Supporting Information

A Scalable Synthesis of the IP_7 Isomer, 5-PP-Ins(1,2,3,4,6)P_5

Honglu Zhang[#], James Thompson[@], Glenn D. Prestwich[#,*]

[#]Department of Medicinal Chemistry, The University of Utah, 419 Wakara Way, Suite 205, Salt Lake City, Utah 84108-1257, USA.

[@]Echelon Biosciences, 675 Arapeen Dr., Suite 302, Salt Lake City, UT 84108.

Phone: +1-801-585-9051. Fax: +1-801-585-9053. Email: gprestwich@pharm.utah.edu

Experimental details for chemical synthesis

General. Chemicals were purchased from Aldrich and Acros Chemical Corporation and used without prior purification. Solvents were reagent-grade and distilled before use: CH_2Cl_2 was distilled from CaH_2 and THF was distilled from sodium wire. TLC was performed with precoated silica gel glass sheets (EM SCIENCE silica gel $60F_{254}$). Flash chromatography (FC) employed Whatman 230~400 mesh ASTM silica gel. NMR spectra were recorded on a Varian INOVA 400 at 400 MHz (^{1}H), 101 MHz (^{13}C), 162 MHz (^{31}P) and 376 MHz (^{19}F) at 25 ºC. Chemical shifts are reported in ppm with TMS as internal standard (δ = 0.00); ^{31}P, 85% H_3PO_4 (δ = 0.00); ^{19}F, $CFCl_3$ (δ = 0.00). Low- and high-resolution mass spectra were obtained on HP5971A MSD and Finnigan MAT95 double focusing mass spectrometer (MS) instruments, respectively.

2-*O*-Benzoyl-1,6:3,4-di-*O*-isopropylidene-*myo*-inositol (5) was prepared according to the published method.[1]

2-*O*-Benzoyl-5-*O*-(4-methoxybenzyl)-1,6:3,4-di-*O*-isopropylidene-*myo*-inositol (6). Intermediate **5** (700 mg, 1.9 mmol) was dissolved in acetonitrile (7 mL) and treated at 0 ºC with BEMP (0.9 mL, 3.4 mmol) and 4-methoxybenzyl bromide (0.6 mL, 4.2 mmol). The mixture was allowed to warm to rt and stirred for overnight before quenching with saturated $NaHCO_3$ solution. The result mixture was diluted with 50 mL EtOAc and washed with 1 *N* HCl solution. After drying over Na_2SO_4, the organic phase was concentrated, and the residue was chromatographed on silica gel (hexanes/EtOAc, 6:1) to afford full protected inositol **6** (786 mg,

84%) as a white solid. ^{1}H NMR(400 MHz, $CDCl_3$) δ 8.05 (d, *J* = 7.2 Hz, 2H), 7.59 (t, *J* = 7.2 Hz, 1H), 7.46 (t, *J* = 8.0 Hz, 2H), 7.40 (d, *J* = 8.8 Hz, 2H), 6.90 (d, *J* = 8.8 Hz, 2H), 6.03 (t, *J* = 2.4 Hz, 1H), 4.83 (s, 2H), 4.18 (t, *J* = 9.2 Hz, 2H), 3.90 (t, *J* = 9.2 Hz, 1H), 3.81 (s, 3H), 3.79 (m, 2H), 1.47 (s, 6H), 1.37 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.5, 159.5, 133.5, 130.3, 130.1, 129.99, 129.9, 129.8, 128.7, 114.0, 113.0, 78.7, 77.1, 75.5, 72.2, 64.7, 55.5, 27.1, 26.6; MALDI-HRMS $[M + Na]^+$ calcd for $C_{27}H_{32}O_8Na$ 507.1989, found 507.1975.

5-*O*-(4-Methoxybenzyl)-1,6:3,4-di-*O*-isopropylidene-*myo*-inositol (7). To a solution of benzoate **6** (280.0 mg, 0.57 mmol) in a mixture of dry methanol and THF (8:1) (9 mL), NaOMe in MeOH (0.3 mL, 25% in methanol, 1.2 mmol) was added at rt. The solution was stirred for 2 h at rt, whereupon it was quenched with saturated NH_4Cl solution (2 mL), and then filtered through a short silica gel column, which was then washed with EtOAc. The organic solvent was removed in vacuo and the residue was chromatographed on silica gel (hexanes/EtOAc, 4:1) to give acetonide **7** (180 mg, 82%). $[\alpha]^D_{20}$ = - 0.9 (*c* 1.10, $CHCl_3$); ^{1}H NMR(400 MHz, $CDCl_3$) δ 7.33 (d, *J* = 8.4 Hz, 2H), 6.86 (d, *J* = 8.4 Hz, 2H), 4.76 (s, 2H), 4.51 (s, 1H), 4.09 (t, *J* = 9.2 Hz, 2H), 3.77 (s, 3H), 3.75 (t, *J* = 8.8 Hz, 1H), 3.53 (dd, *J* = 9.2, 2.4 Hz, 2H), 2.90 (br, -OH, 1H), 1.46 (s, 6H), 1.44 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.3, 130.6, 130.1, 129.9, 129.8, 129.6, 114.0, 113.9, 112.6, 78.7, 77.6, 75.6, 71.7, 63.5, 55.5, 27.2, 26.8; MALDI-HRMS $[M + Na]^+$ calcd for $C_{20}H_{28}O_7Na$ 403.1727, found 403.1718.

5-*O*-(4-Methoxybenzyl)- *myo*-inositol (8). Acetonide **7** (120 mg, 0.32 mmol) was suspended in 2.5 mL of acetone in a small vial, and 63 μL of water was added, followed by 13 mg *p*-toluenesulfonic acid monohydrate. The mixture was shaken for 24 h, and a white precipitate

formed. The white precipitate was washed three times with EtOAc, and the solid pentaol 5-PMB inositol **8** (75 mg, 79%) was obtained as white solid and used without further purification. ^{1}H NMR(400 MHz, DMSO-d^{6}) δ 7.31 (d, *J* = 8.8 Hz, 2H), 6.84 (d, *J* = 8.4 Hz, 2H), 4.66 (s, 2H), 4.45 (br, 5H), 3.71 (s, 3H), 3.68 (t, *J* = 2.4 Hz, 1H), 3.49 (t, *J* = 9.2Hz, 2H), 3.14 (dd, *J* = 9.2, 2.8 Hz, 2H), 2.94 (t, *J* = 9.2 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d^{6}) δ 159.0, 132.5, 129.8, 113.9, 84.5, 73.9, 73.2, 73.1, 72.7, 55.7; MALDI-HRMS [M + Na]$^{+}$ calcd for $C_{14}H_{20}O_{7}Na$ 323.1101, found 323.1104.

