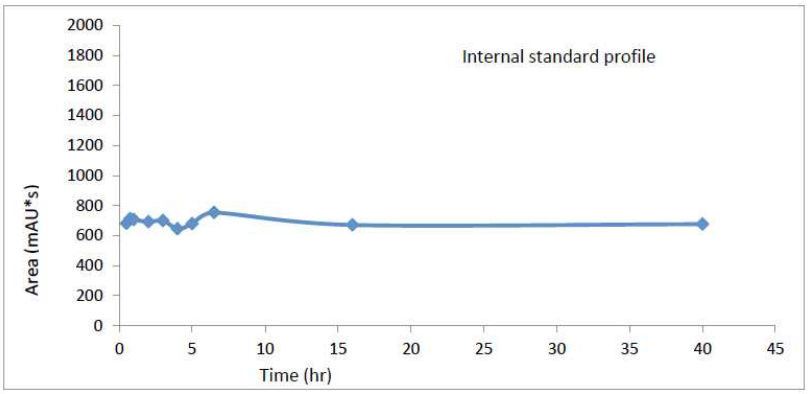
16 (2.8 mg) and 7-(2,3-dihydroxypropyl)theophylline (2.1 mg, Aldrich), an internal standard, were dissolved in DMSO (500 µL). This solution was filtered through a centrifugal 0.45 µm filter (Millex-HN), and the filtrate became the "stock solution". Lyophilized rat plasma (1.0 mL, Sigma) was reconstituted with Milli-Q water (1.0 mL). This plasma solution was incubated at 37 °C for 15 min. The plasma solution was diluted with 0.01 M phosphate buffered saline (0.250 mL), pH 7.0, to afford an 80% plasma solution. The solution was incubated at 37 °C for an additional 5 min. An aliquot (100 µL) of the stock solution was added to the rat plasma (1.0 mL, 80% plasma). The final concentration of compound **16** in the plasma solution was 1.09 mM. Aliquots (10 µL) of this solution were then collected at various time intervals and diluted with acetonitrile (90 µL) to precipitate any protein. The aliquots were thoroughly mixed and then centrifuged at 10,000 rpm for 5 min to precipitate any protein present as a pellet. After centrifugation, a supernatant aliquot (10 µL) was removed from sample and analyzed using Hewlett–Packard 1100 HPLC equipped with a Zorbax SB-Aq 5 µm analytical column (50 x 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). The mobile phase was a linear gradient solvent system: 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)-CH_3CN from 80:20 to 40:60 in 13 min; the flow rate was 0.5 mL/min. Peaks were detected by UV absorption with a diode array detector at 254 nm.

Hydrolytic stability of compound **16** was tested in the presence of pooled mouse liver microsomes (4.6 µg/µL protein). **16** (2.8 mg) and 7-(2,3-dihydroxypropyl)theophylline (2.1 mg), an internal standard, were dissolved in DMSO (500 µL). This solution was filtered through a 0.45 µm filter (Millex-HN). This solution became the "stock solution". Pooled mouse liver microsome (4.6 µg/µL protein) was diluted with 0.01 M phosphate buffered saline, pre-warmed at $37^{O}C$, to obtain 0.5 µg/µL protein concentrations. 50 µL of 10 mM magnesium chloride, 41.6 µL of stock solution of compound **16**, 13 µL of 0.5 µg/µL mouse microsomes and 345.4 µL of phosphate buffered saline were pre-warmed at $37^{O}C$. 50 µL of 10 mM NADPH solution made in phosphate-buffered saline was added to start the reaction. For the control, NADPH was replaced by 50 µL of phosphate buffer. Aliquots (10 µL) of this solution were then collected at various time intervals and diluted with cold acetonitrile (300 µL) and vigorously shaken to precipitate any protein. The aliquots were thoroughly mixed and then centrifuged at 9000 rpm for 15 min to sediment the precipitated protein. After centrifugation, the supernatant (10 µL) of these aliquots was analyzed using Hewlett–Packard 1100 HPLC equipped with Zorbax SB-Aq 5 µm analytical column (50 x 4.6 mm; Agilent Technologies. Inc., Palo Alto, CA). The mobile phase was a linear gradient solvent system: 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)-CH_3CN from 80:20 to 40:60 in 13 min; the flow rate was 0.5 mL/min. Peaks were detected by UV absorption with a diode array detector at 254 nm.