5-*O*-(4-Methoxybenzyl)-1,2,3,4,6-enta-*O*-(*o*-xylylene)phosphate-*D*-*myo*-inositol (9). To a solution of 5-PMB inositol **8** (100 mg, 0.36 mmol) in dry CH_2Cl_2 (15 mL) at 0 ºC was added *O*-xylylene *N*,*N*-diethylphosphoramidite (0.8 g, 3.6 mmol) and 1*H*-tetrazole (0.4 g, 5.71 mmol). The mixture was stirred at 0 ºC for 1 h, and then warmed to rt and stirred for an additional 4 h. The solution was cooled to –78 ºC , and the phosphites oxided with *m*-CPBA (1.77 g, 7.2 mmol) at –78 ºC for 30 min, followed by warming to rt for 40 min. The solution was diluted with CH_2Cl_2 (50 mL) and washed with 10% sodium bisulfite (10 mL × 2). The organic layer was concentrated and the residue purified by flash chromatography (EtOAc/MeOH, 10:1) to give protected pentakisphosphate **9** (400 mg, 99%) as a white solid. $[\alpha]^{D}_{20}$ = - 0.7 (*c* 0.8, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 (m, 22H), 6.87 (d, *J* = 8.8 Hz), 5.76-4.99 (m, 26H), 4.77 (s, 2H), 3.80 (s, 3H); ^{31}P NMR (162 MHz, $CDCl_3$) δ 1.26 (2P), -2.41 (1P), -2.85 (2P); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.3, 135.9, 135.8, 135.4, 134.8, 130.0, 129.8, 129.7, 129.6, 1229.5, 129.3, 129.2, 128.9, 128.8, 128.5, 113.7, 69.6, 69.3, 68.4, 55.5; MALDI-HRMS [M + Na]$^{+}$ calcd for $C_{54}H_{55}O_{22}P_5Na$ 1233.1765, found 1233.1816.

1,2,3,4,6-Penta-*O*-(*o*-xylylene)phosphate-*myo*-inositol (10). Removal of the PMB group was achieved by exposing the pentakisphosphate ester **9** (70 mg, 0.058 mmol) to trifluoroacetic acid/CH_2Cl_2/H_2O (5:1:1, 4 mL) for 30 min at rt. The reaction was concentrated and the residue washed with hexanes to give alcohol **10** (63 mg, 99%) as a white solid, which was directly used for next step.

5-*O*-(Benzyl-2-cyanoethyl)phosphate-1,2,3,4,6-penta-*O*-(*o*-xylylene)phosphate-*myo*-inositol (11). To a solution of alcohol **10** (70 mg, 0.064 mmol) in dry CH_2Cl_2 (3 mL) at 0 ºC was added benzyl-2-cyanoethyl *N*,*N*-diisopropylphosphoramidite (110 mg, 0.35 mmol) and 1*H*-tetrazole (50 mg, 0.71 mmol). The mixture was stirred at 0 ºC for 2 h, and then warmed to rt and stirred for 7 h. Oxidation was then performed with *m*-CPBA (131 mg, 0.53 mmol) at –78 ºC for 30 min, and then warmed to rt for 30 min. The solution was diluted with CH_2Cl_2 (50 mL) and washed with 10% sodium bisulfite (10 mL × 2). The organic layer was concentrated and the residue purified by flash chromatography (EtOAc/MeOH, 8:1) to give the benzyl cyanoethylphosphate **11** (69 mg, 82%) as a white solid. $[\alpha]^{D}_{20}$ = - 2.0 (*c* 0.5, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46-7.04 (m, 25H), 5.77-5.71 (m, 2H), 5.67-5.60 (m, 3H), 5.52-5.37 (m, 5H), 5.29-5.03 (m, 16H), 4.98-4.87 (m, 2H), 4.28-4.21 (m, 1H), 4.15-4.08 (m, 1H), 2.67-2.53 9m, 2H); ^{31}P NMR (162 MHz, $CDCl_3$) δ -0.67 (1P), -1.86 (1P), -2.04 (1P), -2.23 (1P), -2.55 (1P), -2.63 (1P); ^{13}C NMR (101 MHz, $CDCl_3$) δ 135.8, 135.73, 135.72, 135.6, 135.5, 135.1, 134.9, 134.8, 129.7, 129.6, 129.4, 129.38, 129.3, 129.2, 129.1, 129.98, 128.9, 128.8, 128.5, 117.3, 77.5, 76.5, 73.9, 70.4, 70.3, 69.6, 69.5, 69.0, 68.9, 62.7, 62.6, 19.4, 19.3; MALDI-HRMS $[M + Na]^+$ calcd for $C_{56}H_{57}NO_{24}P_6Na$ 1336.1589, found 1336.1625.

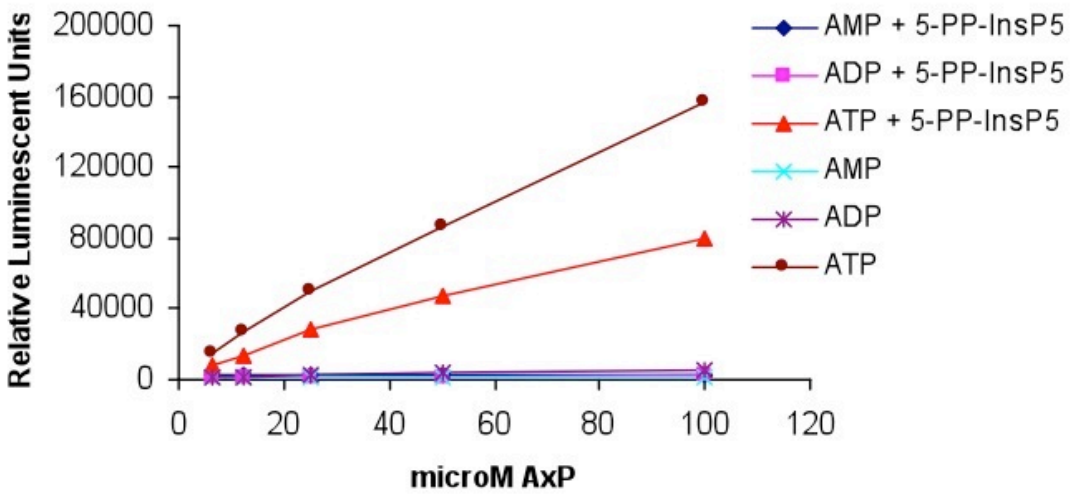
5-*O*-(Benzylhydoxyl)phosphate-1,2,3,4,6-penta-*O*-(orthoxylylene)phosphate-*myo*-inositol (12). To a solution of benzyl cyanoethyl phosphate **11** (50 mg, 0.038 mmol) in CH_3CN (0.5 mL) under Ar was added triethylamine (0.25 mL) followed by the addition of bis(trimethylsilyl)trifluoroacetamide (0.25 mL). After 24 h, the reaction mixture was concentrated and the residue was filtered through a short Dowex-H^+ column afforded benzyl monophosphoric acid **12** (44 mg, 92%).$[\alpha]^D_{20}$ = - 0.6 (*c* 1.2, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ 3:1) δ 7.41-7.08 (m, 25H), 5.67-5.24 (m, 12H), 5.18-4.89 (m, 16H); ^{31}P NMR (162 MHz, $CDCl_3/CD_3OD$ 3:1) δ -1.16 (1P), -1.47 (2P), -2.26 (2P), -2.44 (1P); ^{13}C NMR (101 MHz, $CDCl_3/CD_3OD$ 3:1) δ 136.5, 136.4, 135.6, 135.5, 135.47, 135.4, 134.9, 129.8, 129.63, 129.6, 129.4, 129.3, 129.28, 129.1, 128.6, 128.5, 128.0, 76.0, 73.9, 69.7, 69.6, 69.5; MALDI-HRMS $[M + Na]^+$ calcd for $C_{53}H_{54}O_{24}P_6Na$ 1283.1323, found 1283.1337.

5-*O*-[(Phosphonooxy)hydroxyphosphinyl]-1,2,3,4,6-penta-*O*-phosphono-*myo*-inositol sodium salt (5-PP-InsP_5, 13). To a solution of benzyl monophosphoric acid **12** (50 mg, 0.04 mmol) and triethylamine (13 μL, 0.09 mmol) in anhydrous CH_2Cl_2 (0.5 mL) at 0 ºC was added dibenzylphosphoryl chloride (0.25 mL, 10% in benzene, 0.08 mmol). After 2 h at rt, the volatiles were removed *in vacuo*, and the residue was completely dried below 0.1 mm Hg. The residue was dissolved in t-BuOH/H_2O (6:1, 14 mL) in the presence of PtO_2 (100 mg) and stirred under H_2 (60 psi) for 4 h. The catalyst was removed by filtration and the filtrate was concentrated under *vacuum*. Then the residue was dissolved in 10 mL water and washed with 10 mL each EtOAc and CH_2Cl_2. The aqueous solution was concentrated and purified through ion exchange chromatography (Dowex 50W × 8 – 200 Na^+ exchange resin) by elution with water to afford the 5-PP-Ins(1,2,3,4,6)P_7 sodium form (33 mg, 81%) as white solid. ^{1}H NMR (400 MHz, D_2O) δ

4.80-4.71 (m, 1H), 4.45-4.32 (m, 2H), 4.24 (t, J = 9.6 Hz, 1H), 4.18-4.09 (m, 2H); ^{31}P NMR (162 MHz, D_2O) δ 1.40 (d, 2P), 1.02 (d, 2P), 0.41 (d, 1P), - 9.40 -10.20 (m, 2P); ESI-MS $[M-H]^-$ calcd for $C_6H_{18}O_{27}P_7^-$ (acid form) 738.8, found 738.7.

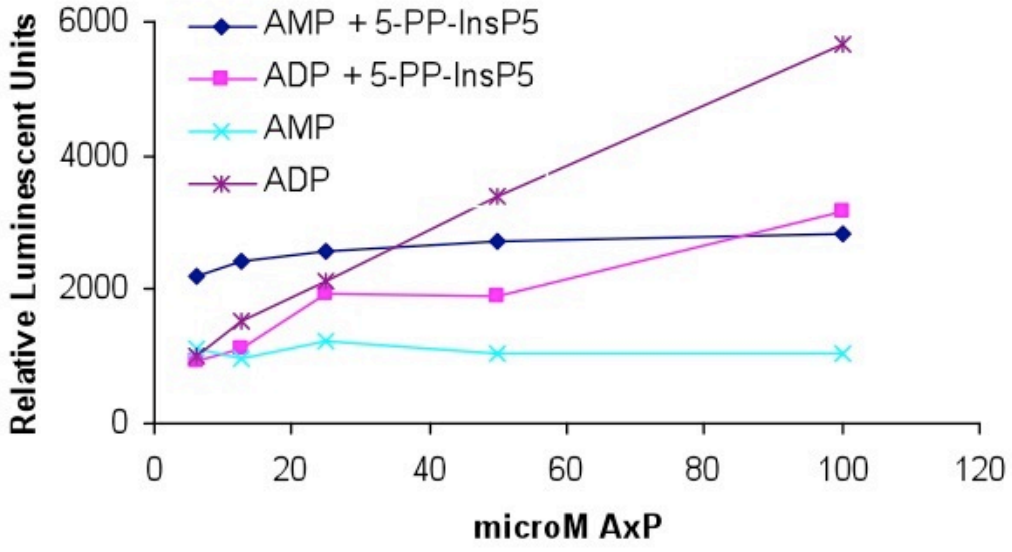
Experimental details for IP6K assay

ATP Synthase Activity of 5-PP-Ins(1,2,3,4,6)P_5 in presence of IP6K1. The ATP synthase activity of the IP6K1 enzyme with synthetic 5-PP-Ins(1,2,3,4,6)P_5 as a substrate was determined using an ATP-dependent luciferase assay. The enzyme IP6K1 (construct obtained from Dr. R. Bhandari in the laboratory of Dr. S. Snyder, Johns Hopkins University) was expressed in *E. coli* and purified to 50% (as measured by SDS-PAGE). A 50 μM solution of ADP, 62.5 μM 5-PP-InsP5 and 10 μg/mL IP6K1 enzyme was prepared in 10 mM Tris-HCl, 30 mM KCl, 6 mM $MgCl_2$ and 0.9% dithiothreitol pH 7.6, and a 50 μM ADP standard was prepared in water. The mixtures were incubated for 30 minutes at 37 ºC with shaking. Twenty μL of an ATP detection buffer containing luciferase (Lonza PKLight Assay Kit) and 10 μL of kinase stop solution (included in the Lonza PK Light Assay Kit) was added to 40 μL of the 5-PP-Ins(1,2,3,4,6)P_5 + ADP + IP6K1 reaction mix or 50 μM ADP. The mixture was allowed to incubate for 10 minutes at room temperature with shaking. Luminescence was determined using Molecular Devices SpectraMax M2 plate reader.



Supporting Figure 1. 5-PP-Ins(1,2,3,4,6)P_5 does not phosphorylate AxP in the absence of IP6K1

ATP Synthase activity of 5-PP-Ins(1,2,3,4,6)P_5 with ADP or AMP only. 5-PP-InsP5 has been known to pyrophosphorylate protein substrates in the absence of kinase catalysis. To confirm synthetic 5-PP-Ins(1,2,3,4,6)P_5 requires the enzyme IP6K1 for ATP synthase activity 100 microM of synthetic5-PP-Ins(1,2,3,4,6)P_5 was added to titrated concentrations of ATP, ADP and AMP. ATP, ADP and AMP was diluted 2X from 100 μM to 6.25 μM in 100 μM synthetic 5-PP-InsP5, 10 mM Tris-HCl, 30 mM KCl, 6 mM $MgCl_2$ pH 7.6. The mixtures were incubated for 30 min at 37 ºC. After incubation, 10 μL of an ATP detection buffer containing luciferase (Promega KinaseGlo Plus) was added to 10 μL of reaction mixture and allowed to incubate for 10 min at rt with shaking. Luminescence was determined using Molecular Devices SpectraMax M2 plate reader.

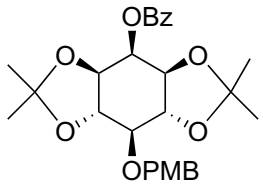


Supporting Figure 2. Magnification of low RLU region of Supporting Figure 1. 5-PP-Ins(1,2,3,4,6)P_5 does not phosphorylate AxP in the absence of IP6K1

(1) Chung, S.-K.; Chang, Y.-T.; Kwon, Y.-U. *J. Carbohydr. Chem.* **1998**, *17*, 369-384.