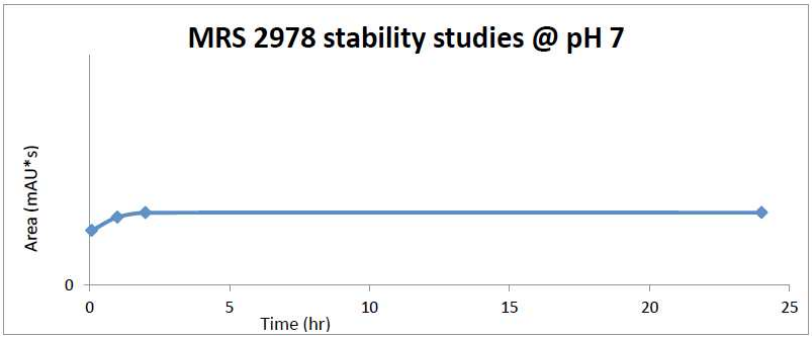
Hydrolytic stability of **16** was tested in the presence of pooled rat liver microsomes (4.6 µg/µL protein, Sigma). **16** (2.8 mg) and 7-(2,3-dihydroxypropyl)theophylline (2.1 mg), an internal standard, were dissolved in DMSO (500 µL). This solution was filtered through a 0.45 µm filter (Millex-HN). This solution became the "stock solution". Pooled mouse liver microsome (20 µg/µL protein) was diluted with 0.01 M phosphate buffered saline, pre-warmed at 37^{O}C, to obtain 1 µg/µL protein concentrations. 50 µL of 10 mM magnesium chloride, 41.6 µL of stock solution of compound **16**, 20 µL of 1 µg/µL rat microsomes and 345.4 µL of phosphate buffered saline were pre-warmed at 37^{O}C. 50 µL of 10 mM NADPH solution made in Phosphate buffered saline was added to start the reaction. For the control NADPH was replaced by 50 µL of phosphate buffer. Aliquots (10 µL) of this solution were then collected at various time intervals and diluted with cold acetonitrile (300 µL) and vigorously shaken to precipitate any protein. The aliquots were thoroughly mixed and then centrifuged at 9000 rpm for 15 min to sediment the precipitated protein. After centrifugation, a supernatant aliquot (5 µL) of each sample was analyzed using a Hewlett–Packard 1100 HPLC equipped with Zorbax SB-Aq 5 µm analytical column (50 x 4.6 mm; Agilent Technologies, Inc., Palo Alto, CA). The mobile phase was a linear gradient solvent system: 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)-CH_3CN from 80:20 to 40:60 in 13 min; the flow rate was 0.5 mL/min. Peaks were detected by UV absorption with a diode array detector at 254 nm.

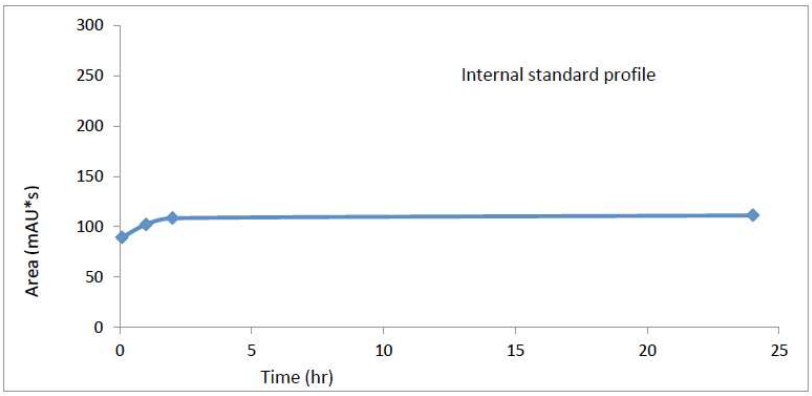
In rat plasma (compd. 16):



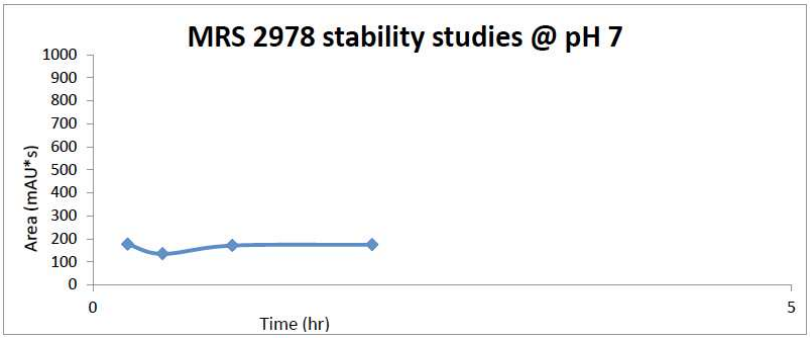


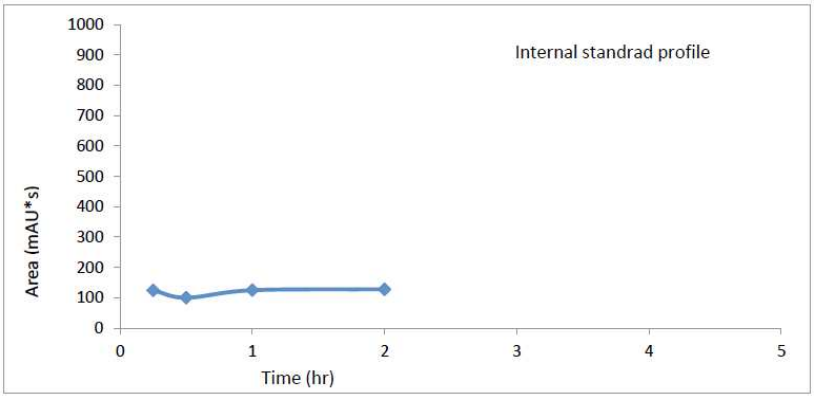
In mouse liver microsomes (compd. 16):