64

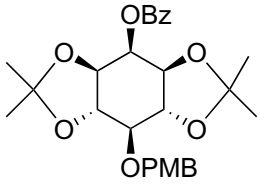
zh31951



6

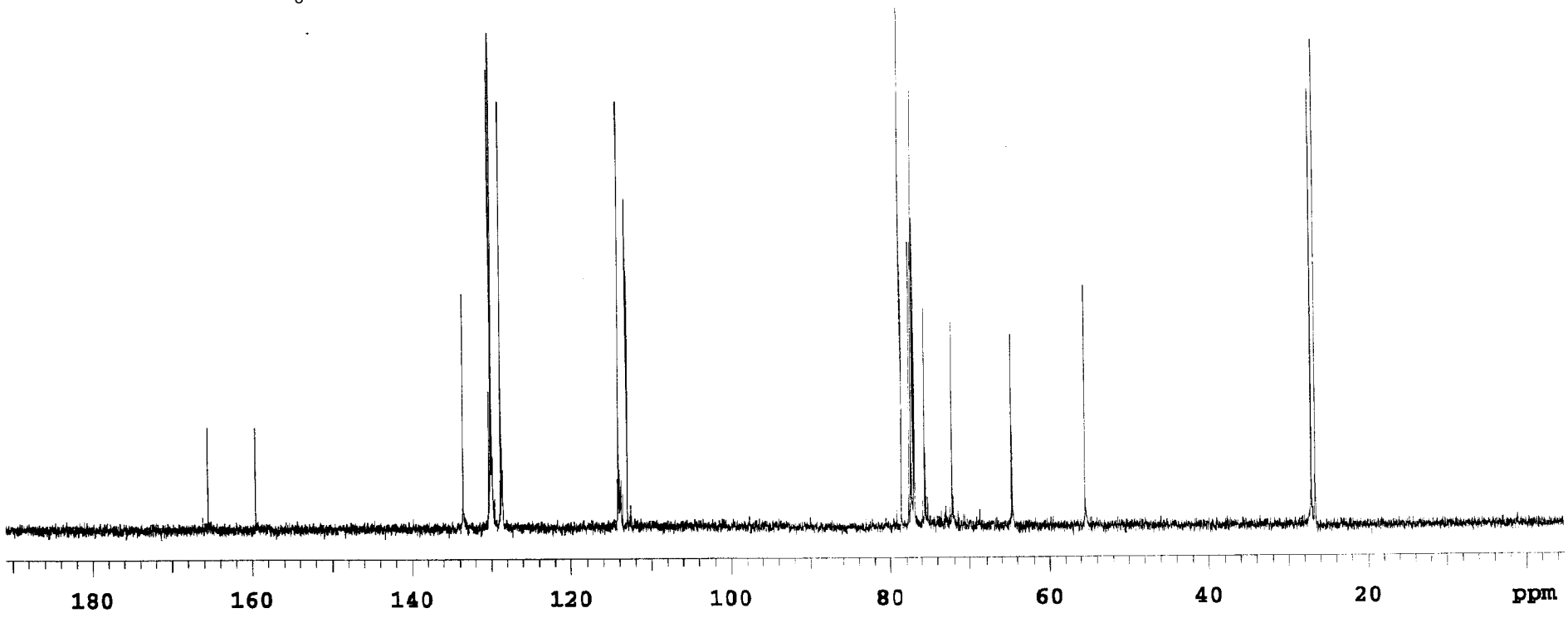
^{1}H NMR $CDCl_3$

6C



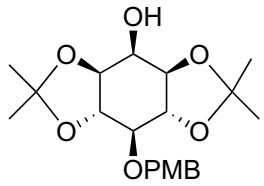
6

^{13}C NMR $CDCl_3$



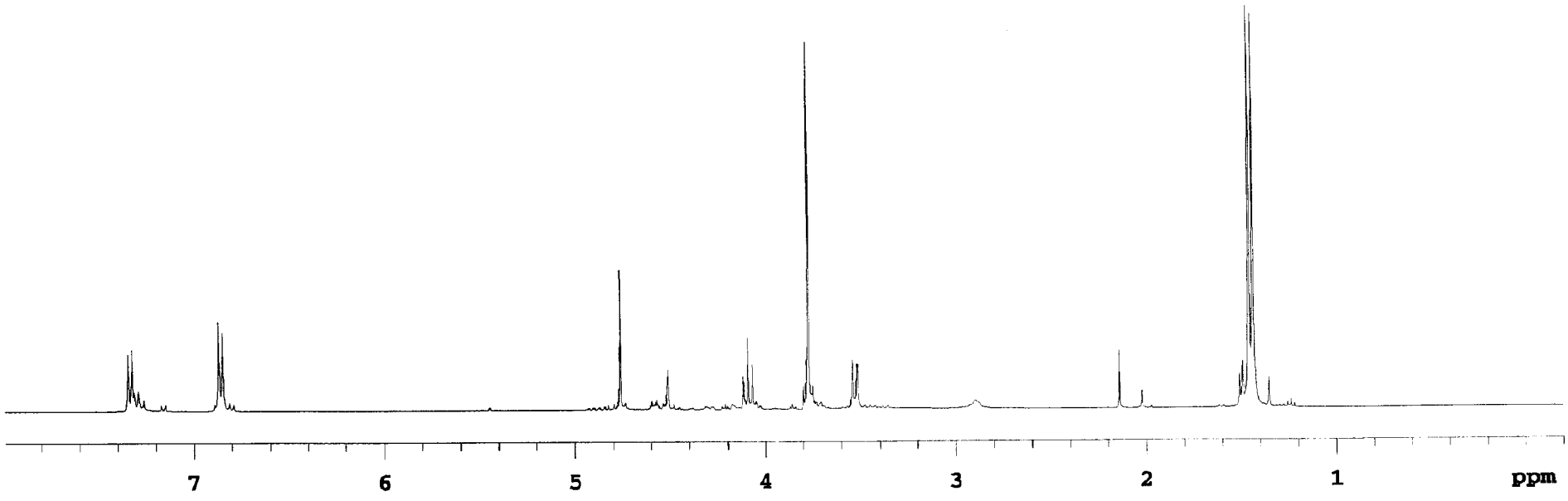
7H

zh31971

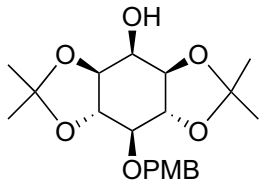


7

^{1}H NMR $CDCl_3$



7c

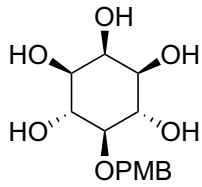


7

^{13}C NMR $CDCl_3$

8H

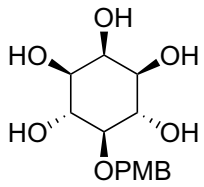
.31991



8

^{1}H NMR DMSO-d6

8c

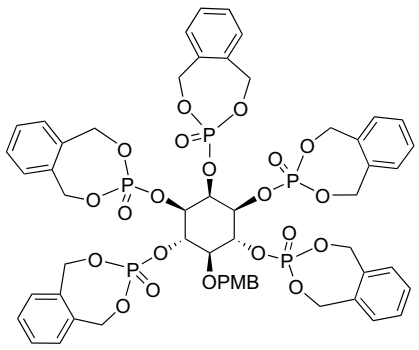


8

^{13}C NMR DMSO-d616

9H

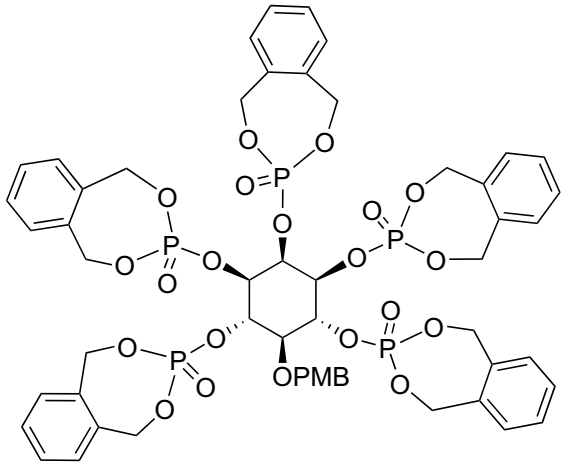
zh33051



9

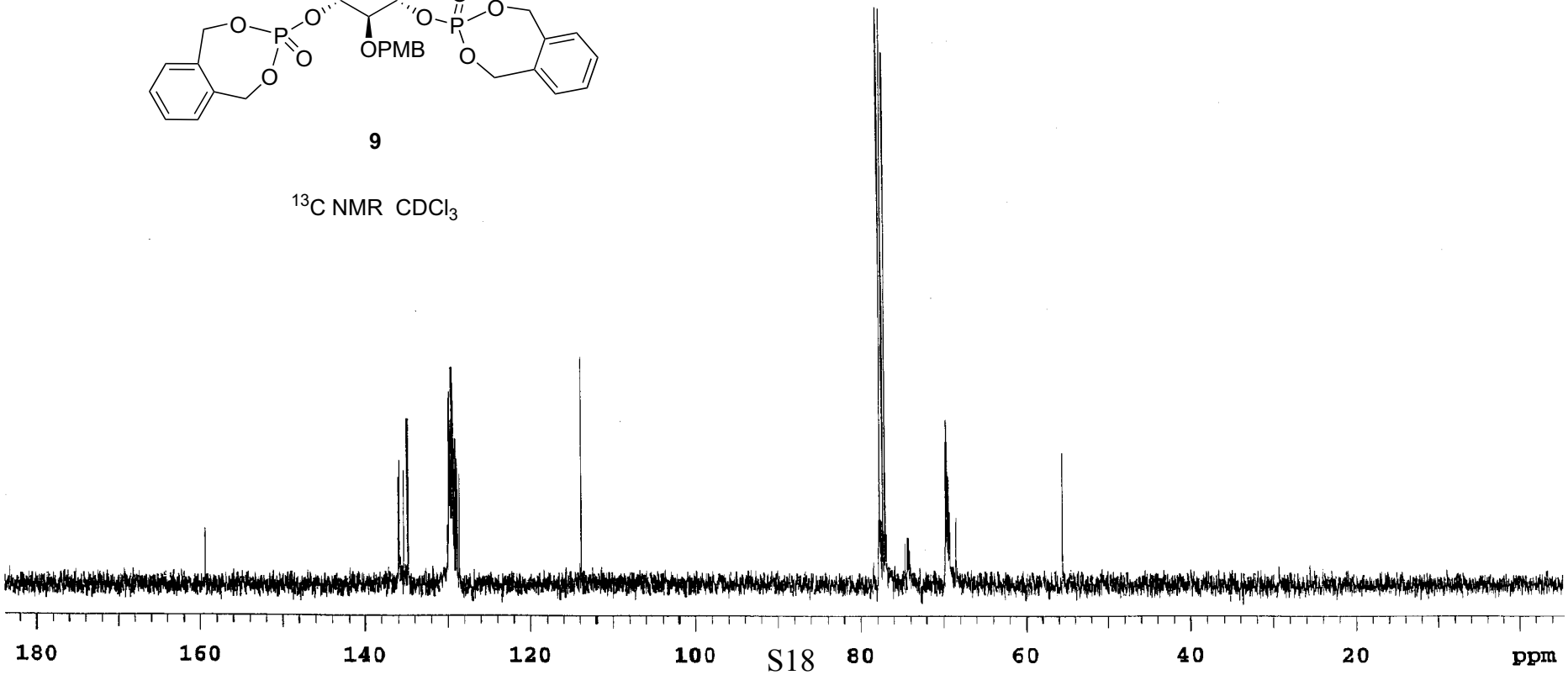
^{1}H NMR $CDCl_3$