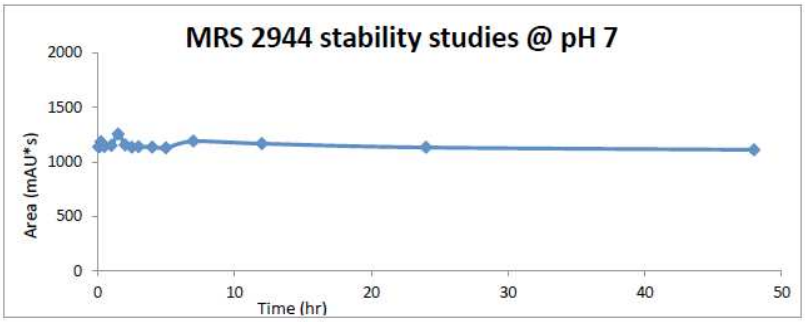


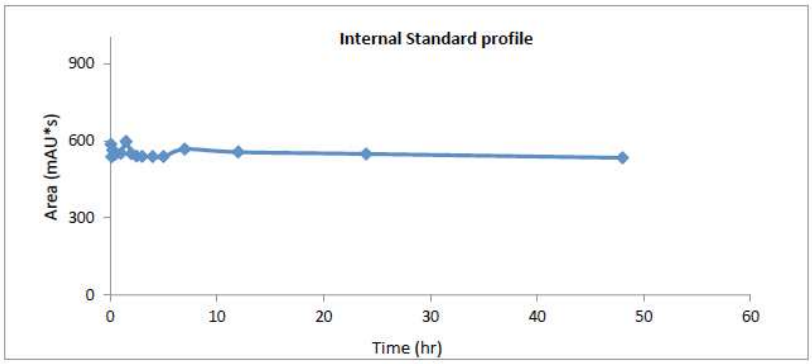
In rat liver microsomes (compd. 16):





Compound 14 in rat plasma:





References:

1. Jones, L.R.; Suzuki, Y.J.; Wang, W.; Kobayashi, Y.M.; Ramesh, V.; Franzini-Armstrong, C.; Cleemann, L.; Morad, M. Regulation of Ca^{2+} signaling in transgenic mouse cardiac myocytes overexpressing calsequestrin. *J. Clin. Invest*. **1998***, 101*, 1385-1393.
2. Knollman, B.C.; Knollmann-Ritschel, B.E.C.; Weismann, N.J.; Jones, L.R.; Morad, M. Remodeling of ionic currents in hypertrophied and failing hearts of transgenic mice overexpressing calsequestrin. *J. Physiol*. **2000,** *525*, 483-498.
3. Hu, B.; Mei, Q.; Smith, E.; Barry, W.H.; Liang, B.T. A novel cardiac inotropic phenotype with cardiac transgenic expression of human $P2X_4$ Receptor transgenic mouse. *FASEB J*. **2001**, *15*, 2739-2741.
4. Curcio, A.; Noma, T.; Prasad, S.V.N.; Wolf, M.J.; Lemaire, A.; Perrino, C.; Mao, L.; Rockman, H.A. Competitive displacement of phosphoinoside 3-kinase from □-adrenergic receptor kinase-1 improves postinfarction adverse myocardial remodeling. *Am. J. Physiol. Heart Circ. Physiol*. **2006**, *291*, H1754-H1760.
5. Kumar, T.S.; Zhou, S.Y.; Joshi, B.V.; Balasubramanian, R.; Yang, T.; Liang, B.T.; Jacobson, K.A. Structure activity relationship of (N)-methanocarba phosphonate analogues of 5'-AMP as cardioprotective agents acting through a cardiac P2X receptor. *J. Med. Chem.* **2010**, *53*, 2562-2576.
6. Silvestri, M. A.; Nagarajan, M.; De Clercq, E.; Pannecouque, C.; Cushman, M. Design, Synthesis, Anti-HIV Activities, and Metabolic Stabilities of Alkenyldiarylmethane (ADAM) Non-nucleoside Reverse Transcriptase Inhibitors. *J. Med. Chem.* **2004**, *47*, 3149-3162.
7. Sun, H. Capture hydrolysis signals in the microsomal stability assay: Molecular mechanisms of the alkyl ester drug and prodrug metabolism. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 989-995.
8. Singh, J. K.; Solanki, A.; Shirsath, V. S. Comparative *in-vitro* Intrinsic clearance of imipramine in multiple species liver microsomes: Human, Rat, Mouse and Dog. *J Drug Metab Toxicol*. **2012**, *3*, 4.