9c



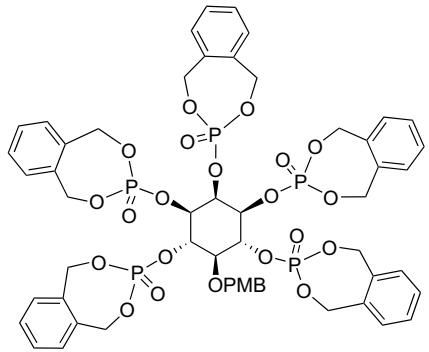
9

^{13}C NMR $CDCl_3$



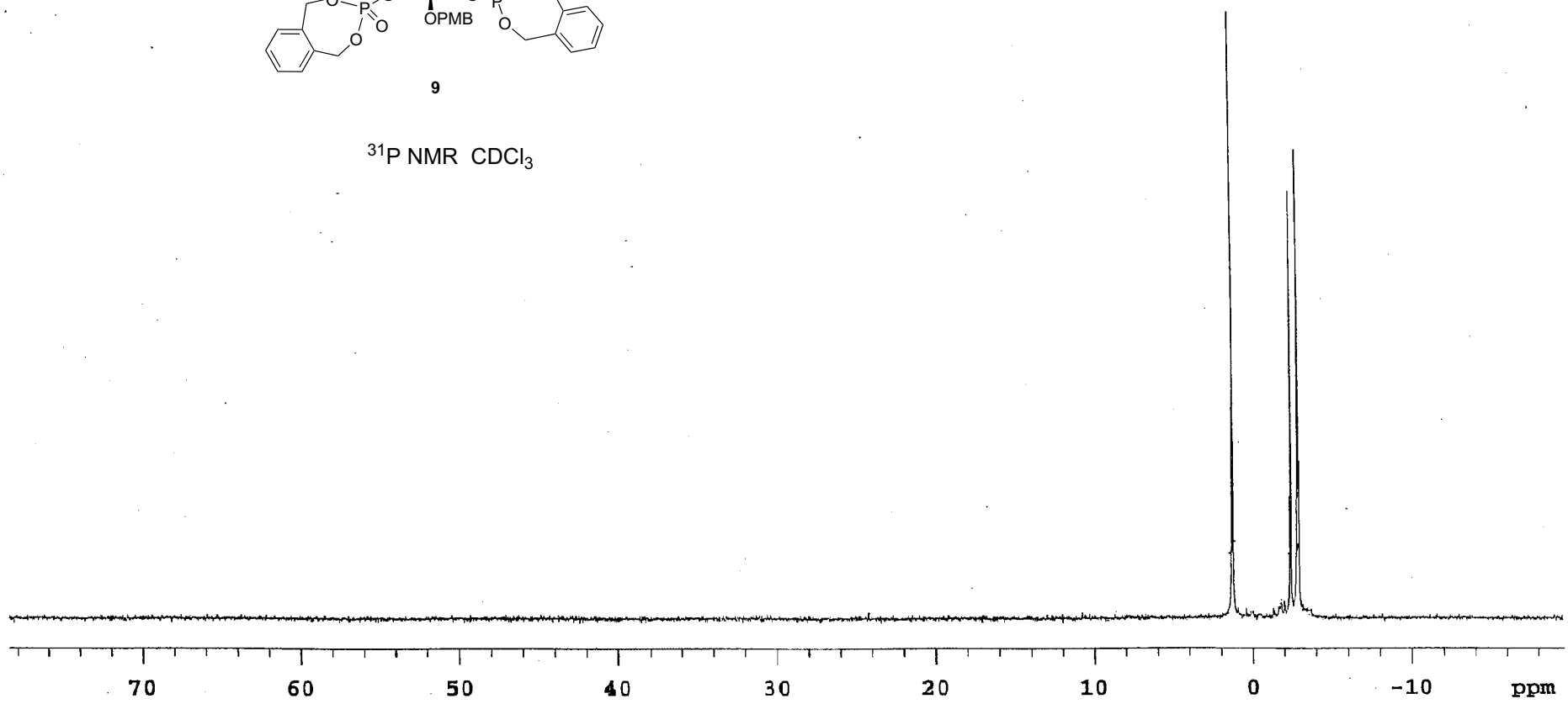
9P

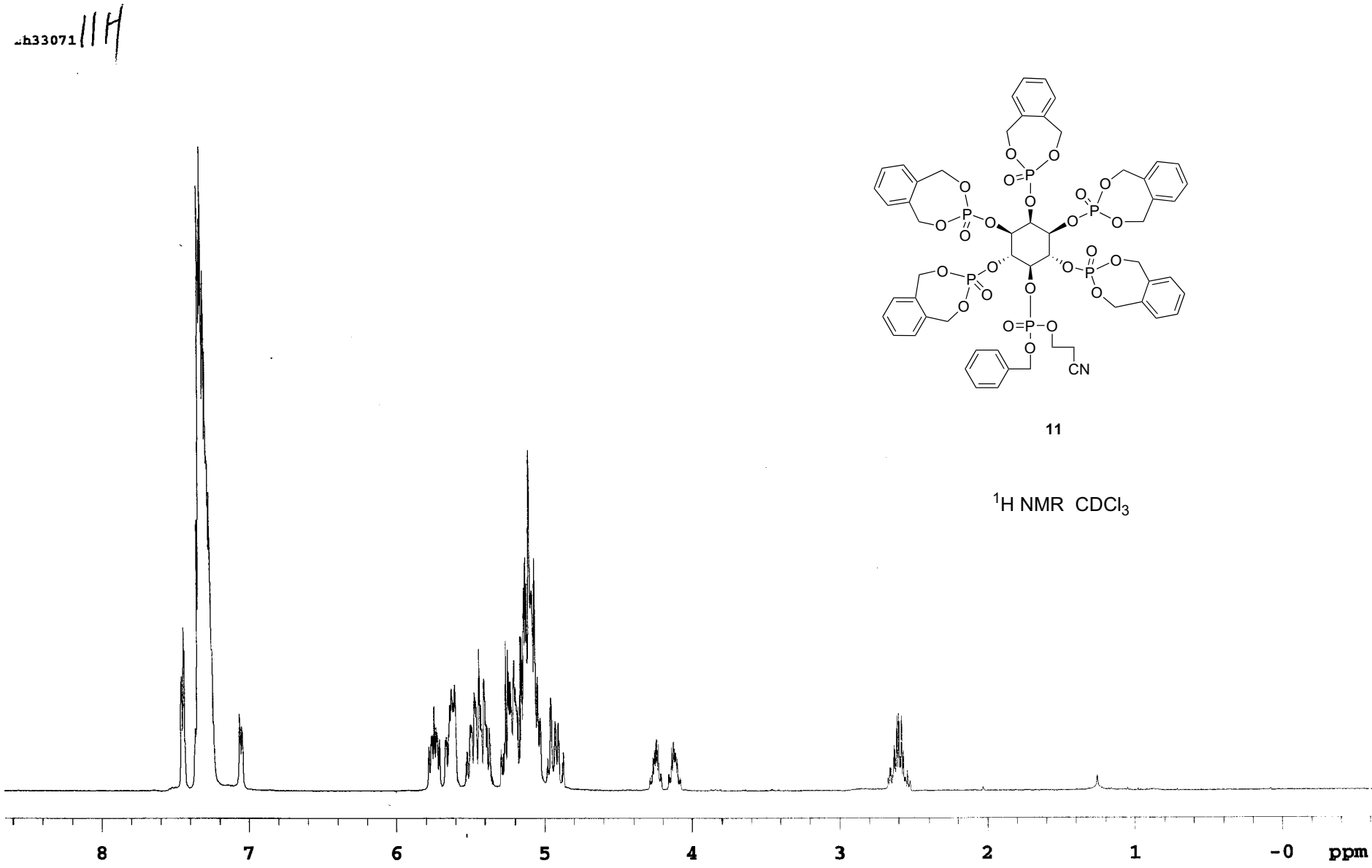
..3051P



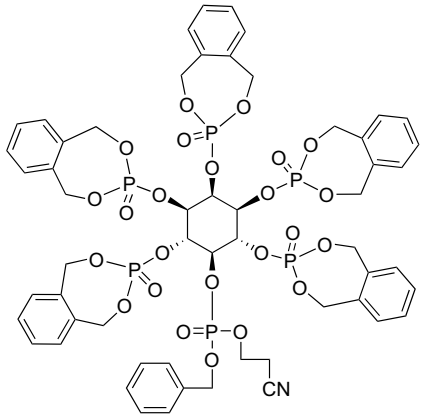
9

^{31}P NMR $CDCl_3$




11
^{1}H NMR CDCl$_3$
8
7
6
5
4
3
2
1
-0
ppm

11C

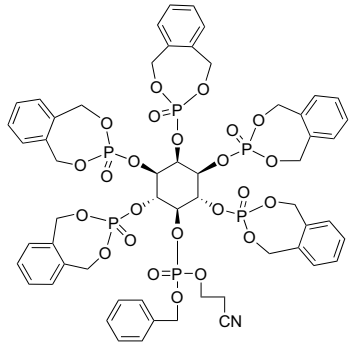


11

^{13}C NMR $CDCl_3$

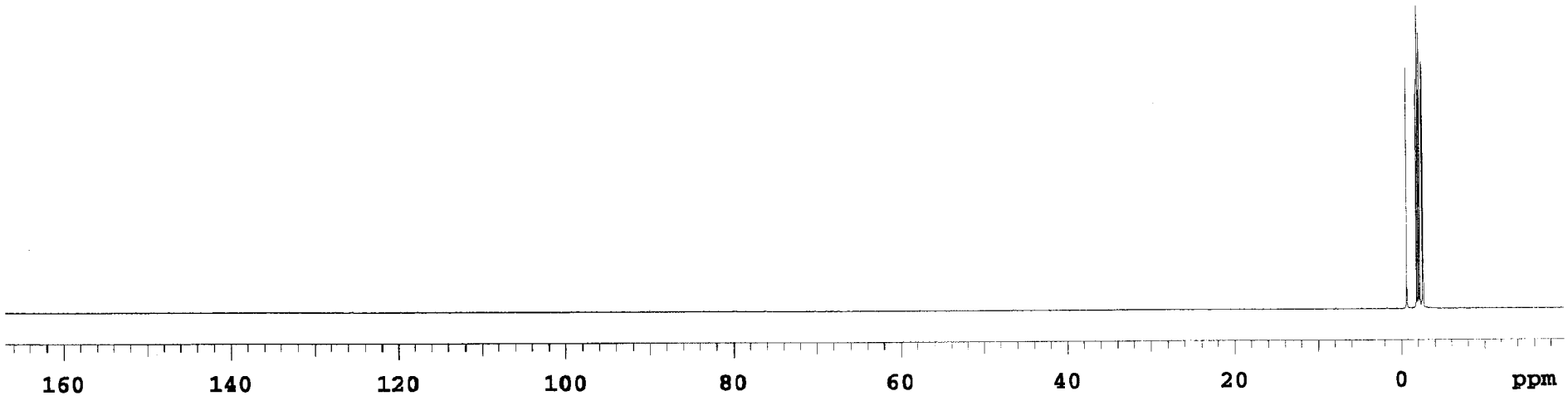
11P

zh33071P



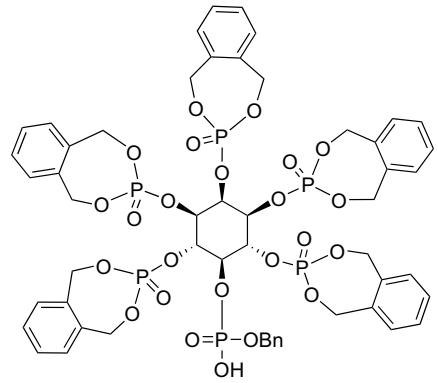
11

^{31}P NMR $CDCl_3$



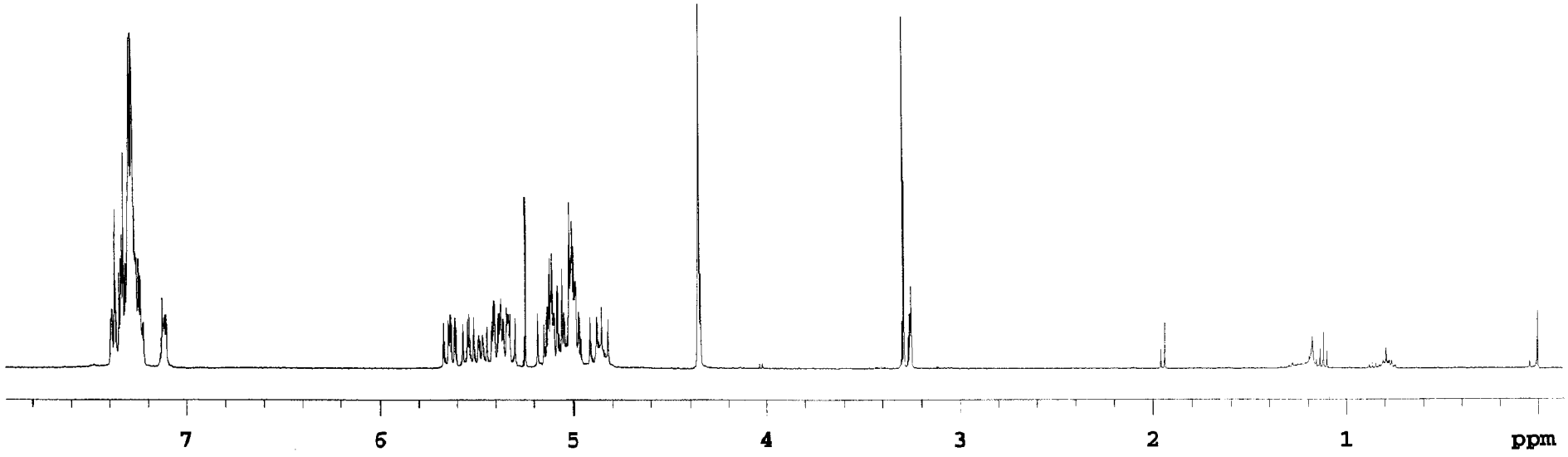
12H

zh33091
CDCl3/CD3OH 3:1

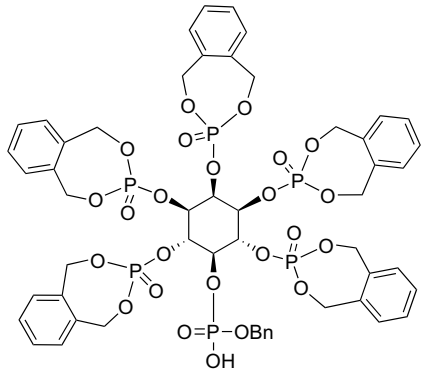


12

^{1}H NMR $CDCl_3$

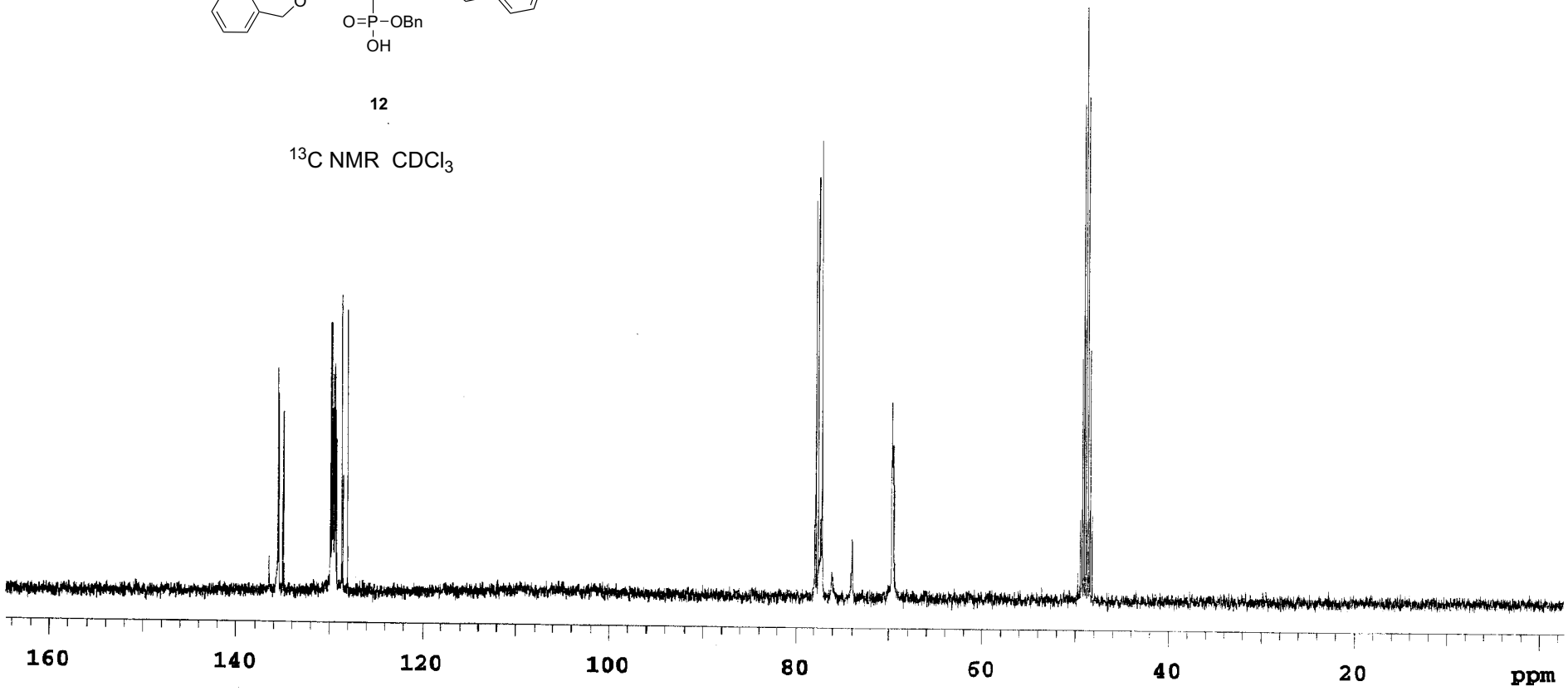


12C

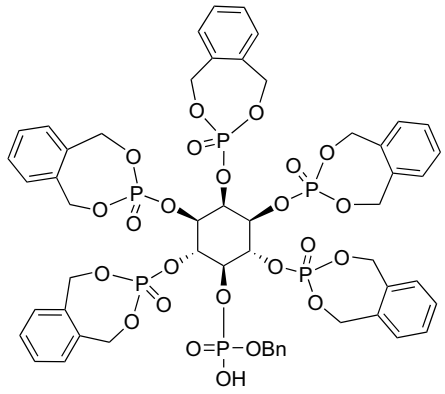


12

^{13}C NMR $CDCl_3$

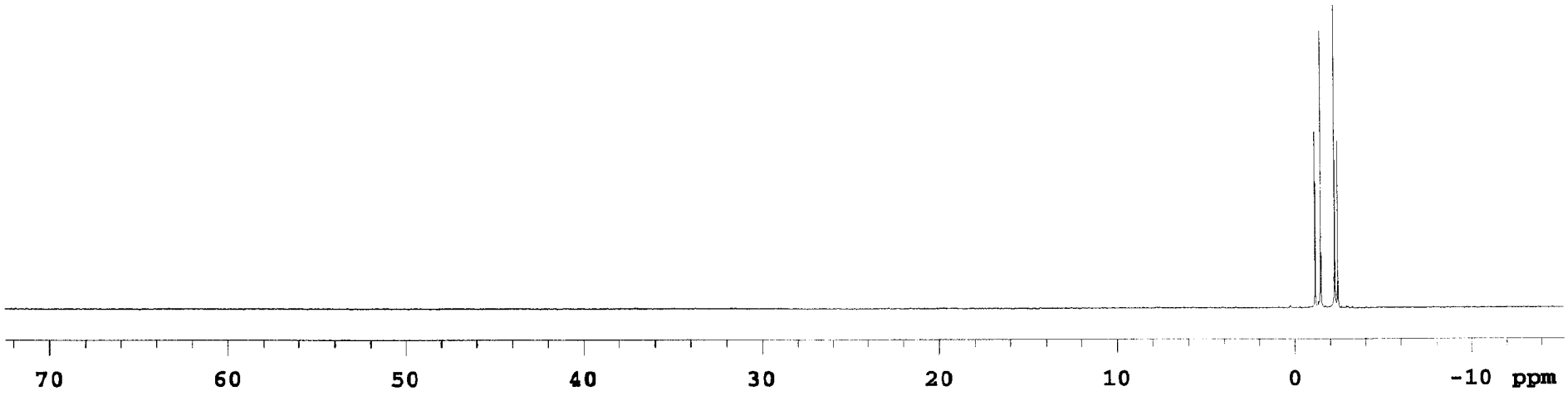


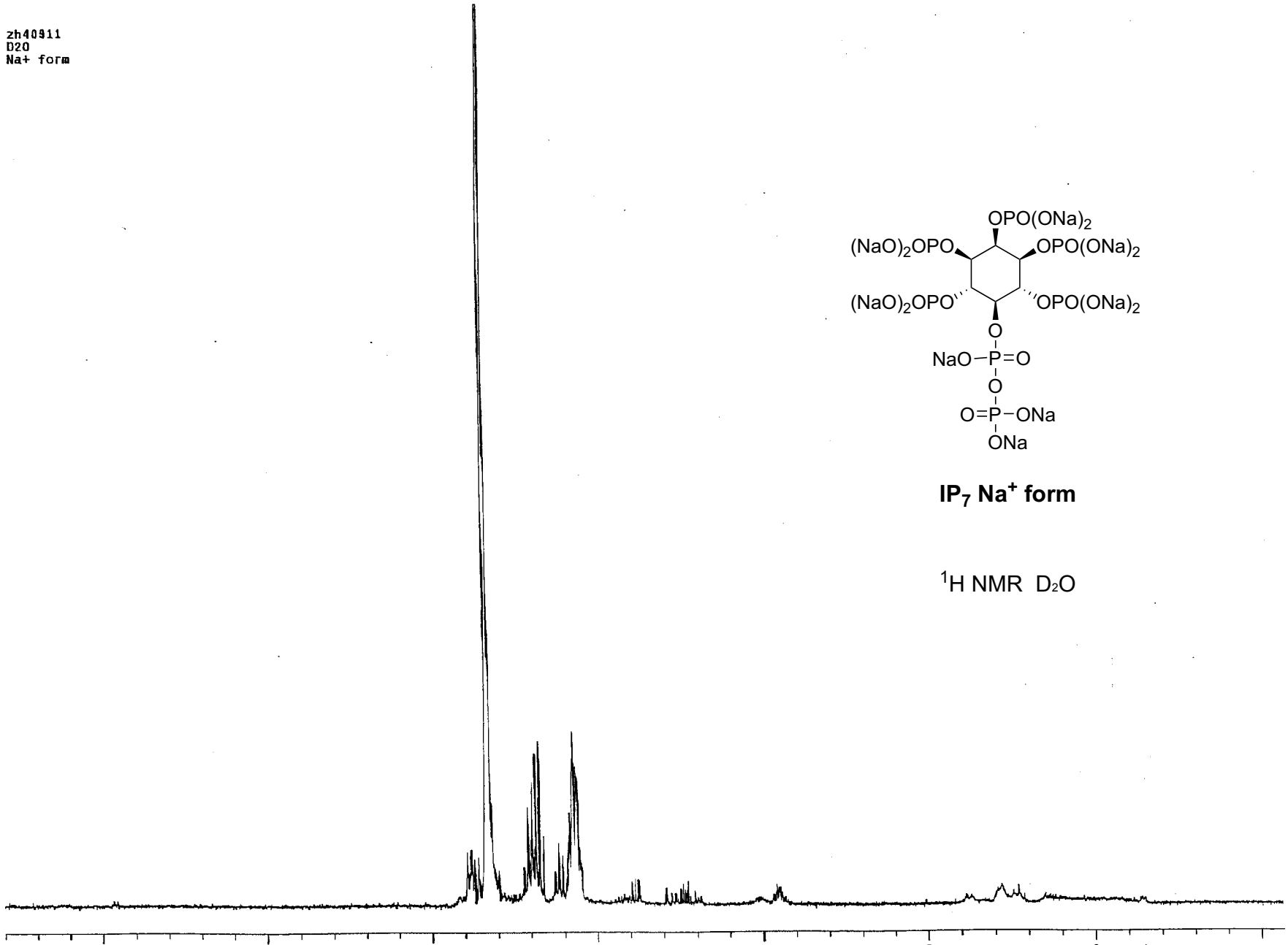
12?



12

^{31}P NMR $CDCl_3$

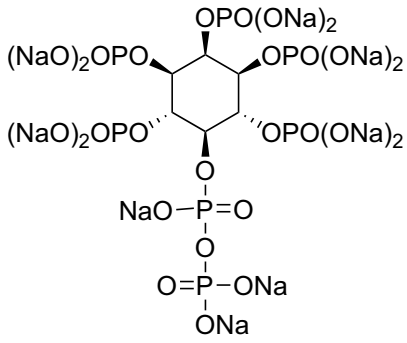




IP_7 Na^+ form

^1H NMR D_2O

zh40911P
Na+ form



IP_7 Na^+ form

^{31}P NMR D_2O